Hypothalamic peptides in the regulation of feeding and peripheral metabolism.

Thesis submitted to the University of London for the degree of Doctor of Medicine (M.D.)

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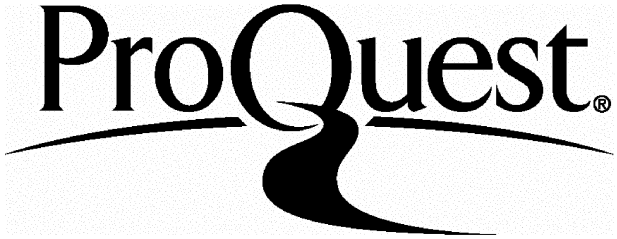

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Abstract.

The central nervous system is essential to the normal control of metabolic processes. Maintenance of energy balance is an important part of homeostasis and involves the control of food intake (input) and of peripheral metabolism (output). Loss of this homeostatic mechanism can lead to either obesity or cachexia.

Neuropeptide Y (NPY) is a 36 amino acid peptide that is found in several hypothalamic regions involved in the control of food intake. It potently stimulates feeding following intracerebroventricular (ICV) injection. In addition it has several effects on peripheral metabolism including stimulation of ACTH release from the pituitary gland and an increase in peripheral insulin resistance. Using analogues of NPY with differential binding to the known NPY receptors, I have shown that there are at least two novel central NPY receptors that mediate these effects of NPY. I have shown that a recently discovered NPY receptor antagonist, BIBP 3226 inhibits NPY induced feeding but that it has no effect on NPY mediated stimulation of ACTH release.

It has been postulated that foetal malnutrition can program peripheral glucose metabolism in later life. I have studied the effects of early malnutrition on pregnant and weanling rats and have shown that such programming does not occur at the level of hypothalamic neuropeptide Y messenger RNA.

I have also shown that glucagon like peptide-1 (GLP-1) is a novel central satiety factor, and that chronic blockade of GLP-1 receptors can lead to an increase in weight of rats. In addition I have shown that the effects of ICV GLP-1 as a satiety factor do not occur by any change in hypothalamic NPY, suggesting that the GLP-1 satiety system works independently of NPY. I have also shown that ICV GLP-1 causes a fall in peripheral plasma glucose and insulin concentrations, suggesting that central GLP-1 may have a role in the modulation of peripheral insulin sensitivity.

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Abbreviations:

ACTH	Adrenocorticotrophic Hormone
cAMP	cyclic AMP
CAPD	Continuous Ambulatory Peritoneal Dialysis
CGRP	Calcitonin Gene Related Peptide
CHO cells	Chinese Hamster Ovary cells
CNS	Central Nervous System
CoPP	Cobalt Protoporphyrin
CRH	Corticotrophin releasing hormone
CSF	Cerebrospinal Fluid
DMN	Dorsomedial nucleus of the hypothalamus
DNA	Deoxyribose nucleic acid
FSH	Follicle Stimulating Hormone
GHRH	Growth hormone Releasing Hormone
GLP-1	Glucagon-like peptide-1 (7-36) amide
HPA axis	Hypothalamo-Pituitary-Adrenal axis
IAPP	Islet Amyloid Polypeptide
ICV	Intracerebroventricular
LH	Luteinising hormone
NPY	Neuropeptide Y
PP	Pancreatic Polypeptide
PVN	Paraventricular Nucleus of the Hypothalamus
PYY	Peptide YY.
RIA	RadioImmunoAssay
RNA	Ribose nucleic acid
RPMS	Royal Postgraduate Medical School
SLE	Systemic Lupus Erythematosus
TSH	Thyroid Stimulating Hormone
USA	United States of America
VMH	Ventromedial nucleus of the Hypothalamus

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Chapter 1

General Introduction

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) (Bernard, 1849). Maintenance of energy balance is an important part of homeostasis and involves the control of input (food intake) and output (energy expenditure). Loss of this homeostatic regulation can result in either obesity or cachexia.

Obesity is the most common metabolic disease in the industrial world and affects more than 30% of the adult population in the United States of America (USA). Furthermore, obesity is a major risk factor for type 2 diabetes and more than 80% of patients with type 2 diabetes in the USA are obese (Bennett, 1990). Up to 6% of total health care expenditure is attributable to the effects of obesity, such as diabetes mellitus and ischaemic heart disease (Dryden et al. 1994). In experimental animals, insulin resistance, diabetes, and dyslipidaemia can be corrected by weight loss (Brownell et al. 1986; Brindley and Russell, 1995). Current behavioural and pharmacological strategies are largely ineffective at controlling body weight. If this could be achieved in humans, the benefits to the community would be immense.

Body weight and metabolic state are under hypothalamic control. The hypothalamus receives afferent neural and hormonal signals, and influences body weight and peripheral metabolism via effects on food intake, energy expenditure, and hormone secretion (Williams and Bloom, 1989). Understanding the role of the central nervous system in the regulation of body weight may lead to the development of new therapeutic

approaches directed towards the prevention of obesity, insulin resistance and diabetes mellitus.

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

Appetite, energy balance and body weight are modulated by a variety of neurochemical and neuroendocrine signals which originate from different organs in the body and diverse regions of the brain. The hypothalamus which lies at the base of the forebrain plays an important integrative role in the control of many homeostatic processes receiving input from, and acting through, a variety of systems that involve a close interaction between nutrients, amines, neuropeptides and hormones (Leibowitz, 1992). The hypothalamus influences a number of metabolic functions including 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 (Williams and Bloom, 1989). Control of food intake and peripheral energy expenditure are closely linked. The net result of the integration of peripheral signals, central nervous system (CNS) signalling, feeding and autonomic activity is that most animals are in a state of energy balance.

Perturbation of this system results in abnormal eating patterns such as those seen in anorexia nervosa, bulimia, diabetes and obesity (Brady et al. 1990; Leibowitz, 1992).

Control of food intake

Experiments carried out in the 1940's and 1950's first identified the hypothalamus as playing an important role in the control of food intake. Lesions of the ventromedial hypothalamus were found to produce uncontrolled hyperphagia and obesity (Hetherington and Ranson, 1940) and lesions of the lateral hypothalamic area, aphagia and weight loss (Brobeck, 1951). These findings 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 (VMH) 'satiety centre'. 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, the area postrema in the brainstem (Contreras et al. 1984) and the amygdala (Morgane, 1969). 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, their site of synthesis (Gold et al. 1977).

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 (Jensen et al. 1983; Stewart et al. 1985), and it is interesting to note 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 (Tempel et al. 1989). 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 large number of neurotransmitters may be involved in the control of food intake. Most of the experiments that have looked at the effects of various neurotransmitters on feeding involve the injection of the compound into the cerebrospinal fluid (CSF) or localised brain areas via stereotactically implanted cannulae. Stimulatory neurotransmitters are best assessed using satiated animals, and in this thesis, most studies were carried out in the early light phase when animals will normally eat only very little. Inhibitory neurotransmitters are best assessed in either food deprived animals (after a 24 hour fast) or in a phase of the diurnal rhythm when animals eat significantly: the early dark phase in the case of the rat. Examples of compounds that are known to stimulate food intake when injected into the CSF include neuropeptide Y, galanin, noradrenaline and dynorphin. Compounds that inhibit feeding include leptin, adrenomedullin (see chapter 10) and glucagon-like peptide-1 (see chapter 7).

Leptin, the protein product of the ob gene (Zhang et al. 1994) is now known to be an important satiety factor. It is synthesised exclusively in adipocytes (Ogawa et al. 1995; Halaas et al. 1995; Saladin et al. 1995), and may be important in the regulation of fat mass. In this thesis I have focussed on neuropeptide Y (chapters 3-6), glucagon like peptide-1 (chapters 7-9) and adrenomedullin (chapter 10).

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. Cyanide would appear to be a potent inhibitory transmitter! 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 receptor antagonists. Such an antagonist would be expected to increase food intake following intracerebroventricular injection. For many of the peptides studied, these antagonists are not available.

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 that affect food intake have significant effects on the hormonal and autonomic output which influence energy expenditure.

Thermogenesis

Excess energy may be expended in the form of work as useful muscular or metabolic activity, or dissipated as heat. Diet induced thermogenesis describes the adaptive

phenomenon whereby waste energy can be dissipated as heat in a regulated manner. In animals fed highly palatable diets, there is a compensatory rise in thermogenesis, particularly in brown adipose tissue, which limits weight gain (Rothwell and Stock, 1979). Conversely genetically obese animals and those with hypothalamic (VMH or PVN) lesions appear to have a reduction in thermogenesis (Himms-Hagen, 1985). Studies of autonomic nervous system activity suggest that this phenomenon is under nervous control, with the integrating centre of that control being in the appetite regulating areas of the hypothalamus (Vander Tuig et al. 1982). Stimulation of the ventromedial hypothalamus increases sympathetic activity to brown adipose tissue, whereas its destruction causes the reverse (Sakaguchi et al. 1988; Holt et al. 1987). 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 (Lupien et al. 1986).

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 (Bernard, 1849). 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. All of these pituitary hormones are under hypothalamic control.

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 (fa/fa) rat, ob/ob mouse) and acquired obesity (animals fed cafeteria or palatable diets). The Zucker (fa/fa) rat is known to have a defect in the leptin receptor (Phillips et al. 1996) and I have used this model in a set of studies in chapter 7. The acquired obesity model fed a highly palatable diet is used in studies in chapter 6.

The role of early nutrition.

Recent studies in humans suggest that low weight at birth, and at one year old, correlates with impaired glucose tolerance and type 2 diabetes in later life (Barker et al. 1993; Phipps et al. 1993; Fall et al. 1992; Hales et al. 1991). Type 2 diabetes may thus result from poor early nutrition.

Programming of peripheral metabolism by early nutrition has been proposed (Hales and Barker, 1992), and the hypothalamus is an obvious site for this to occur. Poor nutrition in early life may result in a permanent and sustained change in the hypothalamic set point for neuropeptides that control feeding and metabolism, resulting in obesity and insulin resistance in later life. In societies where man has ad libitum access to food, insulin resistance or relative insulin deficiency may result in overfeeding which in turn exacerbates insulin resistance in a vicious circle. Patients have usually been insulin resistant for a number of years before diabetes becomes apparent (Eriksson et al. 1989; Warram et al. 1990). Thus insulin resistance may be the initiating factor, with

hyperinsulinaemia occurring as a consequence, and diabetes becoming evident when the pancreatic beta cell fails (Jarrett, 1992).

Poor early nutrition has been shown in rodents to permanently affect the pancreatic beta cell (Dahri et al. 1991; Swenne et al. 1992; Swenne et al. 1987). Whether hypothalamic programming can cause insulin resistance in addition to insulin deficiency has not been previously addressed.

This thesis concentrates on the effects of NPY (chapters 3-6), GLP-1 (chapters 7-9) and a newly discovered member of the calcitonin family of peptides, adrenomedullin (chapters 10 and 11) on feeding and peripheral metabolism. In addition the role of early nutrition (chapter 6) on hypothalamic NPY is investigated in rats. Detailed consideration is given to the NPY receptor subtypes that mediate feeding and ACTH release. (chapter 3).

Aims.

The aims of this thesis are to establish:

1. The role of neuropeptides, in particular neuropeptide Y (NPY), glucagon like peptide-1 (7-36) amide (GLP-1) and adrenomedullin in the control of food intake.
2. The effect of these neuropeptides on the hypothalamo-pituitary-adrenal (HPA) axis.
3. The hypothalamic NPY receptor subtype that mediates feeding.
4. The hypothalamic NPY receptor subtype that mediates ACTH release.
5. The effect of these neuropeptides on peripheral glucose metabolism and insulin sensitivity.
6. The role of early nutrition on the modulation of hypothalamic neuropeptides and hence insulin sensitivity in later life.

Chapter 2

Experimental Protocols

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.

Peptide synthesis

Some of the peptides used in the studies described were purchased from Sigma (Poole, Dorset, UK.). Adrenomedullin, CGRP (8-37), GLP-1 and novel fragments of NPY were synthesised by Dr Peter Byfield and Miss Wendy Callinan at the RPMS using an Applied Biosystems 431A peptide synthesiser. Mass determination by electrospray mass spectrometry was performed to confirm the identity of the peptide.

Use of experimental animals

All animal procedures were performed under licence in accordance with the Home Office (Scientific Procedures) Act, and for which I currently hold a Home Office Personal Licence No 90/02211 under project licence No. 90/00316 for the investigation of the role of regulatory peptides in the control of food intake and metabolism.

Adult male Wistar or Zucker rats (200-250 grams) were housed in individual cages under controlled temperature (22°C) and with an 11-hour light/13-hour dark cycle. Lights were turned on at 9am and off at 8pm. Food and water were available ad libitum at all times except where animals were fasted for 24 hours. In one study (chapter 8), animals were fasted for 72 hours.

CNS cannulation and injection

Rats were anaesthetized with xylazine (20 mg/kg) (Rompun, Bayer, Suffolk, UK) and ketamine (100 mg/kg) (Ketalar, Parke Davis, Pontypool, Gwent, UK) and placed in a Kopf stereotaxic frame. In this thesis cannulae were implanted in either the third ventricle, the lateral ventricle or the paraventricular nucleus of the hypothalamus. Cannulation of the third ventricle involved use of permanent 22 gauge stainless steel guide cannulae (Plastics One inc. Roanoke, VA, USA) which were stereotactically placed 0.8mm posterior to bregma on the midline and implanted 6.5mm below the outer surface of the skull. For lateral ventricular cannulation a similar procedure was used but the coordinates for implantation were as follows: 1.0mm posterior to the bregma, 1.5mm lateral to the midline and 2.5mm below the skull surface. For PVN cannulation 26 gauge cannulae were implanted 1.8mm posterior to the bregma, 0.4mm lateral to the midline and 7.0mm below the skull surface. These co-ordinates were calculated using a rat brain atlas (Paxinos and Watson, 1982). The guide cannulae were held in place by dental cement glued to three stainless-steel screws driven into the skull.

After surgery a wire stylet was inserted into the guide cannula to prevent blockage and to avoid infection. The rats were then allowed to recover for four days. Following this recovery period, rats were given a one week period of daily handling, in order to habituate the animals to experimental procedures and minimise stress. The positioning of the cannulae was verified by a positive dypsogenic response to 150 ng of angiotensin II. Only animals which showed a sustained drinking response within 2 minutes were used for the study. The rats were given at least a two-day rest between studies to recover

from injections, but were still handled during this time. All animals were injected on two occasions with normal saline to acclimatise them to the procedure prior to the first study day.

The injection of substances was by a stainless steel injector placed in, and projecting 0.5mm below, the tip of the guide cannulae. The required volumes (see below) were injected over one minute and a further period of 30 seconds was allowed before removing the injector, to ensure complete diffusion into the brain region. A Hamilton gas tight syringe was connected to an infusion pump to ensure an accurate and constant volume delivery. The tubing was filled with saline solution and a small air bubble (5µl) was drawn up at the distal end to separate it from the test solution. The entire injection process lasted under 2 minutes, and the rats were returned to their cages with the minimum of disruption. Measurement of food intake involved the weighing of the food at the start of the study, and re-weighing the food two hours after injection, with the rats left undisturbed during the intervening period. All compounds were dissolved in 0.9% saline except in chapters 4 and 5 where the compounds studied were only soluble in ethanol. The usual injection volume for injection into the third ventricle was 10 µl. The placement of cannulae were verified at the end of each study by the injection of ink (blue food colouring), removal of the brain, snap freezing and visual examination of coronal brain slices.

Decapitation

Killing of animals by decapitation is the least stressful method for blood collection and is particularly important when assessing hormones that respond to stress. It also allows immediate collection of fresh brain tissue. Animals to be decapitated were put through a regimen of sham decapitation on several occasions before the study to ensure absolute minimal external stress and its metabolic consequences on the day of the study. On the study day, decapitation was followed immediately by collection of trunk blood into a heparinised container containing 0.6 mg aprotinin. The blood was centrifuged and the plasma separated and frozen on dry ice within ten minutes.

Intravenous cannulation.

Rats were anaesthetised as for ICV cannulation. An incision was made in the neck and the internal jugular vein was exposed. Polythene tubing (28 gauge) was cut to length (40 cm) and a mark made 3.2 cm from its end. A minute incision was made in the vein. The tubing was inserted into the incision and passed towards the heart up to 3.2 cm. The tubing was tied to the vein with a sterile 4/0 silk suture. The tubing was tunnelled subcutaneously to the animals back and passed through a steel button which was sutured to the animals back (Figure 2.1). The button was attached to a tether and the cannula attached to a swivel at the top of the cage so that the animal could move around the cage relatively freely, while receiving a continuous intravenous infusion. Up to 1 ml of blood could be safely taken from the line without disturbing the animal.

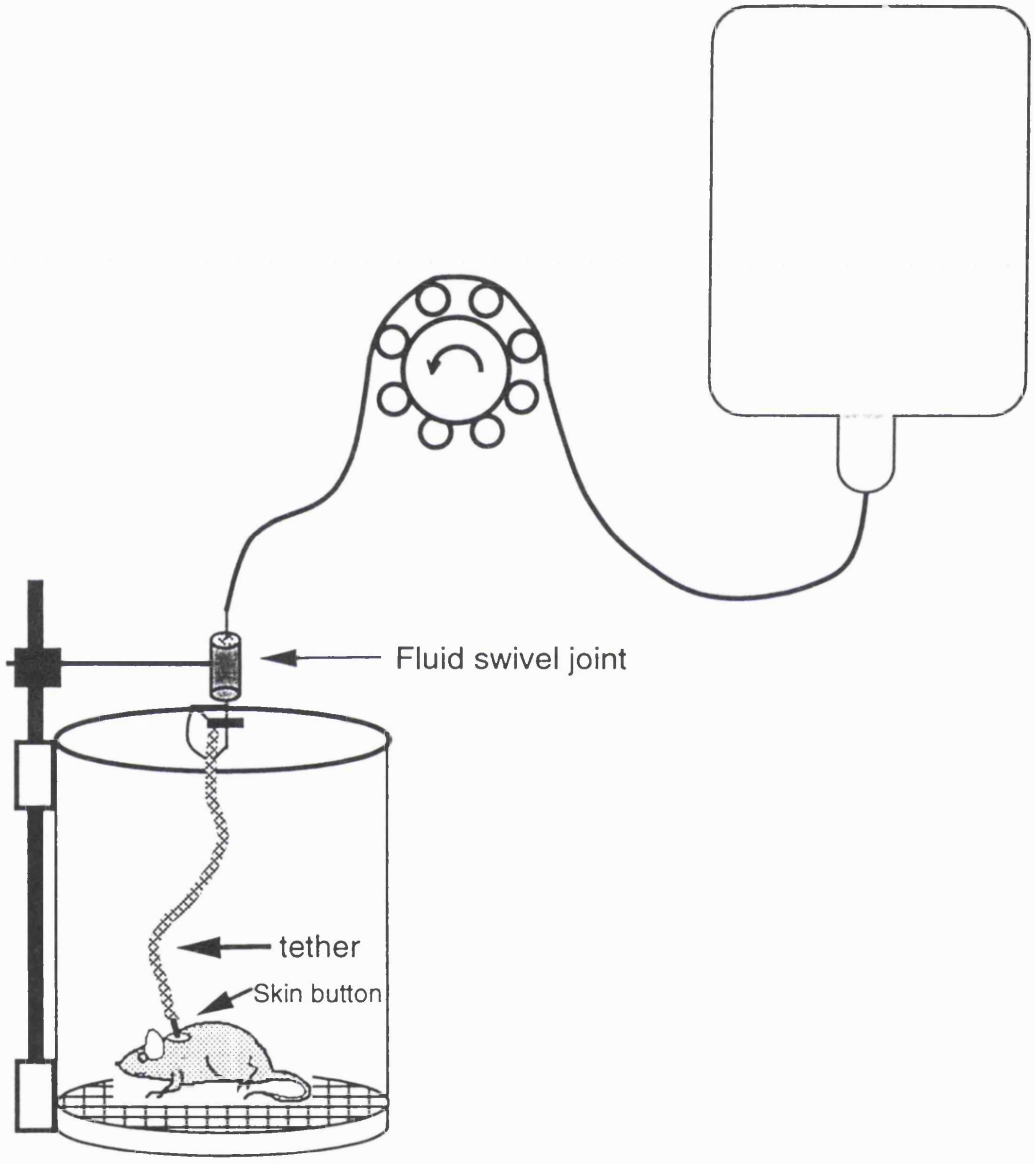


Figure 2.1

Measurement of hypothalamic NPY mRNA by Northern blot analysis

Immediately after death, the hypothalami were rapidly dissected and frozen in liquid nitrogen. Total RNA was extracted using acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Briefly each hypothalamus was added to 3ml denaturing solution (solution D: 4M guanidinium thiocyanate, 25mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1M 2-mercaptoethanol) and homogenized in a glass-Teflon homogenizer at room temperature. Ribonuclease is inhibited by the presence of 4M guanidinium, protecting the RNA from degradation. The mixture was transferred to another tube and 0.3 ml 2M sodium acetate pH 4.0, 3ml of phenol (water saturated) and 1ml chloroform-isoamyl alcohol mixture (49:1) were added to the homogenate, with thorough vortexing. The samples were allowed to stand on ice for 15 minutes and then centrifuged at 10,000g for 20 minutes at 4°C. After centrifugation, RNA was present in the aqueous phase, which was transferred to a fresh tube and mixed with an equal volume of isopropanol at -20°C to precipitate the RNA. The RNA was sedimented, resuspended in solution D and precipitated again in isopropanol. The RNA was approximately quantified by dissolving in distilled water and measuring its absorbance at 260 nm. The RNA was again washed and precipitated in 70% ethanol, vacuum dried and dissolved in water to make a final concentration of 5μg/μl. Approximately 60μg RNA was extracted from each hypothalamus. The integrity of this RNA was established by gel electrophoresis of 5μg RNA, staining with ethidium bromide, and visualisation under UV light. Fifty micrograms total RNA from each sample were then separated by electrophoresis on a 1% formaldehyde-agarose gel and transferred to a Hybond nylon membrane (Amersham International). The filter was baked for 2h at 80°C and prehybridised for 2h at °42 C in 50 mmol/litre sodium

phosphate buffer (pH 6.8), 50% formamide, 5 x SSC (1x SSC= 0.15M NaCl and 0.015M sodium citrate pH 7.0), 5 x Denhardt's solution (polyvinyl-pyrrolidone, 0.02% ficoll, and 0.02% BSA), and 100 μg/ml denatured sonicated herring sperm DNA.

Radioactive probes were prepared with [^{32}P] deoxy-CTP by random primer extension of the appropriate rat cDNA to a specific activity of 1.5 x 10^9 dpm/μg. In this thesis probes were made to NPY, galanin, somatostatin, pre-pro-tachykinin A and neurotensin. The cDNA for each of these was available in our laboratory. The filter was then hybridised for 16 h at 42°C in the prehybridisation solution to which had been added dextran sulphate (100 μg/ml) and labelled probe at a concentration of 1ng/ml. Finally the filter was washed in a solution of 2 x SSC-0.2% sodium dodecyl sulphate (SDS) at 22°C for 1h, followed by a further wash at 60°C for 1h. Quantitative estimates of relative levels of mRNA were made using a phosphorimager and image analysis software. All results were normalised to the amount of mRNA loaded by stripping the filters in 0.5% SDS-1% triethanolamine at 80°C for 30 minutes and reprobing with oligo dT. Final results are presented as a ratio of specific counts for NPY mRNA/counts for oligo(dT) by comparison with control animals which are given an arbitrary count of 100%.

Peptide radioimmunoassay (RIA)

RIA is based upon the binding of antigen (Ag) to a specific antibody (Ab). A standard curve is constructed using known quantities of unlabelled antigen, and unknown samples can then be compared to the standard curve. Two standard curves were constructed for each assay. Assay conditions for each specific RIA (pH, temperature and incubation period) were optimised. Antibodies were obtained from rabbit immunization with peptide

+ adjuvant. Radiolabelled peptides were prepared by iodination (Fraker and Speck, 1978) using radioactive ^{125}I. Throughout these studies all assays for a particular experiment were carried out in a single assay to remove any possibility of interassay variation. In this thesis, RIA was used to measure insulin (Albano et al. 1972), glucagon (Ghatei et al. 1983) and adrenomedullin (Meeran et al. 1997).

Measurement of plasma ACTH

The immunoradiometric assay for ACTH was carried out using an ACTH-Fast kit, supplied by Euro-Diagnostica B.V. (Hodgkinson et al. 1984). Several standard solutions with known concentrations of up to 1150 pg/ml of ACTH were supplied. A solution containing a radioactive sheep anti ACTH antibody which bound to the N-terminal of the ACTH and a rabbit anti ACTH antibody which bound to the C-terminal was supplied. One hundred microlitres of this solution were added to the samples. The mixture was incubated overnight at 4°C. The bound radioactive complex was separated from the free radioactive antibody by adding a second antibody (sheep anti-rabbit IgG). After two washing steps, the complex of ACTH with radioactive antibody was centrifuged and the pellet was counted using a gamma counter for 60 seconds. The amount of ACTH in the samples was calculated by comparing with the standards.

Statistical analysis.

Feeding experiments were analysed by analysis of variance, and pairwise differences between groups by Tukey's post hoc test, using the SYSTAT computer package. The use of summary measures (Matthews et al. 1990) was used to compare serial data, when looking at changes in body weight or food intake over time.

Chapter 3.

Neuropeptide Y

Neuropeptide Y (NPY) is a 36 amino-acid peptide first isolated from porcine brain by Tatemoto in 1982 (Tatemoto et al. 1982). 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 3.1).

The structural similarities between the various members of the pancreatic polypeptide family of peptides appear to be related to a common genetic ancestry. Recent studies show strong evolutionary conservation across a wide variety of species (Blomqvist et al. 1992), implying a crucial role.

Figure 3.1

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

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

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 (Tatemoto et al. 1982), 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 (Adrian et al. 1983; Allen et al. 1983).

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 (Allen et al. 1983). 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 arcuate nucleus and from the brainstem (O'Donohue et al. 1985). An anatomically and functionally distinct NPY system projects from the ventrolateral geniculate nucleus of the thalamus to the suprachiasmatic nucleus of the hypothalamus (Chronwall et al. 1985) and has been postulated as being involved in the regulation of circadian rhythms (Mikkelsen and O'Hare, 1991).

Central actions of NPY

Effects on pituitary hormone release

NPY administered ICV influences the release of a number of pituitary hormones, notably luteinizing hormone (LH), adrenocorticotrophic hormone (ACTH), growth hormone (GH), and thyroid stimulating hormone (TSH). The secretion of the GH and TSH are inhibited by NPY (Kerkerian et al. 1985). LH secretion is also inhibited under most circumstances (Catzeflis et al. 1993), although secretion is increased in oestrogen primed

females (McDonald et al. 1985; Kalra and Crowley, 1984). In males, NPY causes an acute increase in LH release (Allen et al. 1985) but chronic administration of ICV NPY causes suppression of LH release with an associated decrease in the weight of LH dependent tissues (Pierroz et al. 1996). NPY affects several parts of the HPA axis, including an increase in CRF mRNA (Suda et al. 1993), and CRF immunoreactivity (Haas and George, 1987) in the hypothalamus. There is also an increase in anterior pituitary pro-opiomelanocortin mRNA, ACTH synthesis and ACTH release following ICV and PVN administration of NPY (Wahlestedt et al. 1987; Hanson and Dallman, 1995; Inui et al. 1990; Inoue et al. 1989). NPY has been shown to be as potent as CRF in inducing ACTH release in dogs (Inoue et al. 1989). The effect of NPY on plasma ACTH is blocked by a CRF receptor antagonist, alpha-helical CRF, implying that the release of ACTH is mediated by an increase in portal CRF (Inoue et al. 1989). It has been suggested that endogenous NPY has a role in activation of the HPA axis in the presence of hypoglycaemia since HPA activation is prevented when an antibody against NPY is administered ICV immediately before insulin induced hypoglycaemia (Inui et al. 1990).

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 and sustained increase in food intake. This was first observed by Clark & Kalra in 1984, shortly after the demonstration of NPY in the hypothalamus (Clark et al. 1984), and has since been confirmed by many laboratories, and in many species (Pau et al. 1988; Miner et al. 1989; Morley et al. 1987). 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 (Stanley et al. 1993; Stanley and Leibowitz, 1985). Repeated injection of NPY into the PVN of the hypothalamus results in a sustained increase in food intake, and ultimately obesity (Stanley et al. 1986). 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 (Stanley and Leibowitz, 1985). The feeding 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)(Stanley and Leibowitz, 1985), than for noradrenaline (3 minutes) (Allen et al. 1985). The explanation for the long latency is the subject of much debate and is discussed below. The NPY response is longer lasting however, and effects are observed for up to two hours following injection (Stanley and Leibowitz, 1985). Like noradrenaline, the feeding response produced by NPY is predominantly for carbohydrate (Stanley et al. 1989; Stanley et al. 1985). Rats are most sensitive to the feeding stimulatory effects of NPY at the beginning of the dark phase (Tempel and Leibowitz, 1990), a time of day when their food intake is normally at its greatest (Jensen et al. 1983; Stewart et al. 1985).

Why is there a latency to NPY induced feeding ?

The latency to the onset of feeding following ICV NPY may occur because NPY induces

the production of other mediators which may have to be synthesised de-novo. These may be dopamine or opioid analogues although no such agent has yet been demonstrated. Another possible explanation is that ICV NPY induces the production of some satiety factor with a short duration of action, which inhibits feeding for a short period. Corticotrophin releasing factor (CRF) is one such possible factor. ICV CRF has been shown to inhibit NPY induced feeding (Morley et al. 1987). The CRF receptor antagonist, α-helical CRF, enhances NPY-induced feeding and also reduces the latency to begin feeding following the stress of a tail pinch (Heinrichs et al. 1992). These results suggest that endogenous CRF, and possibly the associated release of ACTH may be important in modulating the feeding response to NPY.

NPY is regulated by nutritional state.

The presence of NPY in the hypothalamus, and the demonstration of its potent effects on food intake and metabolism suggests an important role in the regulation of energy homeostasis. NPY content is specifically increased in the hypothalamus in animals with diabetes (Williams et al. 1989; Williams et al. 1988). These changes are mainly confined to known appetite regulating areas, including the arcuate nucleus and the PVN (Williams et al. 1989). This increase in NPY content is 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 (White et al. 1990). Similar changes are observed following food deprivation, and that the increase in NPY is rapidly reversed by refeeding, suggesting that NPY synthesis is determined by nutritional state (White and Kershaw, 1989; Sanacora et al. 1990; Sahu et al. 1988).

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. Intrathecal administration of NPY results in hypotension whereas its administration into the third ventricle increases blood pressure (Vallejo and Lightman, 1986; Westfall et al. 1988). 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 (Westfall et al. 1988). These effects seem to be related to a general increase in sympathetic nervous system activity (Martin et al. 1991).

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-striatal system (Morley et al. 1987). NPY may also be involved in memory (Flood et al. 1987). NPY and NPY Y2 receptors (see below for a description of NPY 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 (Heilig et al. 1988; Widdowson and Halaris, 1991).

Effects on pancreatic hormone release and glucose metabolism

The effects of central NPY injection on peripheral metabolism have also been investigated. The increase in plasma ACTH caused by the ICV administration of NPY causes an increase in circulating corticosterone concentrations (Leibowitz et al. 1988; Inoue et al. 1989; Wahlestedt et al. 1987; Haas and George, 1987). This is not associated with any change in circulating glucose, but there is an increase in plasma insulin, suggesting that peripheral insulin sensitivity may be modulated by hypothalamic NPY (Moltz and McDonald, 1985). Other important metabolic effects observed after central administration of NPY include a reduction in activity in sympathetic nerves innervating brown adipose tissue (Egawa et al. 1991) and alterations in the respiratory quotient favouring metabolism of carbohydrate (Menendez et al. 1990). ICV NPY has been shown to have divergent effects on glucose utilisation by adipose tissue and skeletal muscle, apparently directing energy towards lipogenesis rather than glycogenesis (Zarjevski et al. 1994).

NPY is thus one of the most abundant brain peptides and influences many physiological systems.

NPY Receptors

One of the main aims of the project was to identify the receptor subtype involved in the control of food intake. Only two NPY receptors (Y1 and Y2) had been identified at the start of the project although several others have since been cloned. In an *in-vivo* model of the classical Y1 receptor, NPY and [Pro^{34}]NPY increase blood pressure markedly compared with C-terminal fragments such as NPY (2-36) and NPY (3-36) (Grundemar et al. 1992). The Y2 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 (Wahlestedt and Hakanson, 1986). The development of NPY analogues that stimulated peripheral but not central Y1 receptors (Kirby et al. 1995) is evidence that the receptor involved in feeding is not a classical Y1 receptor. The possibility that the full feeding effect of NPY was due to a combination of Y1 and Y2 receptor activation had not been properly studied.

The Y1 receptor binds with high affinity only full length NPY or full length analogues such as [Pro^{34}]NPY or [$Leu^{31}Pro^{34}$]NPY (Fuhlendorff et al. 1990). The loss of a single N-terminal amino acid (NPY(2-36)) results in significant loss of biological potency and binding affinity at Y1 receptors (Grundemar et al. 1993). NPY Y1 receptors have a much reduced affinity for C-terminal fragments such as NPY(13-36) (Grundemar et al. 1992) and NPY(3-36)(Grundemar et al. 1993). The ability of the C-terminal fragments NPY(2-36) and NPY(3-36) to fully stimulate feeding despite a much reduced Y1 affinity (Stanley et al. 1992) opposes a crucial role for Y1 in the control of feeding. This receptor has been cloned from the human (Larhammar et al. 1992) and rat (Eva et al.

1990) CNS and is the only NPY receptor expressed in the human neuroblastoma cell line SK-N-MC (Larhammar et al. 1992). The Y2 receptor, in contrast, has a much higher affinity for C-terminal fragments such as NPY(13-36) and NPY(3-36)(Grundemar et al. 1993) than [Pro^{34}]NPY (Wahlestedt et al. 1990). This receptor has been cloned from human hippocampus (Gerald et al. 1995), and from another human neuroblastoma cell line, SMS-KAN (Rose et al. 1995). The human neuroblastoma cell lines are considered the *de facto* standards for the characterisation of ligands for Y1 and Y2 receptors. PYY binds with high affinity to both the Y1 and Y2 receptor subtypes, whereas human and rat PP shows low affinity for them (Gerald et al. 1996; Gerald et al. 1995; Larhammar et al. 1992).

The Y3 and Y4 receptors are not involved in food intake.

The Y3 receptor is characterised by its failure to recognise PYY (Wahlestedt et al. 1992), and has therefore been classified as an NPY preferring receptor. Since PYY stimulates feeding (Stanley et al. 1985), the Y3 receptor is unlikely to be involved in the control of food intake. It has been postulated that such an NPY preferring receptor has been cloned (Rimland et al. 1991), although the specificity of this receptor for NPY has been questioned (Herzog et al. 1993). The Y4 receptor has been cloned by screening of a human placental genomic library and mRNA is expressed in the human brain, coronary artery and ileum (Bard et al. 1995). This receptor is characterised by its high affinity for human, bovine and rat PP (Gerald et al. 1996), and by a reduced affinity for NPY(13-36) (Lundell et al. 1995; Bard et al. 1995). Full length NPY does not activate the Y4 receptor (Gerald et al. 1996). Rat PP activates the Y4 receptor (Gerald et al. 1996) but does not stimulate feeding (Clark et al. 1985). Hence the Y3 and Y4 receptors are not

involved in the control of food intake.

Two new NPY receptors both designated Y5 were cloned in 1996. They will be referred to as $Y5_{NAT}$ (Gerald et al. 1996) and $Y5_{JBC}$ (Weinberg et al. 1996) in this thesis after the journals in which they were published. The $Y5_{JBC}$ receptor has been cloned from mouse genomic DNA and is expressed within discrete regions of the hypothalamus. This receptor is characterised by its high affinity for NPY and PYY and extremely low affinity for human PP (Weinberg et al. 1996). Since human PP does stimulate feeding (Clark et al. 1984), this receptor is also not the feeding receptor. The $Y5_{NAT}$ receptor has been recently cloned from rat hypothalamus (Gerald et al. 1996). $Y5_{NAT}$ receptor mRNA is found primarily in the central nervous system, including the paraventricular nucleus of the hypothalamus (PVN) and the lateral hypothalamus, areas of the brain that have been implicated in the central control of appetite (Stanley et al. 1986). The extent to which selected peptides inhibited adenylate cyclase through the $Y5_{NAT}$ receptor and stimulated food intake in rats corresponded well and led the authors to conclude that this $Y5_{NAT}$ receptor was the postulated "feeding" receptor (Gerald et al. 1996).

The stimulation of adrenocorticotrophic hormone (ACTH) release by NPY has been thought to require the entire NPY molecule (Miura et al. 1992). While several studies have attempted to elucidate the "feeding" receptor, no such studies have attempted to find the NPY receptor involved in the mediation of ACTH secretion. The aim of the studies in this chapter is to characterise the NPY receptors that mediate both feeding and the increase in plasma ACTH by determining the effect of fragments of NPY with differential binding to the different NPY receptors on both feeding in rats and on the rise

in their plasma ACTH. The fragments used, and their binding characteristics are summarised in table 3.1. Mr David Morgan (in this laboratory) studied the binding affinities of several fragments and the results are summarised in Table 3.1. He has also studied NPY inhibition of adenylyl cyclase activity in SK-N-MC cells and has shown that [Pro34]NPY was as active as full length NPY in inhibiting adenylyl cyclase. NPY (13-36) and [Pro34]NPY(3-36) were inactive (Figure 3.2).

Legend to figure 3.2:

Inhibition of adenylyl cyclase activity in SK-N-MC cells by NPY and some of its analogues. SK-N-MC cells express the Y1 receptor. Isoprenaline was used to stimulate cAMP accumulation in these cells. This effect was inhibited both by full length NPY and by [Pro34]NPY but not by the fragments [Pro34]NPY (3-36) or by NPY (13-36).

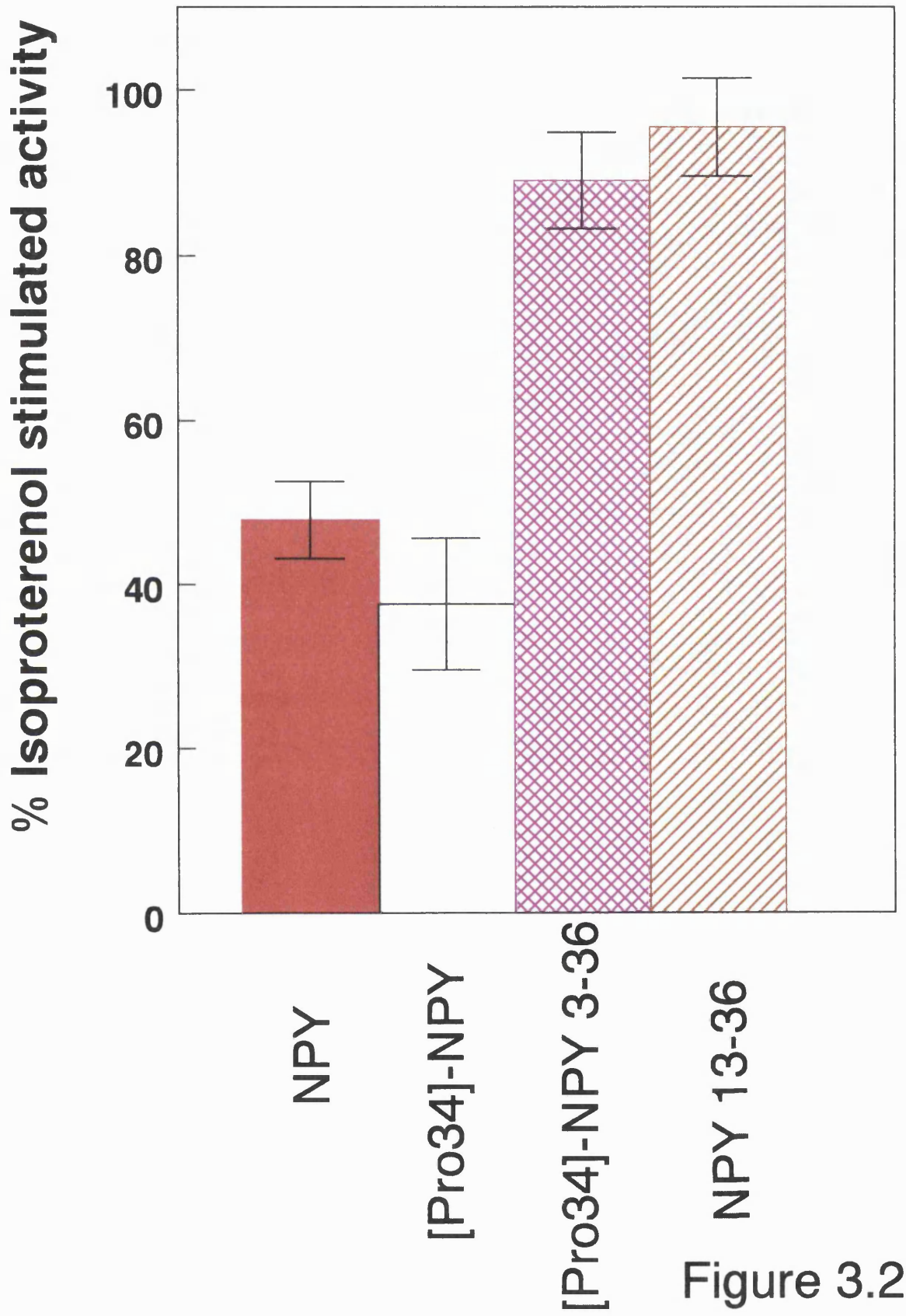

% Isoproterenol stimulated activity
100
80
60
40
20
0
NPY
[Pro34]-NPY
[Pro34]-NPY 3-36
NPY 13-36

Figure 3.2

Table 3.1.

Binding affinities for NPY fragments and analogues in cell membranes expressing Y1 and Y2 receptors.

NPY Fragment	**Y1 (IC_{50})** **(SK-N-MC cells)**	**Y2 (IC_{50})** **(SMS-KAN)**
NPY 1-36	0.5nM	0.5nM
NPY 2-36	1.6nM	1.2nM
NPY 3-36	14.4nM	1.2nM
NPY 13-36	9.6nM	2.8nM
Pro^{34} NPY	0.39nM	> 1000nM
Pro^{34} NPY 3-36	5.6 nM	> 1000nM
Pro^{34} NPY 13-36	4.9 nM	> 1000nM
BIBP 3226	31 nM	>1000 nM

These results are presented again in table 3.3 together with the results of the present chapter.

Table 3.2

Data from (Gerald et al. 1996).

Y1, Y2, Y4 and $Y5_{NAT}$ receptors were stably transfected into 293 cells and activation of each of the receptors was measured using the degree of inhibition of forskolin stimulated [cAMP] production.

Compound	Rat Y1	Rat Y2	Rat Y4	Rat $Y5_{NAT}$
Human NPY	0.14 ± 0.02	1.2 ± 0.2	>1000	0.96 ± 0.19
Porcine NPY	0.15 ± 0.01	2.7 ± 0.1	>1000	0.66 ± 0.15
Porcine NPY (2-36)	3.4 ± 0.9	1.6 ± 0.5	>1000	1.2 ± 0.4
Porcine NPY (3-36)	110 ± 30	2.4 ± 0.5	>1000	2.8 ± 0.8
Porcine NPY (13-36)	300 ± 70	2.2 ± 0.7	>1000	20 ± 3
Porcine [$Leu^{31}Pro^{34}$]NPY *	0.15 ± 0.02	>1000	7.1 ± 2.2	1.2 ± 0.3
Human [D-Trp^{32}] NPY	>1000	>1000	>1000	45 ± 12
Human PYY	0.70 ± 0.15	0.58 ± 0.05	>1000	1.0 ± 0.3
Human PYY (3-36)	>1000	0.64 ± 0.19	>1000	4.2 ± 1.3
Human [Pro^{34}]PYY	0.37 ± 0.11	>1000	6.0 ± 1.2	1.3 ± 0.4
Human PP	150 ± 10	>1000	0.037	1.4 ± 0.5
Rat PP	>1000	>1000	0.060	170 ± 30
BIBP 3226	4.2 ± 0.6	Inactive	Inactive	Inactive

*The substitution of Leucine for Isoleucine at position 31 does not seem to affect binding to Y1 receptors. In this thesis, only [Pro^{34}] NPY was used.

Experimental procedures

Cannulation, injection, food monitoring and decapitation were carried out as described in detail in chapter 2.

Experiment 1

Five separate groups of rats (n=14 to 18 per group) were studied simultaneously. Each group was studied at one dose for NPY, NPY(2-36), NPY(3-36), NPY(13-36), [Pro^{34}]NPY, and [Pro^{34}]NPY(3-36). The doses used were 0.24, 0.72, 2.4, 7.2, and 24 nmol. A dose of 50 nmol was also used for NPY(13-36). At each dose, each rat received each peptide.

Experiment 2.

Rats were injected ICV with NPY or its fragments (10μl) at the start of the light phase. Each group (n=6-7) was injected with 0.072, 0.72 and 7.2 nmol of NPY, NPY(3-36), [Pro^{34}]NPY, [Pro^{34}]NPY(3-36), NPY(13-36), [Pro^{34}]NPY(13-36), PYY or a peptide control (a random sequence of amino acids) (7.2nmol dose only). A pilot study revealed that the largest rise in plasma ACTH following ICV NPY injection occurred at 10 minutes. Rats were thus decapitated 10 minutes post injection and plasma ACTH was measured.

Experiment 3

To investigate the possibility that Y2, in addition to Y1 activation is needed for the full feeding effect of NPY, equivalent doses (based on the binding data) of the Y1 agonist [Pro ^{34}]NPY and the Y2 agonist NPY(13-36) were co-administered, and the feeding response was compared to that of NPY alone. There were six groups of rats (n=8 to 10 per group). Each received two injections two minutes apart and was studied on one occasion only. Doses of 7.2 nmol (NPY and [Pro^{34}] NPY and 14.4 nmol (NPY (13-36)) were used.

Experiment 4.

[D-Trp^{32}]NPY has been suggested to be a specific $Y5_{NAT}$ receptor agonist (see table 3.2 on page 44 and (Gerald et al. 1996)). To investigate the effects of [D-Trp^{32}]NPY on food intake and on NPY induced food intake, four groups of rats (n=17 per group) were given two ICV injections fifteen minutes apart. The first injection was either [D-Trp^{32}] NPY (12 nmol) or saline and the second was either NPY (2.4 nmol) or saline. Following ICV injection, 1 hour food intake was measured.

Experiment 5.

Following publication of the presence of the Y5 NAT receptor (Gerald et al. 1996), experiment 3 was repeated but with the following compounds in order to further characterise the NPY receptor that mediated the release of ACTH. The compounds used were: NPY, human PP, rat PP, NPY (19-36), NPY (1-24), desamido NPY and [DTrp^{32}] NPY.

Results.

Experiment 1.

Fig 3.3. shows a dose dependent stimulation of feeding by ICV NPY reaching a maximum at 7.2 nmol. NPY(2-36) and NPY(3-36) were as powerful as NPY. [Pro^{34}]NPY and [Pro ^{34}]NPY(3-36) demonstrated a similar reduction in maximum effect to 50% that achieved by NPY. Even 50 nmol NPY (13-36) caused a feeding response of only 2.5 ± 0.8 g.

Experiment 2

Fig 3.4. shows that all NPY fragments studied caused a significant rise in plasma ACTH compared to saline or the peptide control at both 0.72nmol ($F(7,42)=3.7:p<0.005$) and 7.2nmol ($F(7,36)=5.4:p<0.001$). The data from figures 3.3 and 3.4 is combined in figure 3.5 so that the effects of the different NPY analogues on feeding and ACTH secretion can be compared. The activation profile of the NPY receptor that mediates feeding is clearly different from the NPY receptor that mediates ACTH release.

Legend to figure 3.3

Dose responses of different NPY analogues and their effect on feeding.

Legend to figure 3.4

Dose responses of different NPY analogues and their effect on ACTH release.

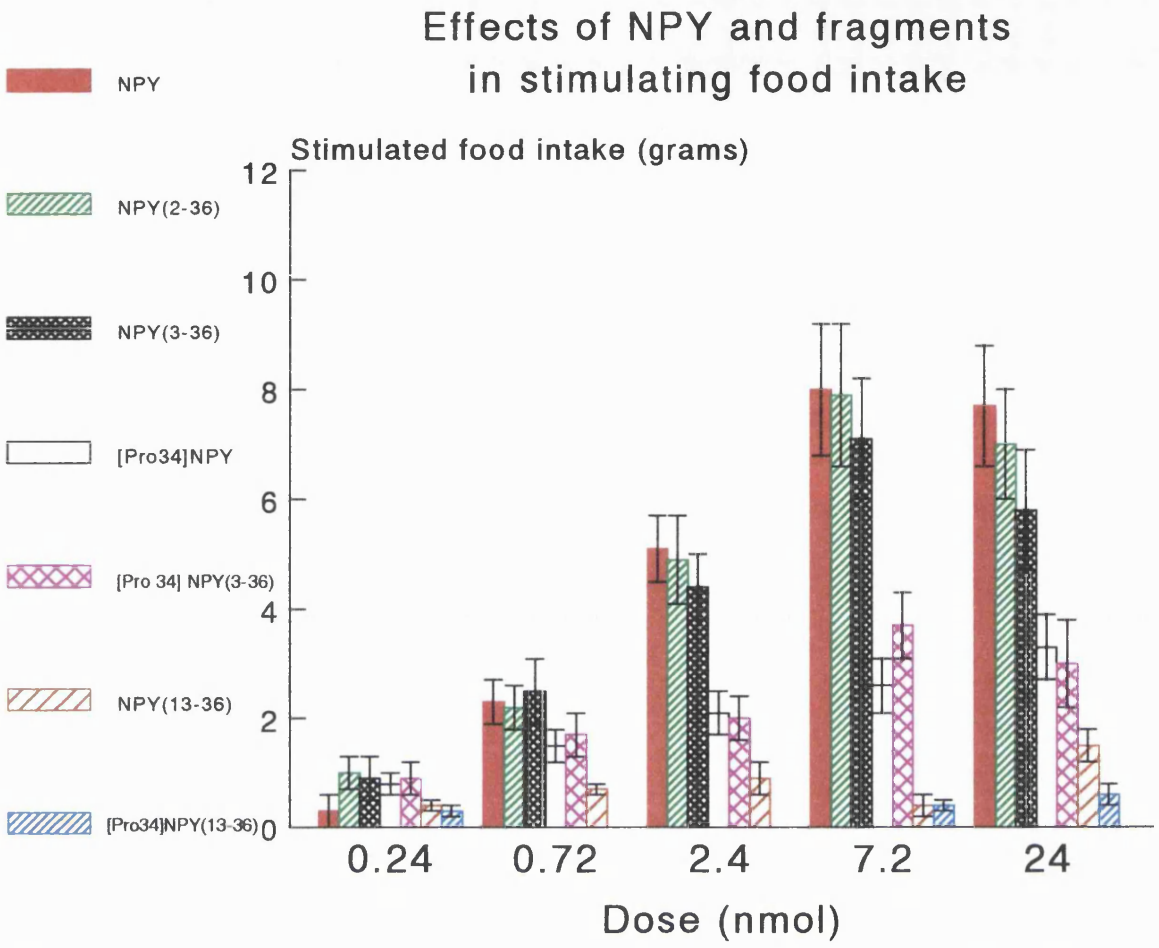


Figure 3.3

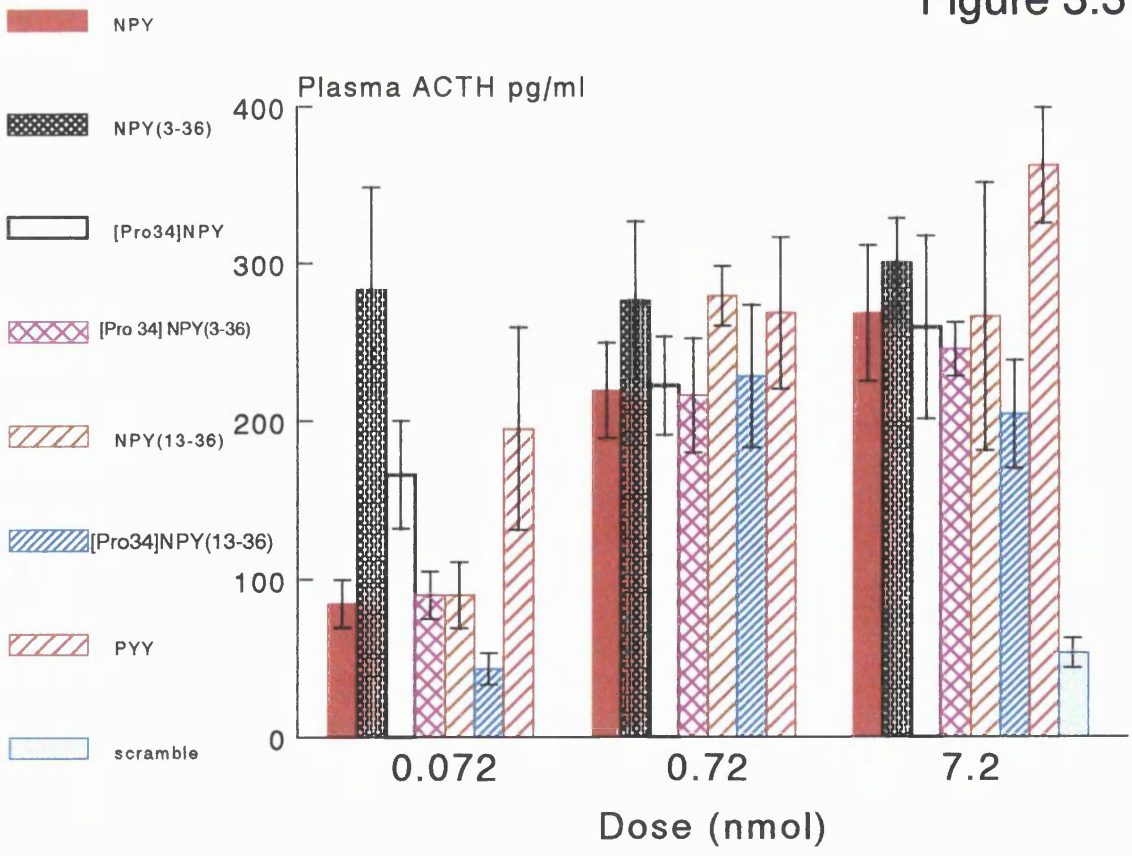


Figure 3.4

Legend to figure 3.5

The data from figures 3.3 and 3.4 have been combined to demonstrate the receptor activation profiles for the NPY receptors that mediate feeding and ACTH release. This shows that these receptors have clearly distinct activation profiles.

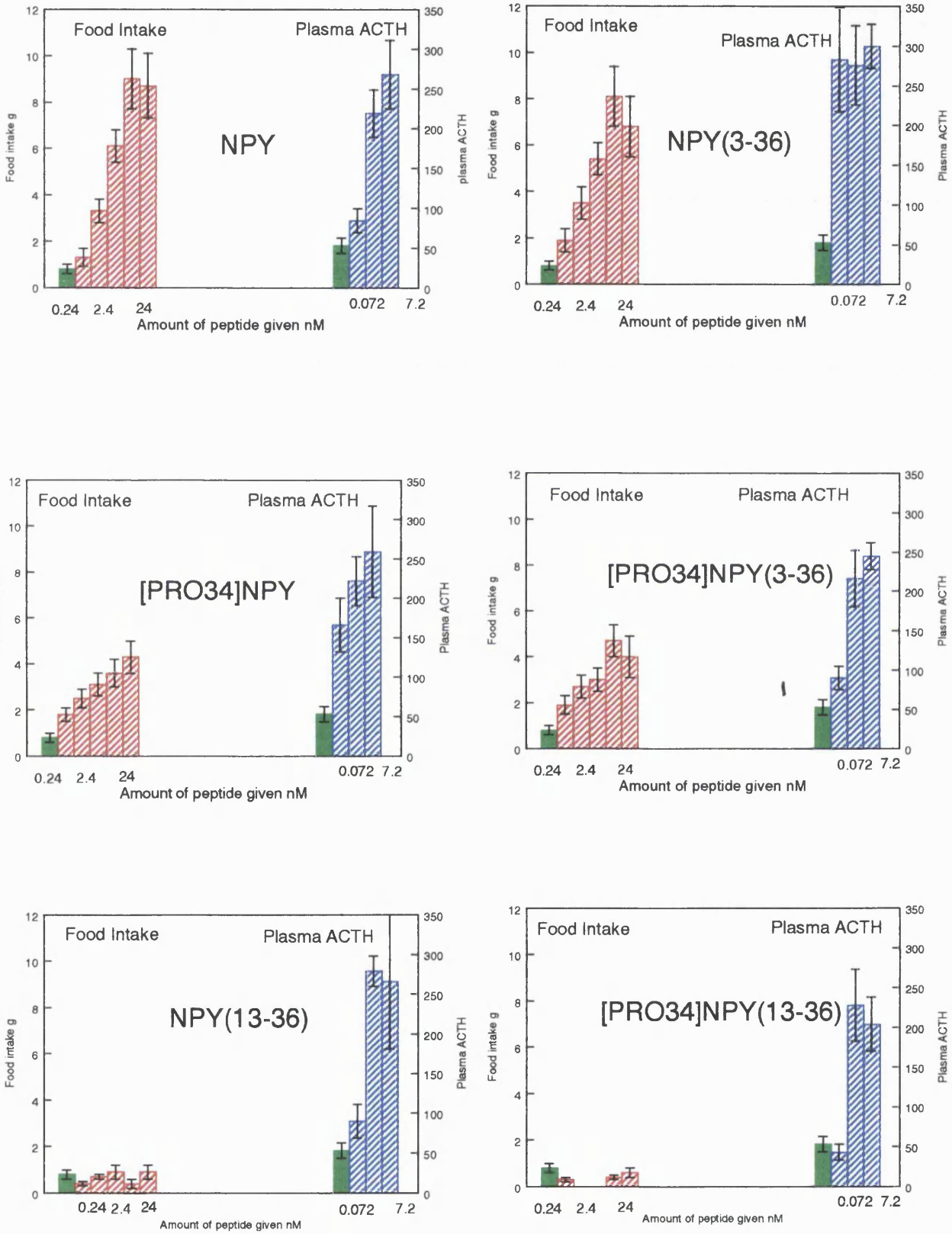

Food Intake
Plasma ACTH
NPY
Food intake g
plasma ACTH
0.24 2.4 24 0.072 7.2
Amount of peptide given nM
Food Intake
Plasma ACTH
NPY(3-36)
Food intake g
Plasma ACTH
0.24 2.4 24 0.072 7.2
Amount of peptide given nM
Food Intake
Plasma ACTH
[PRO34]NPY
Food intake g
Plasma ACTH
0.24 2.4 24 0.072 7.2
Amount of peptide given nM
Food Intake
Plasma ACTH
[PRO34]NPY(3-36)
Food intake g
Plasma ACTH
0.24 2.4 24 0.072 7.2
Amount of peptide given nM
Food Intake
Plasma ACTH
NPY(13-36)
Food intake g
Plasma ACTH
0.24 2.4 24 0.072 7.2
Amount of peptide given nM
Food Intake
Plasma ACTH
[PRO34]NPY(13-36)
Food intake g
Plasma ACTH
0.24 2.4 24 0.072 7.2
Amount of peptide given nM

Figure 3.5

Experiment 3

Fig 3.6 shows that addition of an equivalent dose of the Y2 agonist NPY(13-36) to the classical Y1 agonist [Pro^{34}]NPY does not alter the feeding response. Adding NPY(13-36) to NPY also has no effect.

The binding affinities of each of the compounds on Y1 and Y2 receptors is given in table 3.3 together with an inverse ratio which gives an estimate of the effect that each compound would have on a Y1 and a Y2 receptor if that receptor were mediating a biological effect.

The relative potencies for feeding and ACTH secretion are also given for direct comparison. This table makes it clear that neither the stimulation of feeding nor the stimulation of ACTH secretion is mediated either by a Y1 or a Y2 receptor.

NPY Fragment	**Y1 (IC_{50}) (SK-N-MC)**	**Ratio at Y1**	**Y2 (IC_{50}) (SMS-KAN)**	**Ratio at Y2**	**Potency on feeding**	**ACTH release**
NPY 1-36	0.5nM	1.0	0.5nM	1.0	1	1
NPY 2-36	1.6nM	0.3	1.2nM	0.4	1	1
NPY 3-36	14.4nM	0.03	1.2nM	0.4	0.90	1
NPY 13-36	9.6nM	0.05	2.8nM	0.18	0.05	1
Pro^{34} NPY	0.39nM	1.3	> 1000nM	<0.001	0.5	1
Pro^{34} NPY 3-36	5.6nM	0.09	> 1000nM	<0.001	0.5	1
Pro^{34} NPY 13-36	4.9nM	0.1	> 1000nM	<0.001	0.05	1

Experiment 4.

[D-Trp^{32}]NPY (12 nmol) did not stimulate feeding but did significantly inhibit NPY (2.4 nmol) induced feeding (Figure 3.7).

Legend to figure 3.6

The feeding effect with maximal stimulation of 7.2 nmol [Pro 34] NPY cannot be augmented with co administration of 14.4 nmol NPY (13-36). The feeding effect of NPY is thus not due to a combination of Y1 and Y2 stimulation.

Legend to figure 3.7

12 nmol [D-Trp^{32}]NPY did not stimulate feeding directly but it did inhibit feeding induced by 2.4 nmol NPY.

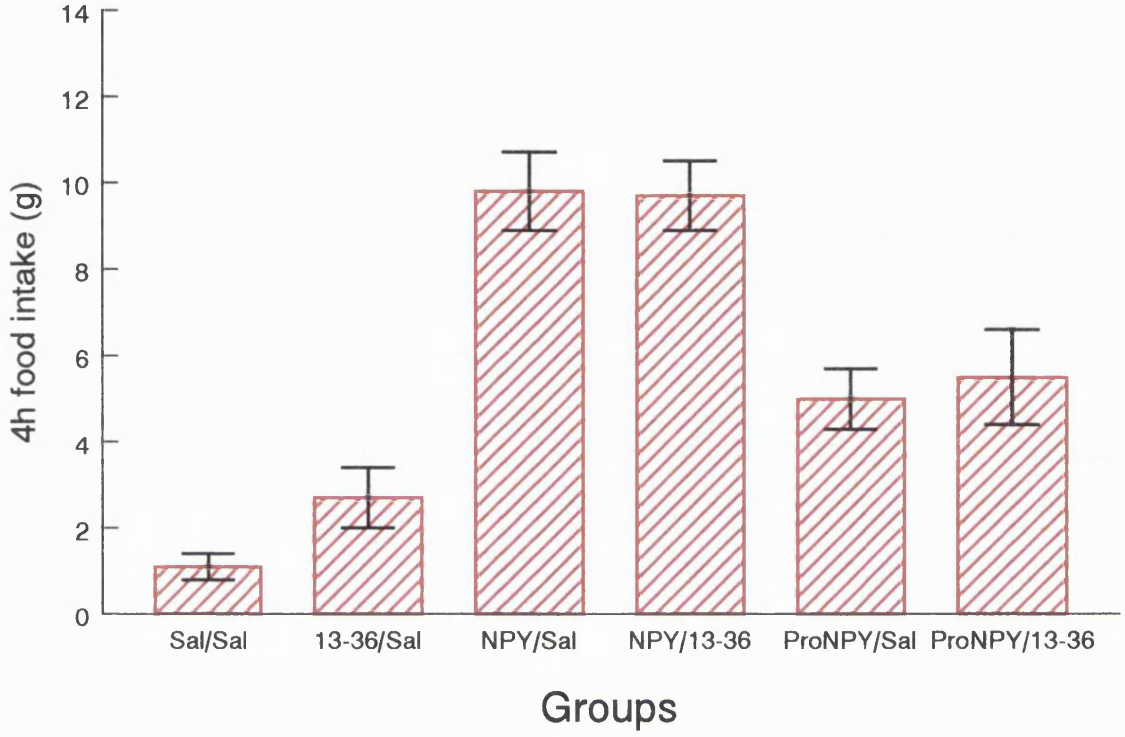


Figure 3.6

Effect of [D-Trp32] (12 nmol)
on NPY (2.4 nmol) induced feeding

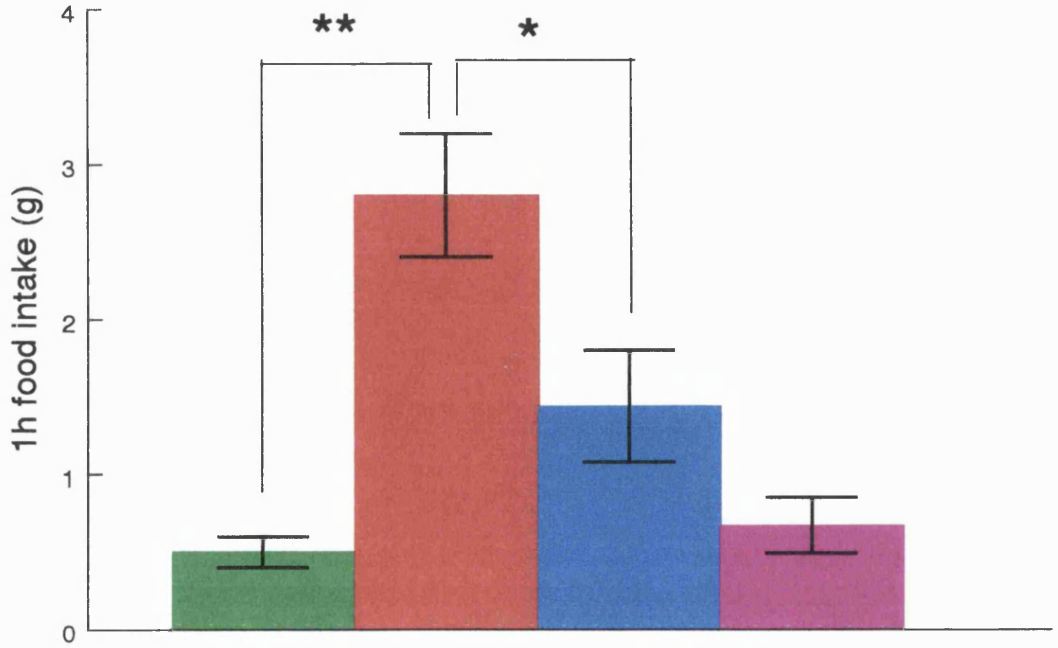


Figure 3.7

Experiment 5.

Following publication of the presence of the $Y5_{NAT}$ receptor, where the claim that this receptor was the "feeding" receptor had been made, further studies were carried out in order to determine whether this receptor was in fact the receptor that mediates ACTH secretion rather than the "feeding" receptor.

Figure 3.8 shows the effect of these compounds on plasma ACTH levels. NPY, [D-Trp32]NPY and human PP potently stimulate ACTH release. Rat PP and desamido NPY cause only a small increase in plasma ACTH when the highest dose (7.2 pmol) is used. The profile of the receptor that mediates ACTH secretion is similar to the $Y5_{NAT}$ receptor (compare figure 3.8 (page 53) with table 3.2 (page 44) which is from (Gerald et al. 1996)).

The possibility that the receptor that mediates ACTH release is not the Y5 receptor is suggested by the fact that I have found that D-Trp is as potent as NPY in stimulating ACTH release.

Legend to figure 3.8.

The effect of further NPY analogues on ACTH release in a separate experiment. Human PP but not rat PP potently stimulates ACTH release.

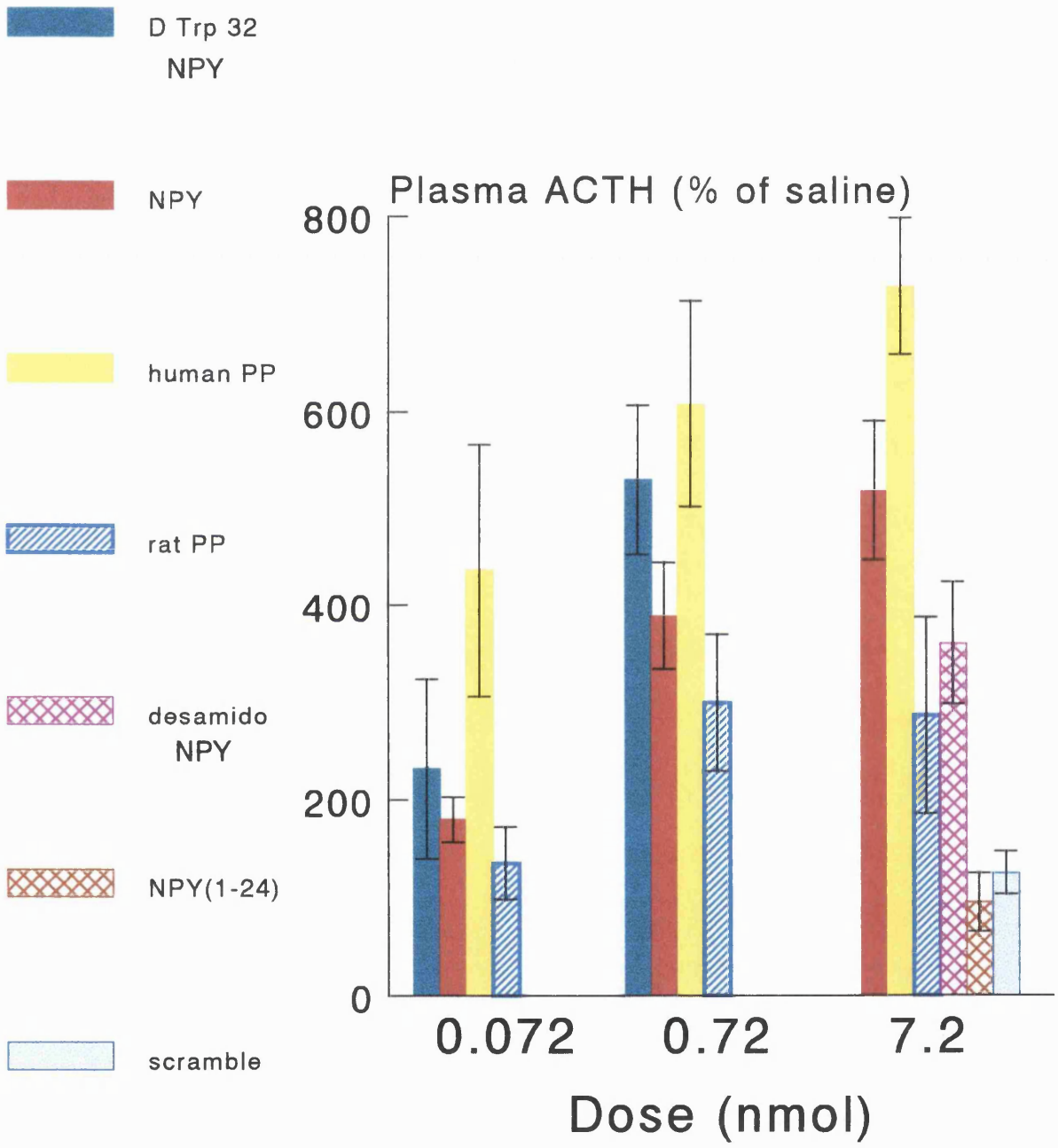

D Trp 32 NPY
NPY
human PP
rat PP
desamido NPY
NPY(1-24)
scramble
Plasma ACTH (% of saline)
800
600
400
200
0
0.072
0.72
7.2
Dose (nmol)

Figure 3.8

Discussion

NPY is the most potent physiological mediator of food intake described (Leibowitz et al. 1992; Lambert et al. 1993) and yet the receptor mediating NPY induced feeding has not been characterised. Before rational therapeutic manipulation of the NPY effect on feeding can be attempted, the pharmacology of the receptor mediating this effect must be fully characterised. Studies attempting to do this have so far given conflicting results. To identify the receptor that mediates NPY induced food intake, we have examined in vitro binding to NPY Y1 and Y2 receptors, and in vivo stimulation of feeding by several NPY analogues.

Y1 receptor activation with [Pro^{34}]NPY, a full agonist at this receptor (Grundemar et al. 1992), gives only 50% of the maximum feeding effect of NPY itself. In addition the C-terminal fragments NPY (2-36) and NPY (3-36), but not NPY (13-36) are as powerful as NPY itself at inducing feeding following ICV injection despite a marked reduction in their Y1 receptor affinities. Of these C-terminal fragments, NPY(13-36) is the most selective for activation of the Y2 receptor in SK-N-BE2 cells (Wahlestedt et al. 1990) and is the fragment which was used in the combination studies we performed here.

The dose response in our study, using Y1 and Y2 agonists, clearly shows that NPY, NPY (2-36) and NPY (3-36) all stimulate feeding equally across a range of doses up to maximum stimulation. This indicates that these compounds are equally potent and efficacious. I have also shown that ICV NPY(13-36), the classical Y2 agonist, has little effect on food intake.

[Pro^{34}]NPY and [Pro^{34}]NPY(3-36) differ from NPY and NPY(3-36) respectively in their greatly reduced affinities for the Y2 but not the Y1 receptor. The decreased feeding in response to both [Pro ^{34}] substituted analogues could thus be explained in two possible ways. Either Y2 receptor activation is essential for the full feeding effect of NPY, or the feeding response is mediated by a novel receptor at which [Pro^{34}]NPY and [Pro^{34}]NPY(3-36) are partial agonists.

Our third in-vivo study (experiment 3) was designed to distinguish between these two possibilities. Co-administration of the Y2 agonist NPY(13-36) with the Y1 agonist [Pro^{34}] NPY did not change feeding compared to [Pro^{34}]NPY alone. This strongly suggests that the feeding induced by NPY is independent of Y2 receptor activation.

Others have reported that NPY (2-36) is either more (Jolicoeur et al. 1991; Kalra et al. 1991) or less (McLaughlin et al. 1991) potent than NPY following central administration, but these widely quoted studies either failed to cover a dose range appropriate to the claims made or were technically flawed. The study by Jolicoeur et al. reports that NPY(2-36) is more potent than NPY (Jolicoeur et al. 1991) but fails to show a significant difference in stimulated food intake following ICV NPY and NPY(2-36). The repeated rectal temperature measurements made throughout the period of monitoring of food intake are likely to account for the poor responses to both NPY and NPY (2-36) in their study. Feeding responses were well below what we and others have demonstrated following equivalent doses of the same peptides (Kalra et al. 1991; Turton et al. 1996).

The highest dose of NPY or NPY (2-36) used by Kalra et al (Kalra et al. 1991) was 0.47 nmol, well below the dose that we found gave 50% of maximum stimulation of feeding. The comprehensive microinjection study by Stanley et al (Stanley et al. 1992) demonstrates that NPY(2-36) is more potent at lower doses than NPY but maximum response to either compound was not assessed. The proposed role for the Y1 receptor in mediating the feeding effect of NPY is based on studies in which only lower doses have been assessed (Kalra et al. 1991; Stanley et al. 1992). Our study clearly shows that at maximum stimulation the Y1 receptor accounts for only 50% of the feeding effect of NPY although at lower doses, this difference is not apparent. While McLaughlin et al claim that NPY and NPY (2-36) stimulate feeding by 486% and 219% respectively, they did not demonstrate any significant difference between these two (McLaughlin et al. 1991). The percentage difference is fully accounted for by a difference in the control groups in their study and the absolute amounts eaten are virtually identical. The 2 hour food intake in animals given 5.0 nmol NPY was 12.6 g compared with 12.2 g in those given 5.0 nmol NPY (2-36).

The $Y5_{NAT}$ receptor is stimulated by NPY (13-36) with an EC_{50} of 20 nM and by [D-Trp^{32}] NPY with an EC_{50} of 45 nM (see page 44 and (Gerald et al. 1996)). We have found only minimal stimulation of feeding with 50 nmol NPY (13-36). In addition, we have shown that 12 nmol ICV [$DTrp^{32}$] NPY does not stimulate feeding, and that it inhibits NPY induced feeding. Direct injection of 2400 pmol [D-Trp^{32}]NPY into the PVN has been shown to significantly inhibit feeding induced by a PVN injection of 240 pmol NPY, and to have no effect on feeding when given alone (Balasubramaniam et al. 1994).

The results in experiments 2 and 5 demonstrate that the NPY receptor controlling the activation of the HPA axis is unlike any previously described with NPY, PYY, NPY(13-36) , NPY(3-36) and [Pro^{34}]NPY all stimulating ACTH release to a similar degree (figure 3.4). The two novel NPY fragments, [Pro^{34}]NPY(3-36) and [Pro^{34}]NPY(13-36), are as effective as NPY despite showing greatly reduced binding affinity for the Y1 and Y2 receptor. The Y3 receptor has a low affinity for PYY (Dumont et al. 1994; Wahlestedt et al. 1992) and the Y4 receptor has a low affinity for full length NPY and NPY(13-36) (Lundell et al. 1995; Bard et al. 1995). Thus the receptor that mediates ACTH release is distinct from the four recognised (Y1-Y4) receptors and from the feeding receptor, which is not stimulated by the novel fragment [Pro^{34}]NPY(13-36). The $Y5_{JBC}$ receptor does not bind human PP which both stimulates feeding (Clark et al. 1984) and, as can be seen from experiment 5, also stimulates ACTH secretion.

Experiment 5 suggests that the stimulation profile of the $Y5_{NAT}$ receptor fits the profile of the NPY receptor that mediates ACTH release rather than the feeding receptor. In addition, this study demonstrates clearly that the feeding and ACTH effects of NPY involve distinct NPY receptors. [D-Trp^{32}]NPY stimulates the $Y5_{NAT}$ receptor (EC_{50}=45nM) and potently stimulates ACTH release. Rat PP is a poor agonist at the $Y5_{NAT}$ receptor (EC_{50}=170nM) and poorly stimulates ACTH release whereas human PP is a better agonist (EC_{50}=1.4 nM) and very effectively stimulates ACTH release. The "feeding" receptor has thus still not been identified.

Current pharmacological strategies are aimed at developing antagonists to the classical Y1 receptor (Rudolf et al. 1994), a receptor that mediates several effects of NPY

including blood pressure and anxiolysis (Kirby et al. 1995). Rational therapeutic manipulation will only be possible with the development of an antagonist to the appetite specific receptor, that does not affect NPY induced HPA axis activation. The dose of NPY and its fragments needed to affect ACTH release is much smaller than that required to stimulate feeding. A dose of 0.72 nmol ICV only minimally stimulates feeding but had an almost maximal effect on ACTH release. Approximately ten times as much ICV NPY is needed for the stimulation of food intake than for the activation of the HPA axis. It seems unlikely that this result is due to differential diffusion to the site of action in the two systems, since NPY is thought to stimulate both systems through the PVN (Liposits et al. 1988; Stanley and Leibowitz, 1985). This has implications for the development of receptor antagonists that manipulate these systems, especially as a tool for the modulation of food intake. An NPY antagonist that affected both feeding and ACTH secretion may not be clinically useful as ACTH secretion is affected at a much lower dose than the desired effect on appetite. Since we have shown that the NPY receptor that mediates ACTH release and the NPY receptor mediating food intake are distinct, it may be possible to target blockade these systems independently.

ICV infusion of NPY but not NPY(13-36) has been shown to profoundly affect two other important pituitary hormones, namely growth hormone (GH) and luteinising hormone (LH) in both male (Pierroz et al. 1996) and female (Catzeflis et al. 1993) rats. Chronic injection of NPY was shown to profoundly inhibit both the somatotrophic and the gonadotrophic axes with complete abolition of GH pulsatility, a fall in plasma IGF-1, a reduction in circulating LH, delayed sexual maturation in female rats and disruption of oestrous cyclicity. In addition, chronic NPY infusion produced a highly significant

decreases in seminal vesical weight, testis weight and plasma testosterone (Pierroz et al. 1996) and atrophy of the ovary (Catzeflis et al. 1993). Acute administration of NPY ICV decreases plasma concentrations of LH in ovariectomized, hormonally untreated rats but stimulates LH release in ovariectomized rats pretreated with oestradiol benzoate and progesterone. The analogue [$Leu^{31}Pro^{34}$]NPY, also elicited the dual response, but NPY(13-36) was completely inactive. It was concluded that the effects of NPY on the secretion of LH is mediated by a Y1 receptor (Kalra et al. 1992), although further studies with other analogues of NPY are now needed to confirm this. The fact that NPY(13-36) was completely inactive suggests that the receptor that affects these axes is distinct from the receptor that mediates ACTH release.

NPY was first identified as a neuropeptide with widespread distribution in the mammalian brain (Adrian et al. 1983; Allen et al. 1983). It has subsequently been established as a highly potent regulator of a number of physiological systems including effects on pituitary prolactin gene expression (Garcia de Yebenes et al. 1995), and on LH secretion (Catzeflis et al. 1993; Pierroz et al. 1996). It is possible that there are several further NPY receptors that subserve these functions. The full implications of this discovery for the management of disorders of feeding, growth, and reproductive function will only become clear with the generation of receptor selective agonists and antagonists.

Chapter 4

The effect of BIBP 3226, a specific non peptide NPY Y1 antagonist on feeding and NPY induced ACTH release.

The work in this chapter was done in collaboration with Mr. Omar Haddo, a BSc. student who carried out a three month project under my supervision at the RPMS. The effect of (R)-N^2-(diphenylacetyl)-((4-hydroxyphenyl) methyl)- argininamide), or BIBP 3226 on feeding and ACTH secretion in vivo was investigated.

(R)-N^2-(diphenylacetyl)-((4-hydroxyphenyl) methyl)- argininamide), or BIBP 3226, is thought to be the first potent non-peptide NPY antagonist, specific for the Y1 receptor. It has been shown to inhibit the increase in intracellular calcium, induced by NPY, and to displace ^{125}I-NPY with high affinity (Ki=7nM) from the human NPY Y1 receptor (Rudolf et al. 1994). BIBP 3226 has also been shown to inhibit the contractile effect of NPY in the rabbit saphenous vein (a Y1 effect) but to fail to antagonise the biological effects of NPY in the rat vas deferens (Y2) and the rat colon (putative Y3) (Jacques et al. 1995).

In view of the finding that the effects of NPY on feeding and on the activation of the HPA axis are mediated by two novel NPY receptors, we investigated the ability of BIBP 3226 to block the effect of exogenously administered NPY to stimulate food intake and ACTH secretion. In order to ascertain that any effect on feeding was not due to a simple toxic effect making the animals feel ill and hence not eat, we also investigated the effect of BIBP 3226 on galanin and noradrenaline induced food intake. Both of these compounds also stimulate feeding when administered ICV (Wellman et al. 1993; Allen et al. 1985; Schick et al. 1993; Tempel et al. 1988).

As mentioned in chapter 3 (see page 34), the latency to feeding with NPY is surprisingly long, namely 10 minutes after ICV injection (Clark et al. 1985; Corp et al. 1990; Sahu et al. 1988). The feeding response to NPY is completed by two hours. Galanin is a 29-amino acid peptide which stimulates feeding seven minutes after ICV administration (Schick et al. 1993). The effect is over by 40 minutes. Galanin stimulates fat intake preferentially (Tempel et al. 1988). Noradrenaline stimulates food intake by binding to α2-adrenoreceptors (Wellman et al. 1993). Its effect starts within three minutes and lasts up to one hour (Allen et al. 1985), with a marked preference for carbohydrate in the diet (Welch et al. 1994).

Materials and methods.

For animals, surgery, peptides and infusions see chapter 2.

Preparation of BIBP.

Receptor binding studies carried out by Mr. David Morgan and Mr. Omar Haddo in this laboratory have shown that to compete for ^{125}I-PYY binding to the Y1 receptor, approximately 60 times the molar concentration of BIBP 3226 was required. (NPY Kd = 0.5nM and BIBP 3226 Kd = 31nM at the Y1 receptor using SK-N-MC cells). This property was not exhibited at the Y2 receptor (NPY Kd =0.5nM and BIBP 3226 Kd > 1000nM; using SMS-KAN cells).

Due to the low solubility of BIBP 3226 in normal saline (our standard injection vehicle for ICV studies) we have used BIBP 3226 dissolved in 70% ethanol in all studies. Initial studies were carried out to examine the toxicity both of BIBP 3226 itself, and of the

70% ethanol vehicle. BIBP 3226 dissolved in 70% ethanol was given at high dose (120 nmol) to four animals, and at medium dose (60 nmol) to two animals. Three animals were given 70% ethanol vehicle alone.

Rats receiving high dose (120 nmol) BIBP 3226 initially barrel-rolled. Subsequently decreased grooming, rearing and movement was observed, lasting for a total of 20 minutes. The second group injected with a medium dose (60 nmol) of BIBP 3226 displayed decreased movement lasting 5 minutes. The control group showed no unusual behavioural response to an ICV ethanol injection.

These results dictated that 60 nmol (27 μg) of BIBP 3226 was the maximum dose to be administered ICV in the following studies. The half maximal stimulation of NPY induced feeding occurs at a dose of approximately 1.2 nmol NPY.

Experimental detail and Results.

1. The effect of BIBP on NPY induced feeding.

Two studies were initially carried out to investigate the effects of BIBP 3226 on NPY. Each experiment was composed of four groups, each receiving two injections according to the following plan:

Injection protocol #1	1st Injection	2nd Injection
injection time	0 minutes	2 minutes
vehicle	70% ethanol	saline
Group 1	-	-
Group 2	-	NPY
Group 3	BIBP 3226	NPY
Group 4	BIBP 3226	-

The first experiment involved giving rats 60 nmol BIBP 3226 or ethanol vehicle followed within one minute by 1.2 nmol NPY or saline vehicle (fig. 4.1). A significant increase in feeding was observed with NPY injection as expected ($p<0.05$). This was almost entirely blocked by pre-injection of BIBP 3226 ($p<0.05$). Injection of BIBP 3226 alone had no significant effect on feeding.

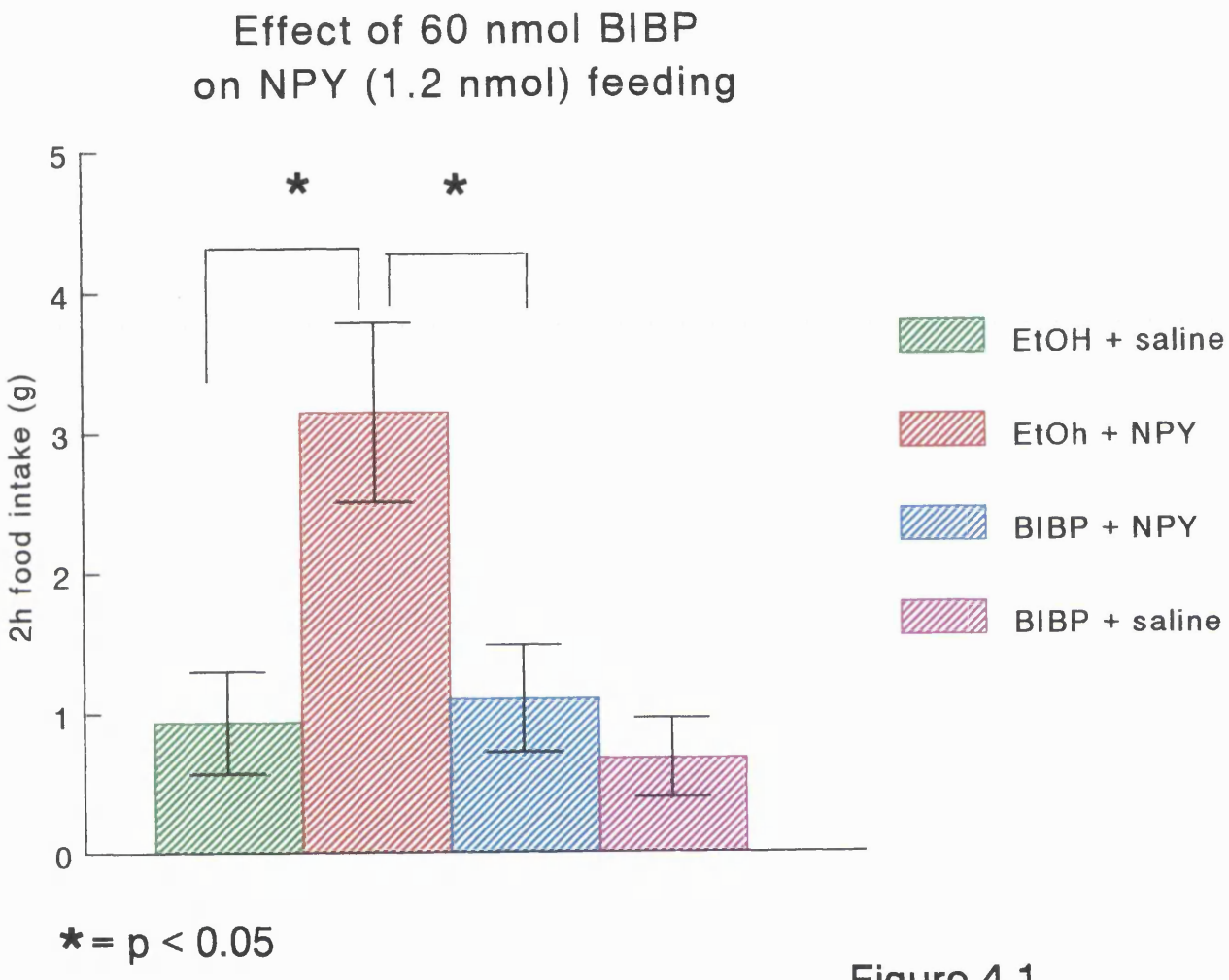


Figure 4.1

Legend to figure 4.1.

Following ICV injection, 60 nmol BIBP 3226 significantly reduced the feeding effect of 1.2 nmol NPY. The BIBP 3226 was injected immediately before the NPY.

In this experiment we still observed some adverse behavioural effects of BIBP 3226 . Since the dose used in this experiment seemed sufficient to completely block the effect of NPY, it was decided to try a lower dose. The second experiment employed 30 nmol BIBP 3226 against the same dose of 1.2 nmol NPY (fig. 4.2). In this experiment a much smaller, non-significant decrease in feeding was seen following BIBP 3226 administration (P=0.357). However, the same behavioural problems were seen as in the previous experiment. Since the behavioural effects were short lived, it was decided to increase the interval between administration of BIBP 3226 and NPY in order to avoid behavioural interference. By doing this, it was hoped that any non-specific effects of BIBP 3226 might have disappeared before NPY injection, whilst specific binding to Y1 receptors might still be able to block NPY induced feeding. Hence a third experiment was carried out using the same concentrations of NPY and BIBP 3226 as the second experiment, but introducing a 30 minute inter-injection interval (fig. 4.3). Since no effects of BIBP 3226 alone on feeding had been seen in the previous experiments, it was also decided to omit this group from the experiment to achieve larger numbers in the other groups.

Injection protocol #2	1st Injection	2nd Injection
injection time	0 minutes	30 minutes
vehicle	70% ethanol	saline
Group 1	-	-
Group 2	-	NPY
Group 3	BIBP 3226	NPY

Legend to figures 4.2 and 4.3

30 nmol BIBP 3226 did not significantly effect NPY induced feeding unless a 30 minute interval was left between injections.

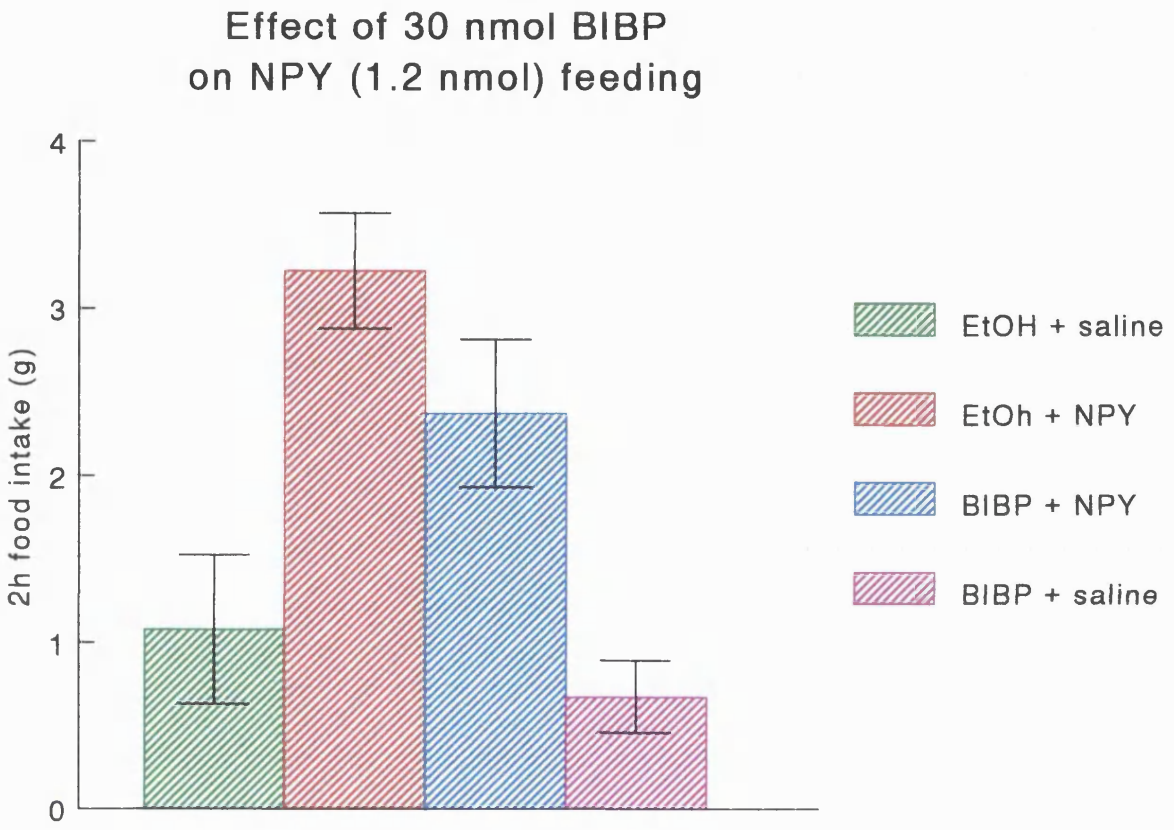


Figure 4.2

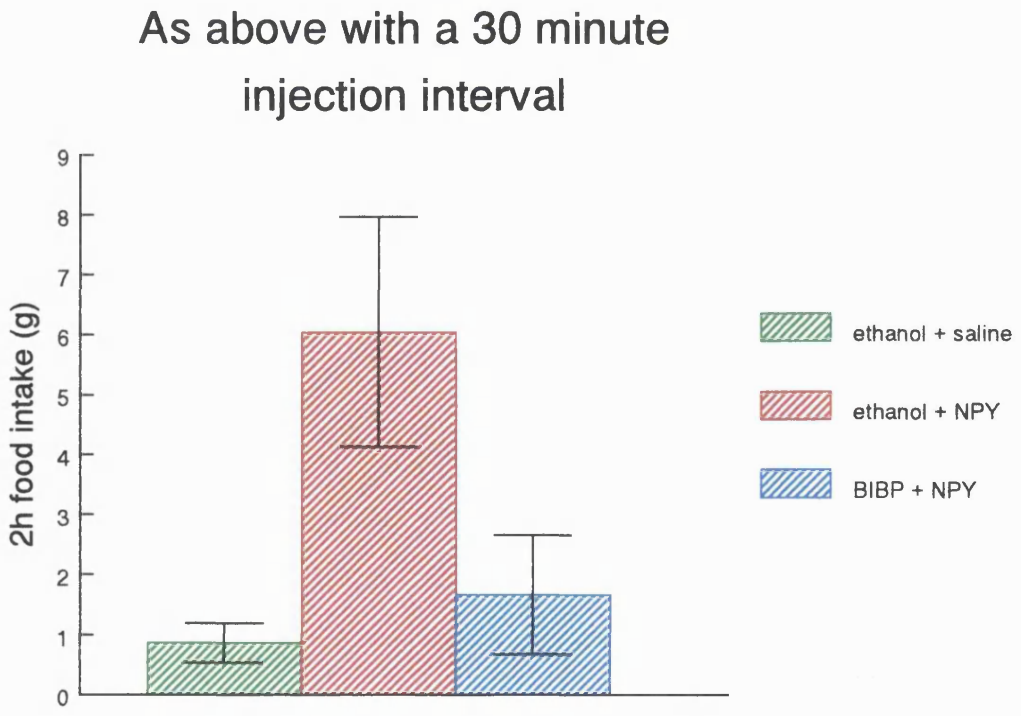


figure 4.3

In this experiment, although a large difference was seen between NPY treated animals and both vehicle treated and BIBP 3226 /NPY treated groups, the 95% confidence limit was not reached due to the small number of animals involved in the experiment ($p=0.152$). Although in only one of the above experiments is a significant reduction in feeding achieved, BIBP 3226 consistently reduces NPY induced feeding.

The effect of BIBP on galanin induced feeding.

Injection protocol #3	1st Injection	2nd Injection
injection time	0 minutes	30 minutes
vehicle	70% ethanol	saline
Group 1	-	-
Group 2	-	GALANIN
Group 3	BIBP 3226	GALANIN

Contrary to our expectations, we found in this experiment that 30 nmol BIBP 3226 was able to completely block the stimulation of food intake by 3 nmol galanin (Fig 4.4; $p<0.001$). To ensure that BIBP 3226 was not in fact acting via the galanin receptor, I investigated the ability of BIBP 3226 to compete for the binding of ^{125}I-Galanin to membranes prepared from RIN 5AH cells. This cell line is derived from a rat insulinoma, and we are presently involved in characterising galanin receptors present on it. No significant difference in ^{125}I-Galanin binding in the presence or absence of 1 μM BIBP 3226 was found (8152 ±191.75 vs 8937.75 ±186.5 counts per minute). In the presence of 200 nM galanin, however, binding was reduced by 92 % (701.25 ± 15.5 counts per minute). This suggests that BIBP 3226 is not interacting directly with the galanin receptor to prevent activation.

Legend to figure 4.4.

30 nmol BIBP 3226 significantly blocked galanin induced feeding.

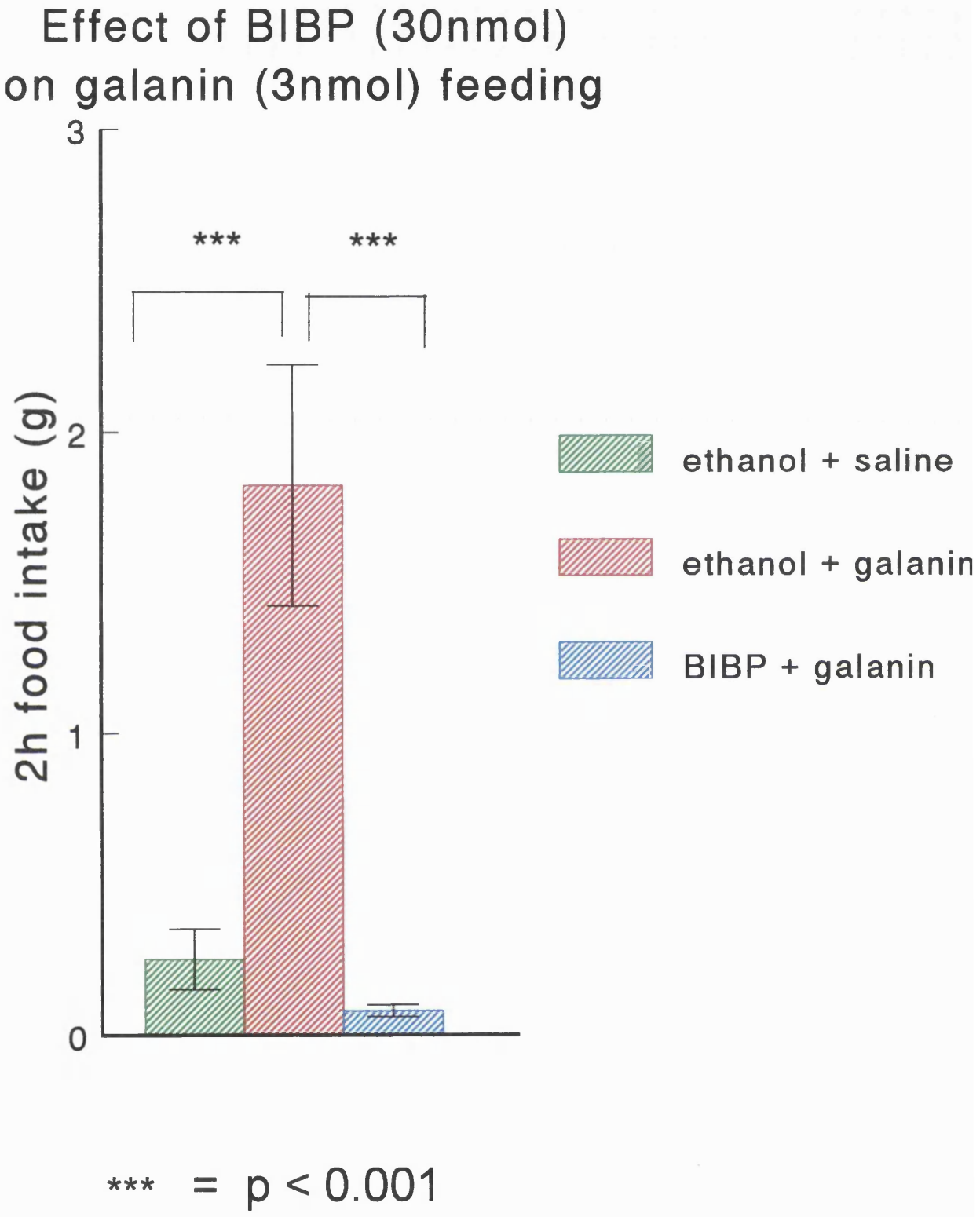

Effect of BIBP (30nmol)
on galanin (3nmol) feeding
2h food intake (g)
3
2
1
0
***
***
ethanol + saline
ethanol + galanin
BIBP + galanin
*** = p < 0.001

Figure 4.4

The effect of BIBP on noradrenaline induced feeding.

To further study the effects of BIBP 3226 on inhibition of feeding stimuli, we decided to look at its effect on noradrenaline stimulated food intake. Again a 30 minute interval was allowed between injections.

Injection protocol #2	1st Injection	2nd Injection
injection time	0 minutes	30 minutes
vehicle	70% ethanol	saline
Group 1	-	-
Group 2	-	NORADRENALINE
Group 3	BIBP 3226	NORADRENALINE

Again we found that BIBP 3226 was able to block feeding (fig. 4.5), reducing noradrenaline-induced food intake by almost 75 % ($p<0.005$).

Legend to figure 4.5

30 nmol BIBP 3226 significantly blocked noradrenaline induced feeding.

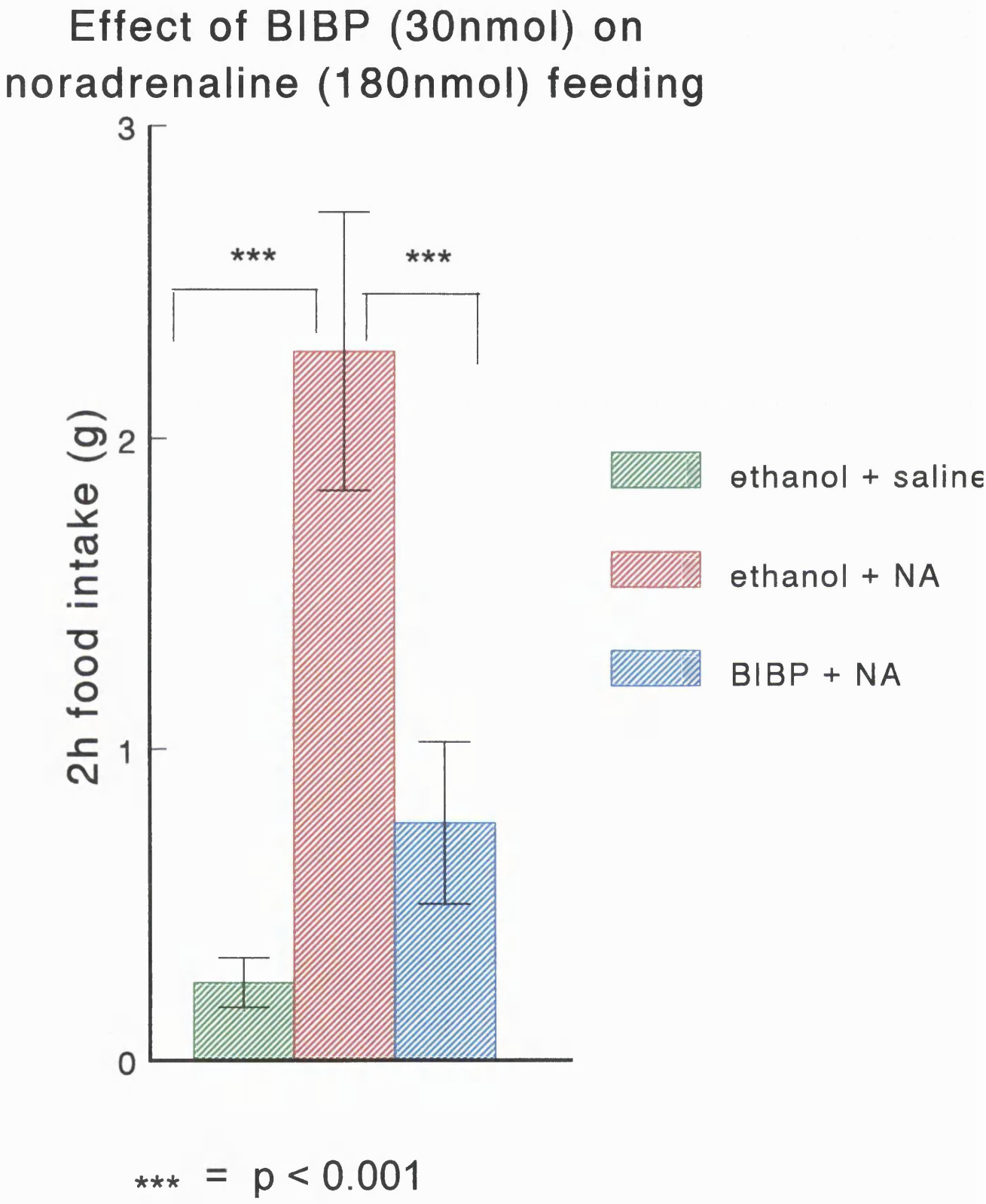

Effect of BIBP (30nmol) on noradrenaline (180nmol) feeding
2h food intake (g)
0
1
2
3
***
***
ethanol + saline
ethanol + NA
BIBP + NA
*** = p < 0.001

Figure 4.5

Effects of BIBP on NPY stimulated ACTH release

We now know that the NPY receptor that mediates ACTH release is different from that which mediates feeding, and that neither is necessarily a Y1 receptor. If NPY induced ACTH secretion is mediated by a Y1 receptor, we would expect this to be blocked by the administration of BIBP-3226. From the NPY fragment studies in chapter 3, I expected ACTH release to be mediated by the $Y5_{NAT}$ receptor, which does not bind BIBP 3226. The purpose of this study was thus to determine whether NPY induced ACTH release could be inhibited by BIBP 3226. As we had seen extraneous behavioural effects in previous experiments, we felt it possible that BIBP 3226 could be having non-specific toxic effects. Such effects might be expected to cause a stress response in the animals and, associated with this, a rise in plasma ACTH. ACTH was measured as detailed in chapter 2. The protocol for this study was identical to the first NPY study.

As can be seen from figure 4.6, the rise in ACTH caused by NPY (20μg) is not blocked by BIBP(30 nmol). In addition, BIBP alone does not cause an increase in ACTH.

These results are consistent with the possibility that the $Y5_{NAT}$ receptor mediates ACTH secretion.

Legend to figure 4.6

ICV injection of 30 nmol BIBP 3226 had no effect on NPY induced stimulation of ACTH release. BIBP alone had no effect on ACTH release.

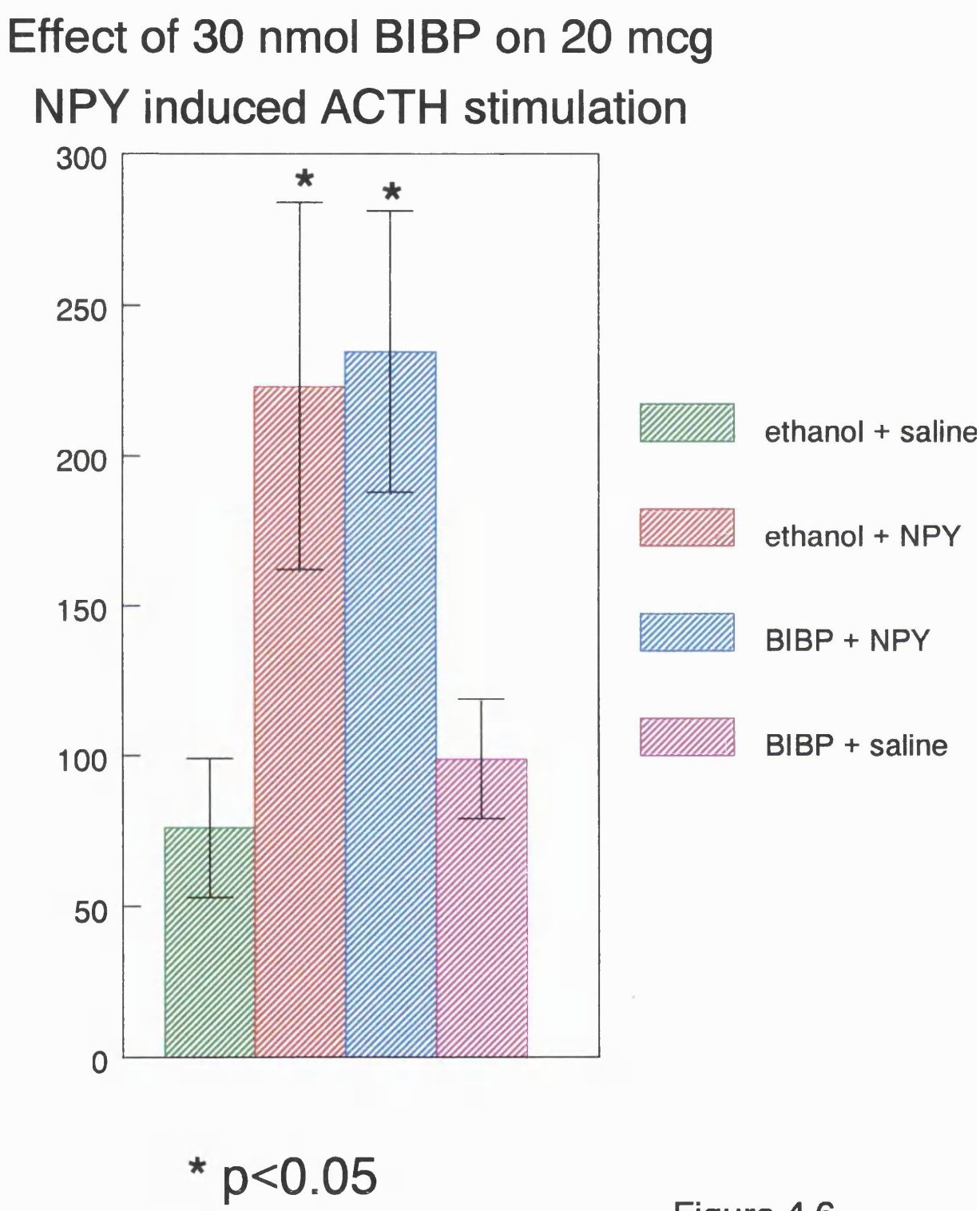

Effect of 30 nmol BIBP on 20 mcg
NPY induced ACTH stimulation
300
250
200
150
100
50
0
*
*
ethanol + saline
ethanol + NPY
BIBP + NPY
BIBP + saline
* p<0.05

Figure 4.6

Discussion

These results show that, as well as inhibiting the feeding response to NPY, BIBP 3226 inhibits galanin and noradrenaline induced feeding. This is an unexpected result since BIBP 3226 is a specific NPY Y1 receptor antagonist (Jacques et al. 1995; Rudolf et al. 1994). We have also shown that BIBP 3226 is unable to bind to galanin receptors on RIN 5AH cell membranes, suggesting that its effect on galanin induced feeding at least, is not a direct, receptor mediated one. There are three possible explanations for the nonspecificity of the action of BIBP 3226 on feeding:

1) We have shown that BIBP 3226 injection causes considerable side effects apart from inhibition of feeding. We have observed a period of barrel rolling as well as decreased grooming, rearing and locomotor activity, lasting approximately ten minutes after the injection of BIBP 3226 . Whether these are nonspecific toxic effects, or whether they are indicative of inhibition of tonic Y1 receptor activation in other systems is unclear. In either case it is possible that inhibition of ingestive behaviour is a symptom of the same effect.

2) It is possible that a low level of tonic NPY receptor activation is required for the stimulation of food intake by galanin and noradrenaline. This might be part of the same pathway(s) as activated during the stimulation of feeding by NPY, or an entirely separate pathway. In either case, inhibition of such tonic activation by an NPY receptor antagonist such as BIBP 3226 might block the effect of galanin or noradrenaline on feeding.

3) It is possible that NPY lies on the same pathway to the activation of feeding as galanin and noradrenaline. Hence, blockade of NPY receptor activation by BIBP 3226 would stop the signal propagated by either galanin or noradrenaline from reaching its target and stimulating food intake.

Since α_2-adrenergic activation is necessary for galanin to increase food intake (i.e. the increase in feeding seen with galanin can be blocked using α_2-adrenergic antagonists (Kyrkouli et al. 1990)) it seems likely that galanin and noradrenaline are at least partially on the same pathway. However there is less evidence for NPY sharing a common pathway with either. This connection would be hard to demonstrate if NPY lies in distal to noradrenaline, since blockade of noradrenaline action might not be expected to affect components of the pathway downstream.

The ACTH study was carried out in order to find out whether the NPY induced rise in ACTH, can be blocked with BIBP 3226. If BIBP was toxic, it is likely that it would cause a rise in ACTH because of the stress of toxicity. In this study we have shown that ACTH levels are not suppressed by BIBP, whereas feeding with any of the stimuli we used was blocked. It is possible that the dose of BIBP we used was not sufficient to block the NPY induced rise in ACTH as the effect of NPY on ACTH occurs at a dose ten times less than that required to see a definite effect on feeding, and I should probably have used a smaller dose of NPY in this study.

Future studies involving BIBP should involve cannulation of the paraventricular nucleus itself. This is technically more difficult than cannulating the third ventricle but there are

two advantages. The total dose of BIBP administered locally will be much smaller than the dose needed for the third ventricle. If BIBP is toxic, the smaller dose should will reduce the chances of barrel rolling. In addition, receptors outside the PVN will not be affected, again reducing the chances of barrel rolling. Studying the effects of BIBP on other central actions of NPY will give further insight into the specificity of the action of BIBP. In particular the suppression of LH secretion induced by NPY is proposed to be a classical Y1 mediated phenomenon (Catzeflis et al. 1993; Pierroz et al. 1996; Kalra et al. 1992). If this is the case and BIBP is a selective Y1 antagonist, then BIBP should block the fall in LH induced by NPY.

In conclusion, these results are consistent with my hypothesis and findings (chapter 3) that the receptors mediating the feeding effect of NPY and the rise in ACTH are different, and in particular, that the NPY receptor that mediates the rise in ACTH is not a Y1 receptor, but perhaps the $Y5_{NAT}$ receptor.

Chapter 5.

Does Intracerebroventricular Cobalt Protoporphyrin specifically inhibit feeding by affecting the Neuropeptide Y system?

The work in this chapter was carried out in association with Dr. Sang Jeon Choi who was studying for an M. Phil. under my supervision at the RPMS. We were investigating the mechanism whereby cobalt protoporphyrin (CoPP) affects feeding.

The search for receptor antagonists to the potent feeding stimulatory effect of NPY has been extensive and to date one of the best candidates is BIBP as discussed in the previous chapter. CoPP, a synthetic metalloprotoporphyrin, has been reported to cause a dramatic and prolonged reduction in food intake. It induces a significant weight loss in normal and genetically obese rats, (Galbraith and Kappas, 1990; Galbraith et al. 1987; Galbraith and Kappas, 1989; Galbraith and Kappas, 1991; Galbraith and Kappas, 1991; Galbraith et al. 1992; Galbraith et al. 1992) and beagle dogs (Galbraith and Kappas, 1991) when administered subcutaneously or ICV but its mechanism of action is unknown. The weight loss caused by CoPP has two stages (Galbraith et al. 1992). There is an initial 2 to 3 days of severe hypophagia and weight loss, followed by a gradual return to normal food intake. The difference in body weight persists for as long as 300 days after ICV injection (Galbraith and Kappas, 1991), implying that animals defend a new body weight following CoPP injection. The likely site of action of CoPP on body weight and food intake is the hypothalamus. When CoPP is given ICV, only 1% of the systemic dose is required for an equivalent effect on food intake and body weight (Galbraith and Kappas, 1989). Following ICV injection, a higher content of cobalt is found in the hypothalamus compared with other brain areas (Galbraith and Kappas, 1991). In addition direct microinjection of CoPP (20 nmol/kg) into the medial, but not the lateral, nuclei of the hypothalamus causes reduced food intake and body weight in

rats (Galbraith et al. 1992). No evidence of structural hypothalamic change is found either macro- or microscopically after CoPP injection (Galbraith and Kappas, 1989). This implies that CoPP induces a biochemical effect on the hypothalamus.

In elucidating the mechanism of action of ICV CoPP on food intake, only NPY-induced feeding has previously been studied and based on these studies a specific effect on central NPY has been proposed as a mechanism of action for CoPP (Turner et al. 1994; Galbraith et al. 1992). To test this hypothesis we assessed the effect of ICV administered noradrenaline and galanin, in addition to NPY on food intake in CoPP treated animals.

Materials and Methods

Animals were cannulated into the third cerebral ventricle as described in chapter 2. Noradrenaline bitartrate was purchased from Sigma (Poole, Dorset, UK).

CoPP

CoPP (Porphyrin Products, Logan, UT) is insoluble at the concentration we required for ICV injection in all aqueous solutions including sodium hydroxide, which has been used for subcutaneous (Muhoberac et al. 1989; Galbraith and Jellinck, 1990; Rosenberg, 1993) and in vitro (Cannon et al. 1985) studies. For this reason, it was dissolved in 70% ethanol and the pH adjusted to 7.5 with 1 M NaOH. The final volume was made up to 6 ml and the solution was filtered through a 0.2 μm millipore filter. The filtered solution was diluted 1:1000 and the optical absorbance was measured at 417 nm to confirm the concentration of the dissolved compound before administration. All other compounds were dissolved in 0.9% saline.

CoPP or vehicle (70% ethanol filtered, pH 7.5) was administered ICV and 24 hour food intake and body weight were monitored daily for two days prior to treatment and until day 7 following injection. The animals were handled daily and observed for any obvious behavioural abnormalities.

In the first two experiments, 1 or 2 hour food intake in response to saline, noradrenaline, galanin or NPY injection was carried out. Each animal received one of the three compounds (including saline) in succession in random order, 7, 10 and 13 days after ICV CoPP injection. Further details of each study are given below. In experiment 3, the effects on NPY mRNA were studied following ICV CoPP.

Experiments.

1. CoPP (0.2 μmol / kg) was injected ICV. NPY (10 μg in 10 μl), galanin (10 μg in 10 μl) or saline (10 μl) was injected ICV on days 7, 10 and 13 by crossover design (CoPP n=14, vehicle n=11). After ICV injection, premeasured food pellets were given in the metal hopper with water available *ad libitum* and 1 hour and 2 hour food intakes were measured.

2. CoPP (0.2 μmol / kg) was injected ICV. NPY (10 μg in 10 μl), noradrenaline (30 μg in 10 μl) or saline (10 μl) was injected ICV on days 7, 10 and 13 by crossover design. (CoPP n=9, vehicle n=6). The one and two hour food intakes were measured as in Experiment 1.

3. CoPP (0.4 μmol/kg) was injected ICV and the hypothalami were collected in liquid nitrogen nine days later. (CoPP n=9, vehicle n=12). Hypothalamic NPY mRNA was measured and quantified by northern blot assay as described in chapter 2.

Results.

CoPP treated animals had reduced locomotor activity for up to 24 hours after ICV CoPP injection. No other obvious differences in behaviour were noted.

Experiment 1

The body weight (fig 5.1) and food intake (fig 5.2) of CoPP treated animals was significantly reduced compared to controls from day 2 following treatment. The 2 hour feeding response to ICV NPY in CoPP treated animals was 4.0 ± 1.0 g compared to 7.1 ± 0.7 g ($p<0.05$) in vehicle treated animals (Fig. 5.3). The 1 hour feeding response to ICV galanin was 1.3 ± 0.5 g in CoPP treated animals compared to 3.2 ± 0.5 g ($p<0.05$) in vehicle treated animals (Fig. 5.3).

Experiment 2

The effect of CoPP on body weight and food intake in this experiment was similar to that in experiment 1. The 2 hour feeding response to ICV NPY in CoPP treated animals was 4.6 ± 0.9g compared to 10.1 ± 2.0g in vehicle treated animals ($p<0.05$) (Fig. 5.4.). The 1 hour feeding response to ICV noradrenaline in CoPP treated animals was 0.6 ± 0.2 g compared to 1.9 ± 0.5g in vehicle treated animals ($p<0.05$). (Fig.5. 4)

Experiment 3

The effect of CoPP on body weight and food intake in this experiment was similar to that in experiment 1. Hypothalamic NPY mRNA measured at day 9 was 104.5 ± 19.9% in CoPP treated animals compared to 100 ± 8.8% in vehicle treated animals (Fig 5.5).

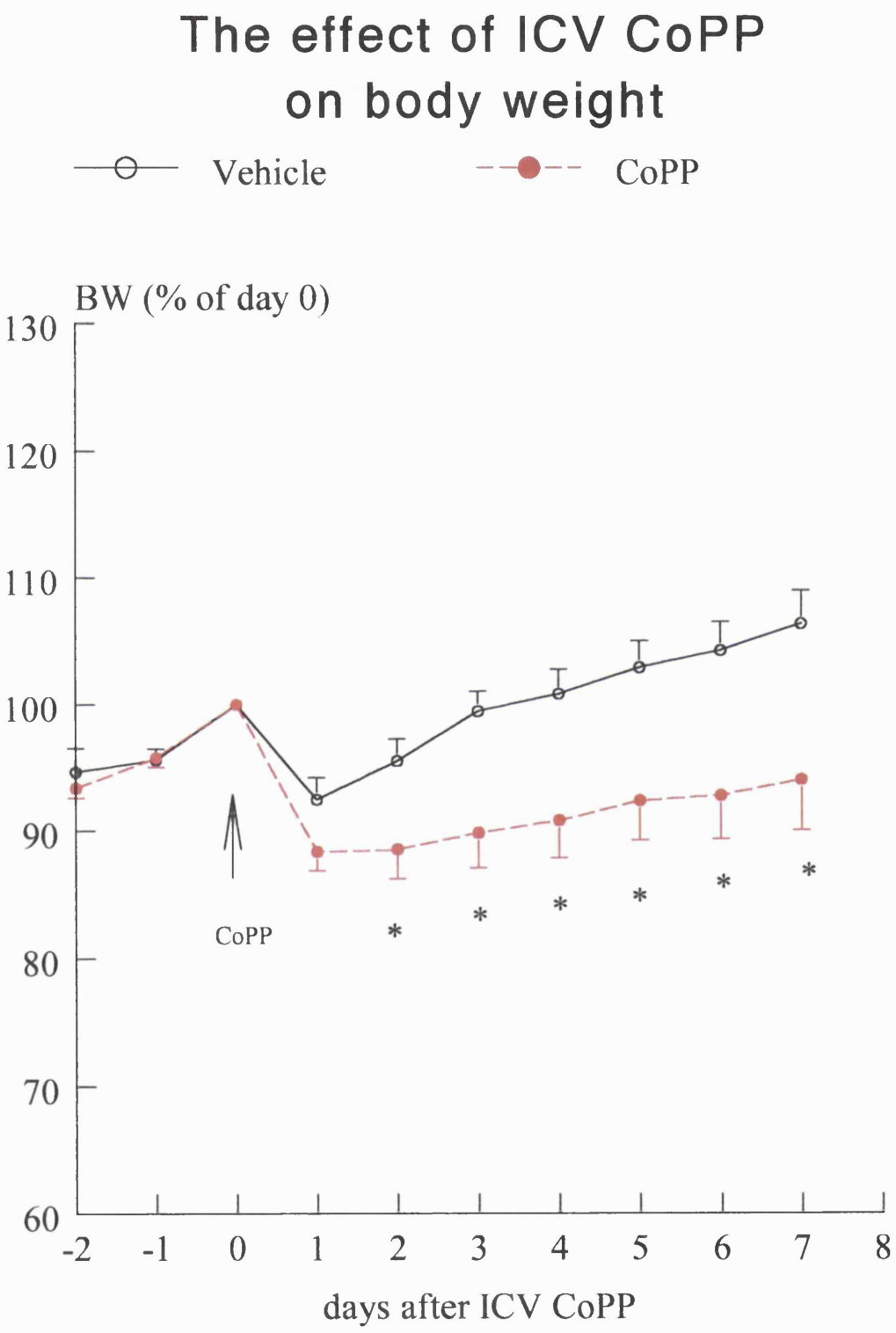

The effect of ICV CoPP
on body weight
Vehicle
CoPP
BW (% of day 0)
130
120
110
100
90
80
70
60
CoPP
*
*
*
*
*
*
-2
-1
0
1
2
3
4
5
6
7
8
days after ICV CoPP

Figure 5.1

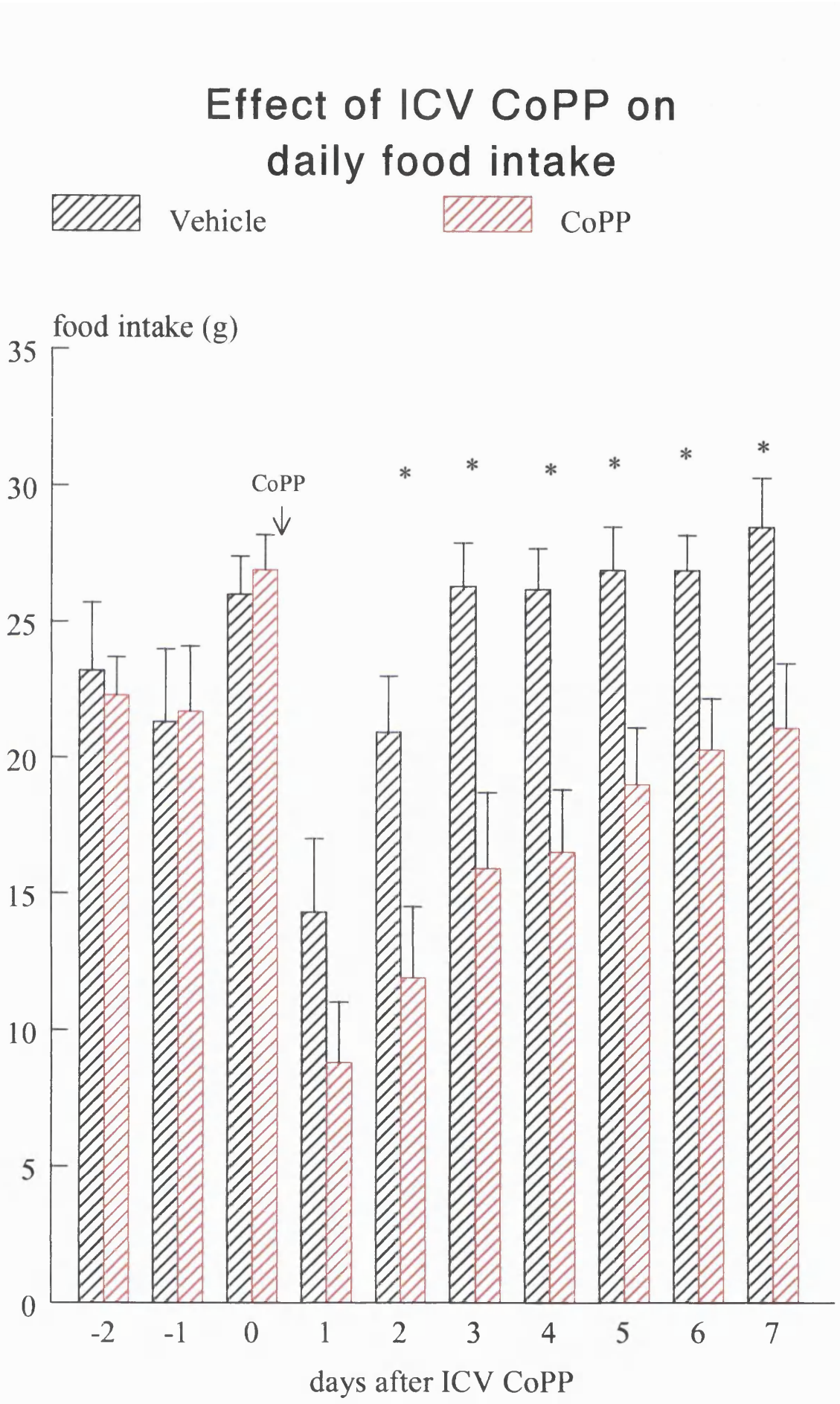

Effect of ICV CoPP on daily food intake
Vehicle
CoPP
food intake (g)
35
30
25
20
15
10
5
0
CoPP
*
-2
-1
0
1
2
3
4
5
6
7
days after ICV CoPP

Figure 5.2

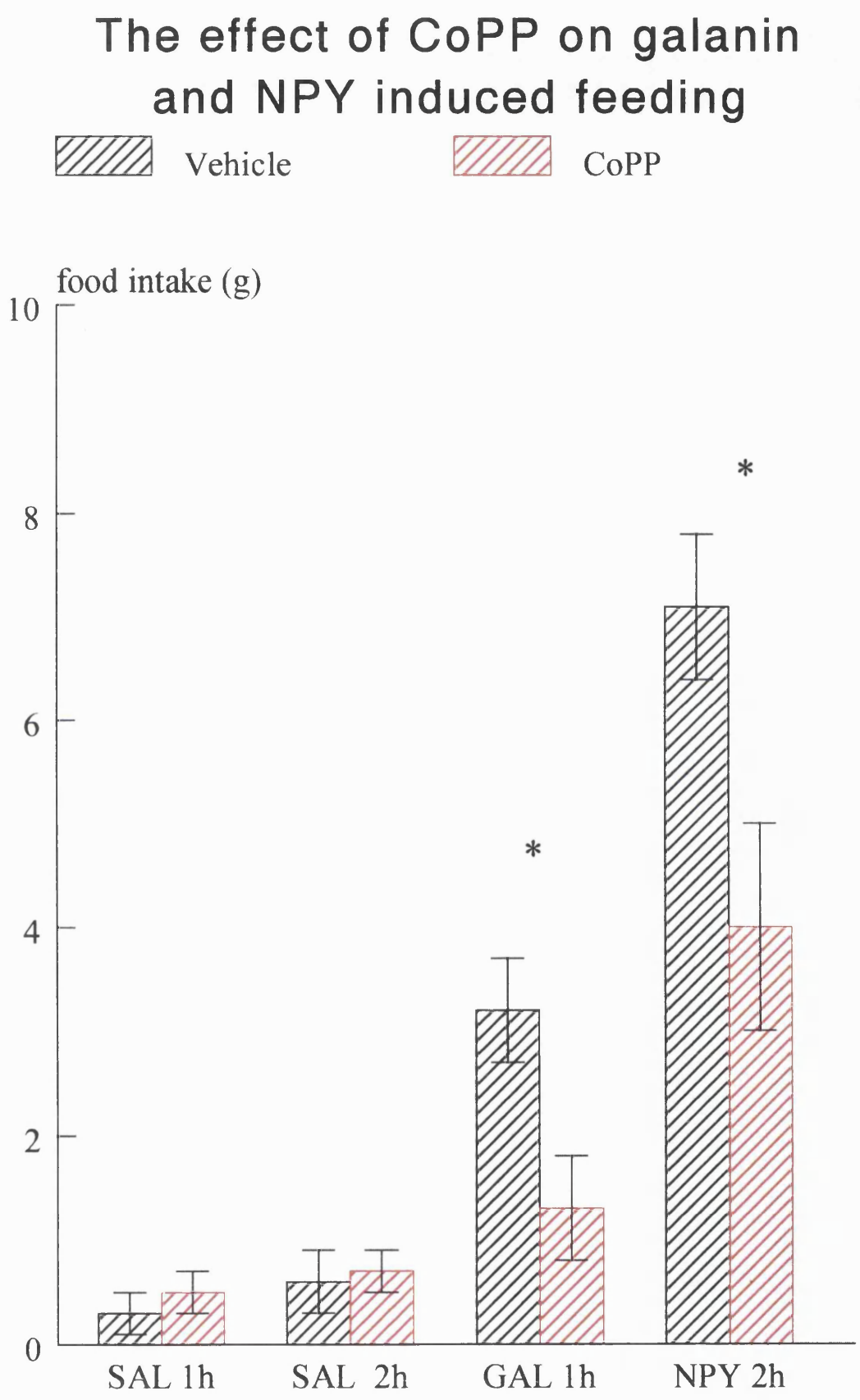

The effect of CoPP on galanin and NPY induced feeding
Vehicle
CoPP
food intake (g)
10
8
6
4
2
0
*
*
SAL 1h
SAL 2h
GAL 1h
NPY 2h

Figure 5.3

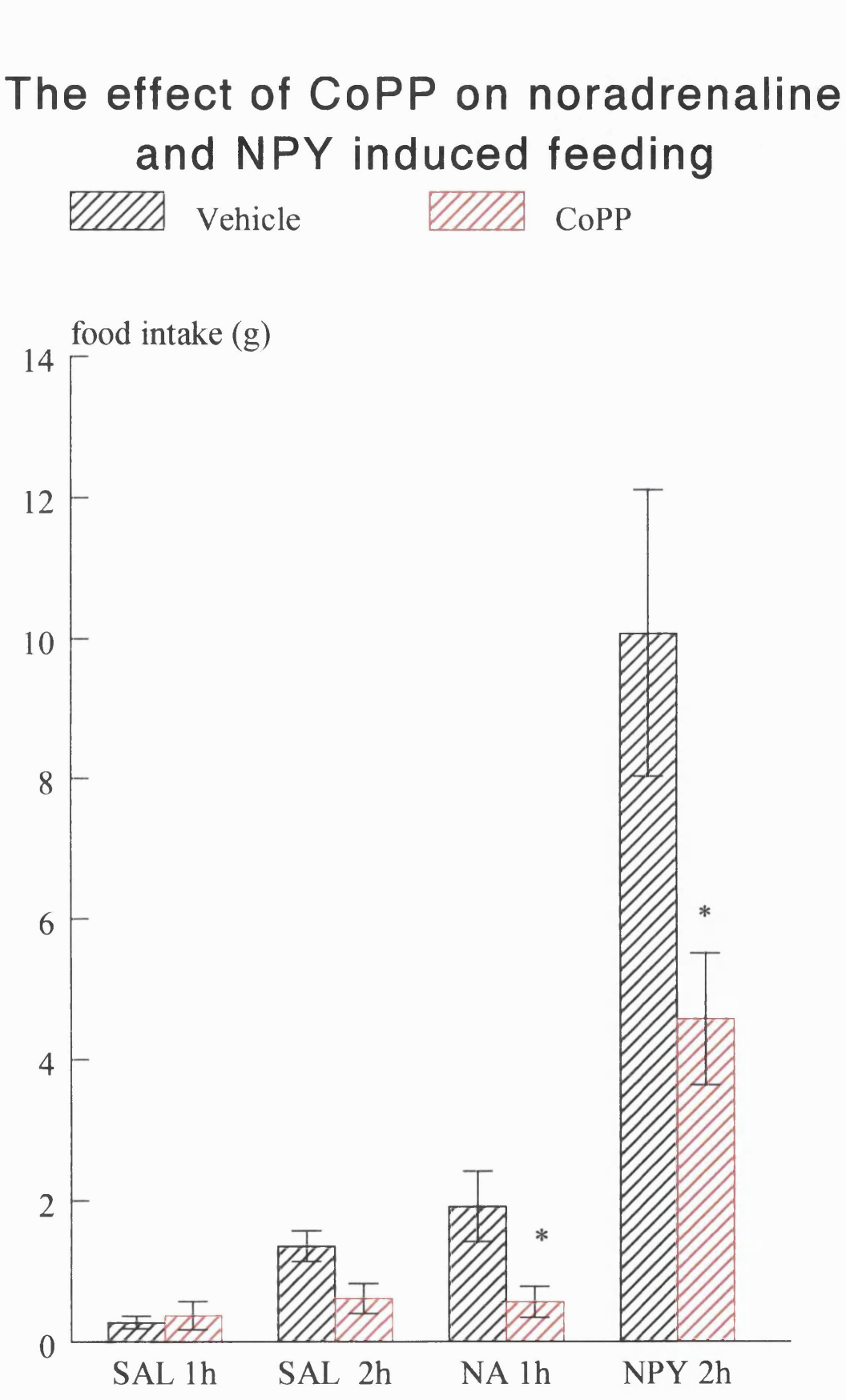

The effect of CoPP on noradrenaline and NPY induced feeding
Vehicle
CoPP
food intake (g)
14
12
10
8
6
4
2
0
*
*
SAL 1h
SAL 2h
NA 1h
NPY 2h

Figure 5.4

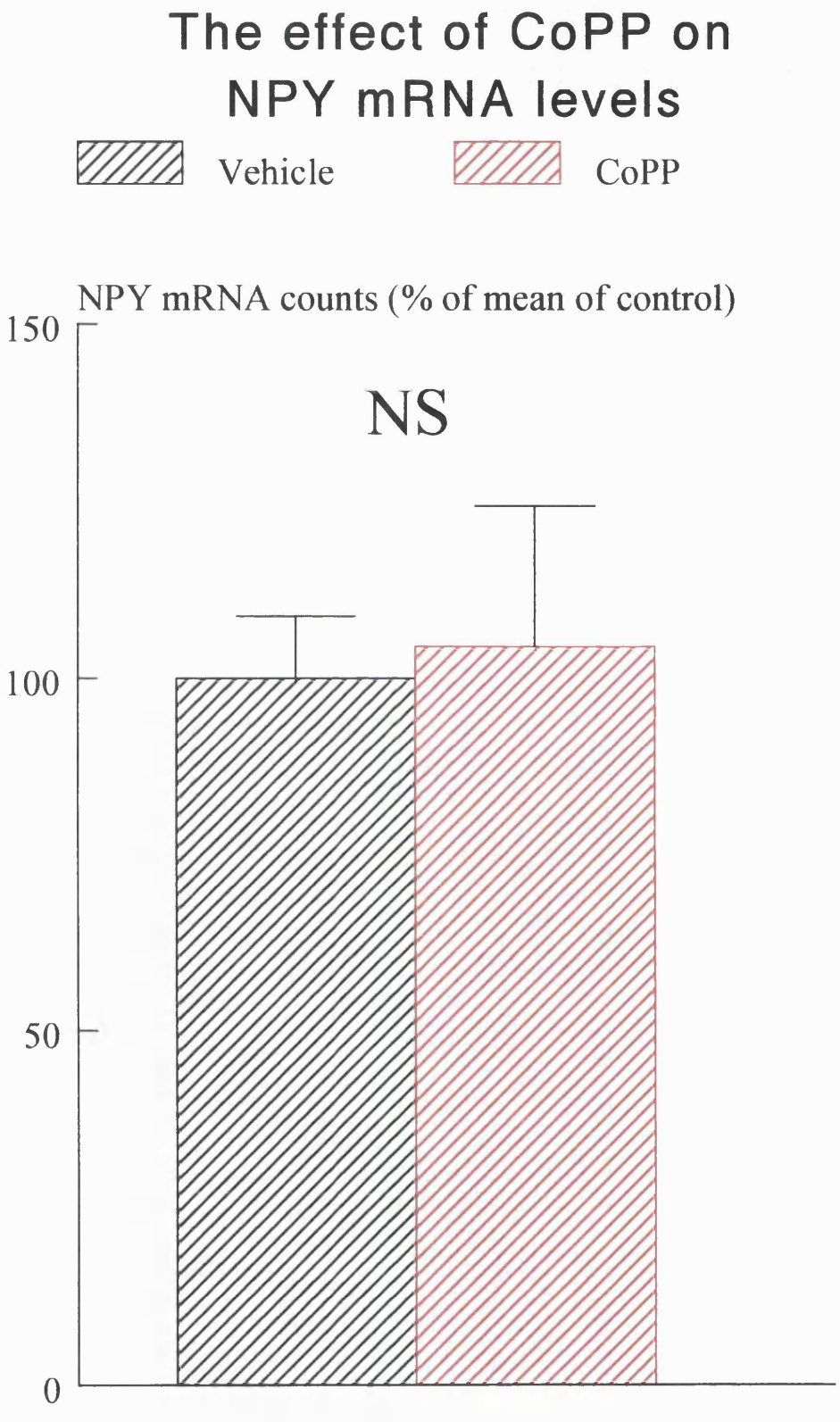

The effect of CoPP on
NPY mRNA levels
Vehicle
CoPP
NPY mRNA counts (% of mean of control)
150
100
50
0
NS

Figure 5.5

Discussion

In this study we confirmed that following ICV CoPP, the body weight and food intake of rats remain decreased for seven days as previously described (Galbraith and Kappas, 1991). We have also found that the feeding response to ICV NPY, galanin and norepinephrine was decreased in CoPP treated animals. It has been suggested that the reduction in feeding in response to exogenous NPY in CoPP treated animals is due to a specific decrease in NPY sensitivity (Turner et al. 1994; Galbraith et al. 1992). This is unlikely given that the three stimulants we studied act through their own distinct receptors and the feeding response to all three was reduced by CoPP administration.

As discussed in chapters 3 and 4, the interaction between NPY, galanin and noradrenaline is complicated. It is known that α-2 antagonists block galanin but not NPY induced feeding, implying that NPY does not act through noradrenaline (Kyrkouli et al. 1990). In addition, the onset of galanin and noradrenaline induced feeding occurs within seven minutes of ICV administration (Allen et al. 1985; Schick et al. 1993) whereas NPY induced feeding is delayed by between 12 and 20 minutes (Clark et al. 1985; Corp et al. 1990; Sahu et al. 1988). It is therefore unlikely that either noradrenaline or galanin have their feeding effect through NPY, unless the hypothesis put forward in chapter 3 regarding latency (see page 34) is correct. The difference in macronutrient selection caused by NPY, which stimulates carbohydarate intake, and galanin, which stimulates fat intake, is further evidence for their independence of action. Our finding of decreased sensitivity to all three compounds following ICV CoPP administration implies that CoPP does not have a specific effect on any individual system. The effect of CoPP may be independent of all three compounds, or it may affect

the final common pathway of feeding.

Galbraith et al reported that NPY mRNA measured at day 2 after ICV CoPP injection was more than doubled and that there was no feeding response to ICV NPY (Galbraith et al. 1992). This increased hypothalamic mRNA at day 2 may reflect a normal compensatory increase, in response to severe hypophagia. Our animals had decreased locomotor activity as well as decreased feeding on the first day following CoPP treatment. It is not clear whether this is because of an early toxic effect, or a compensatory response to decreased energy intake. Hypophagia persisted until day 9 at which time we found no significant change in NPY mRNA levels. Thus the reported rise in hypothalamic NPY mRNA seen at day 2 does not persist despite continued hypophagia.

Since the effect of CoPP has been shown to last for 300 days (Galbraith and Kappas, 1991), it has been suggested that a change in body weight set point may occur. The results of experiments utilising pair fed animals are consistent with this suggestion (Galbraith and Kappas, 1989). When administered systemically in large doses, CoPP has several other metabolic and endocrine effects. These include induction of hepatic heme oxygenase, decreased plasma testosterone without a compensatory rise in luteinising hormone (Galbraith and Krey, 1989; Jellinck and Galbraith, 1991; Galbraith and Jellinck, 1990) and suppression of hepatic cytochrome P- 450 enzyme content and activity (Drummond and Kappas, 1982).

ICV CoPP may exert its effect on food intake and body weight by inducing brain heme-oxygenase or by altering steroid metabolism via inhibition of cytochrome P-450 enzyme activity within the hypothalamus. A change in steroid metabolism may alter food intake as steroids are known to have a permissive effect on the feeding responses elicited by NPY and norepinephrine (Tempel and Leibowitz, 1993).

In conclusion ICV CoPP decreases sensitivity to exogenous NPY, galanin and noradrenaline. Its effect on neurotransmitter systems in the brain is therefore more generalized than was previously recognized. As the peptide systems affected are so widespread it is unlikely that any agent related to CoPP will be a rational therapy for the management of eating disorders.

Chapter 6

The role of early nutrition.

The work in this chapter was carried out in collaboration with Mina Desai and Professor C.N. Hales using the animal facilities at the Dunn Nutritional Laboratory, Cambridge.

Introduction.

Type 2 diabetes is an important cause of morbidity and mortality. Its prevalence is rising worldwide, especially in populations that are becoming increasingly affluent (Bennett, 1990). For example, 30-40% of the adult population of certain groups of American Indians have type 2 diabetes and yet, before 1940 the occurrence of diabetes in these populations was very rare. A similar situation is seen in Nauruan Islanders who suffered severe nutritional deficiency before and during the last World War. After the War, phosphate mining resulted in sudden affluence. Concurrently, the incidence of diabetes on the island increased at an alarming rate (Zimmet et al. 1984; Zimmet, 1979).

Type 2 diabetes or impaired glucose tolerance is found more frequently in subjects with hypertension, hyperlipidaemia, or ischaemic heart disease. This statistical linkage is commonly referred to as "syndrome X" or "the insulin resistance syndrome" (Moller and Flier, 1991; Reaven, 1988). It has been hypothesised that the common factor linking these associations is insulin resistance which leads to raised plasma insulin concentrations with consequent secondary hypertension and hypertriglyceridaemia. The occurrence of insulin resistance is believed to have a genetic basis. Certainly, type 2 diabetes has a tendency to cluster in families and concordance in identical twins approaches 100%. Also there is an increased risk of developing diabetes in the relatives of subjects with type 2 diabetes. However, the inheritance of type 2 diabetes does not follow the clear patterns expected of a dominant or recessive genetically determined condition.

The thrifty genotype hypothesis.

The paradox of the failure of natural selection to eliminate a genetically determined lethal condition was addressed several years ago by Neel's "thrifty genotype" hypothesis (Neel, 1962). This proposes that over a long evolutionary period, human metabolism has had to adapt to low levels of nutrition. Genes that were beneficial to this process would be naturally selected. However, it is postulated that such genes could be detrimental under conditions of normal or excess nutrition, leading to diabetes. It suggests that the high incidence of diabetes in the Western or recently affluent societies results from the existence of diabetogenic genes, which confer a survival advantage at times of subsistence living, though detrimental to survival under conditions of overnutrition. Whilst this concept has been well received there have been no specific suggestion as to which genes might be involved.

Insulin resistance as a marker of hypothalamic or sympathetic activation.

Counter-regulatory hormones to insulin, such as adrenaline, nor-adrenaline, cortisol, growth hormone and glucagon can cause insulin resistance through a direct effect on the enzymes involved in glucose homeostasis in many tissues. The hypothalamic arousal and neuroendocrine dysregulation scheme proposed by Bjorntorp proposes that "stress" may cause insulin resistance by increased hypothalamic arousal (Bjorntorp, 1991; Bjorntorp, 1991). The resulting increase in sympathetic activity raises blood pressure, increases hepatic glucose production, and suppresses glycogen synthesis. Several negative feedback loops involving many neurotransmitters are present in the hypothalamus, and abnormalities in pre- or post-synaptic receptors may affect baseline hypothalamic activity. Genes which cause increased baseline sympathetic outflow will cause

hypertension and insulin resistance simultaneously. Such "thrifty genes" will favour lipogenesis at the expense of glycogenesis and may confer a selective advantage at times of starvation (Neel, 1962; Dowse and Zimmet, 1993), explaining their high apparent prevalence worldwide.

The thrifty phenotype hypothesis.

Barker and Hales have recently hypothesised that insulin resistance may be programmed by changes in the intrauterine and early foetal environment (Barker et al. 1993; Barker et al. 1993). This "thrifty *phenotype*" hypothesis stems from epidemiological evidence linking low birth weight with Reavan's syndrome X in later life (Hales and Barker, 1992). The essence of this hypothesis is that nutrition in the foetal and early neonatal period imposes mechanisms of nutritional thrift upon the growing individual. This can only occur if some form of programming occurs in neonatal life, resulting in a metabolically "thrifty" individual. Such individuals may be insulin resistant, and for example will store excess food as fat rather than as glycogen. Intrauterine and early foetal environment may modulate insulin resistance, perhaps by altering the set point of hypothalamic activity. Insulin resistance may therefore be determined by a combination of genetic influences and early foetal environment, which together determine the set point of hypothalamic activation.

Neuropeptide Y or one of its receptors is an obvious hypothalamic candidate to be involved in such nutritional programming. Administration of NPY into the third cerebral ventricle (ICV) increases plasma ACTH, causes a potent stimulus to feeding, and has been shown to cause peripheral insulin resistance, favouring lipogenesis over glycogen

synthesis in hyperinsulinaemic euglycaemic clamp studies (Zarjevski et al. 1994). A change in the sensitivity of a hypothalamic neuropeptide Y receptor may for instance increase appetite and change insulin sensitivity.

In order to determine if any changes in hypothalamic NPY mRNA occur following neonatal food deprivation in rats, I administered either a control or a protein deficient diet to pregnant rats and studied their offspring. The offspring were weaned onto a normal diet and hypothalamic NPY mRNA levels assessed either 21 days, 6 weeks or 12 weeks later. In order to mimic overnutrition in adults, some of these animals were fed a highly palatable "cafeteria" diet between the ages of 6 and 12 weeks.

Materials and methods.

Animals and diet:

Virgin female Wistar rats with an initial body weight 240 ± 20 g were mated and day 0 of gestation was taken as the day on which vaginal plugs were observed. Thereafter, rats were fed either a diet containing 20% protein or an isocaloric diet containing 8% protein throughout gestation and lactation. The full composition of these diets is given in table 6.1. All ingredients are expressed as grams per 100g of diet. Cerelose is starch and cellulose is fibre.

Table 6.1

	20 % protein diet	**8% protein diet**
	(g/100g)	(g/100g)
Mineral and vitamin mixture	5.05	5.45
Casein (88% protein)	22.00	9.00
dl-methionine	0.20	0.08
Corn starch	8.00	8.00
Cellulose	5.00	5.00
Soybean oil	4.30	4.30
Cerelose	55.15	68.17
Calories	367.4	365.2

Spontaneous delivery took place on day 22 of pregnancy after which, at 3 days of age large litters were reduced to eight pups, thus ensuring a standard litter size per mother. At this stage, half the number of mothers from each diet group continued to nurse their own offspring until 21 days of age. For the remaining mothers, cross fostering techniques were employed, such that the offspring of mothers on a 20% protein diet were nursed by mothers on a 8% protein diet and vice versa. In order to do this, litters

that were born on the same day were used. This allowed a separate evaluation of the effects of maternal protein restriction during either gestation alone or lactation alone. It also allows assessment of maternal protein restriction carried out throughout gestation and lactation. Thus, the 'control' group consisted of offspring of mothers fed a 20% protein diet during pregnancy and were nursed by mothers on a 20% protein diet. The 'low protein' group consisted of offspring of mothers fed a 8% protein diet over the same period of time. The 'postnatal low protein' group consisted of offspring of mothers fed a 20% protein diet during pregnancy but were subsequently nursed by mothers on a 8% protein diet. Finally, the 'recuperated' group was the reverse of the postnatal group, that is, they were the offspring of mothers on a 8% protein diet and were then nursed by mothers on a 20% protein diet. These details are summarised in table 6.2. Some of the offspring were killed at 21 days of age. The remainder were weaned onto a normal laboratory chow (LAD1 diet containing 21 % protein) and killed at 6 weeks of age.

Table 6.2

Percentage of protein in maternal diet when offspring were either in utero or being nursed.

	control	low protein	postnatal low protein	recuperated
during pregnancy (in utero)	20%	8%	20%	8%
during nursing up to age 21 days	20%	8%	8%	20%

At the age of 21 days, all animals were weaned onto a control LAD1 diet which was continued for a further three weeks.

In separate experiments, only the control and low protein offspring were used. This represented five pregnancies per diet group with 4 males and 4 females per litter. At 21 days of age, all offspring were weaned onto normal laboratory chow (LAD1, containing 21% protein, Special diet Services, UK) as in the previous experiment. At 6 weeks of age, 2 males and 2 females from each litter were placed on another standard laboratory chow (PRD pellets, containing 20% protein) and the remaining rats on a highly palatable diet (HP). The recipe for the highly palatable diet is given in table 6.3 and a comparison of the PRD diet with the HP diet is given in table 6.4.

Table 6.3

Ingredients	**Amount (g)**
Powdered PRD pellets	330
Nestlé's full fat condensed milk	330
Sucrose	70
Water	270

Table 6.4. The composition of PRD and HP diets that were used between the ages of 6 and 12 weeks. Values are expressed as a percentage of calories.

	PRD	**HP**
carbohydrate	66	69
protein	25 % of calories	18 % of calories
fat	9	13

The respective diets were continued for a period of 6 weeks and at the age of 12 weeks the animals were fasted for 48 hours after which time they were killed.

Hypothalamic NPY mRNA measurement.

There were thus three sets of experiments, with animals being killed at age 21 days, 42 days or 84 days (3,6 and 12 weeks respectively). All animals were killed using carbon dioxide inhalation. Immediately after death the hypothalami were rapidly dissected and frozen in liquid nitrogen. Hypothalamic RNA was extracted, blotted and probed for NPY mRNA using the method described in chapter 2.

Results.

Body growth till weaning (from 4 to 21 days of age).

As can be seen from figure 6.1, the offspring of animals on the low protein diet were significantly smaller than animals whose mothers were given a control diet. In animals that were crossed over, the postnatal low protein group grew significantly more slowly than the recuperated group.

The body weight at 6 weeks of age reveals that recovery does not occur especially in animals that were exposed to maternal low protein diet while weaning, even though these animals have all been on the same diet for the three weeks before death. (See table 6.5)

Table 6.5: Weight of animals at age 6 weeks.

Group	Control	Low Protein	Recuperated	postnatal low protein
Weight (g)	215.8	168.5	207.3	184.5
SEM	5.01	3.91	5.01	3.71

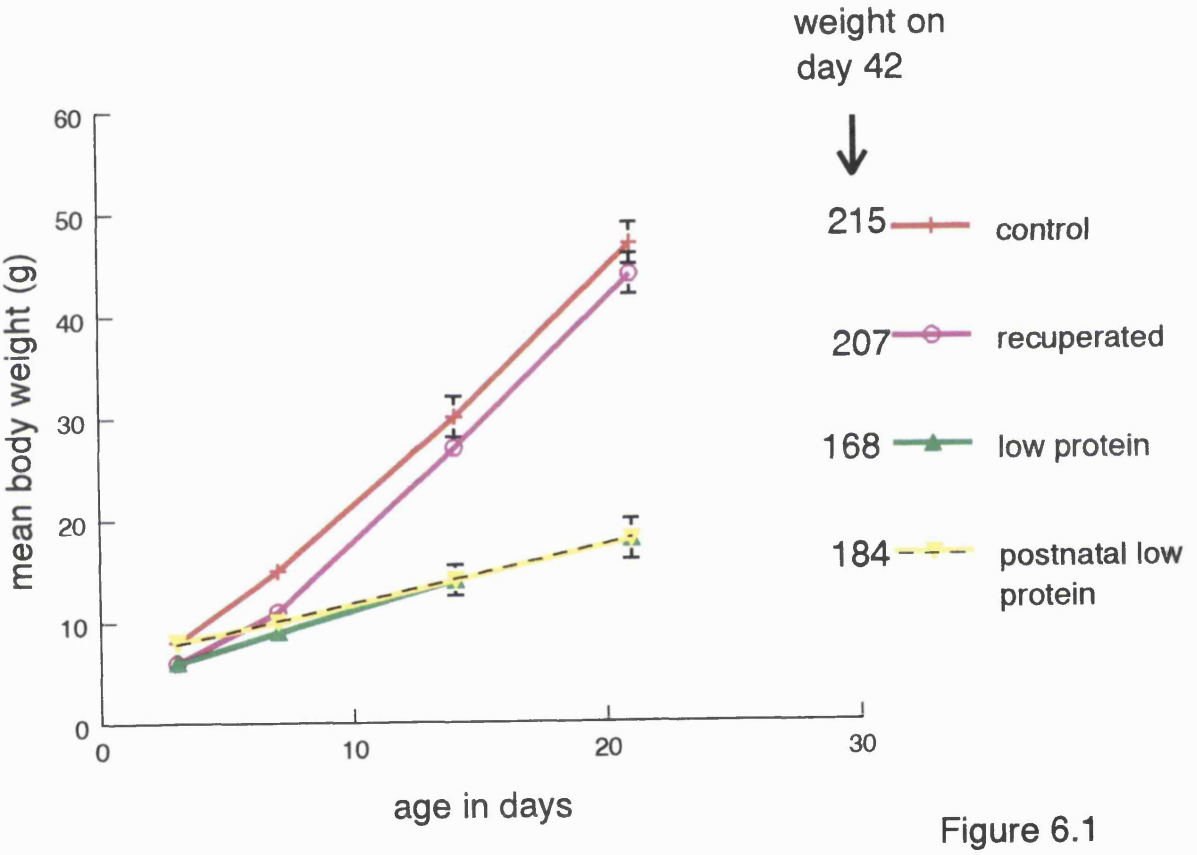


Figure 6.1

Legend to figure 6.1

Mean bodyweight of animals whose mothers were fed either a normal diet or one low in protein from 4 days after birth until aged 21 days.

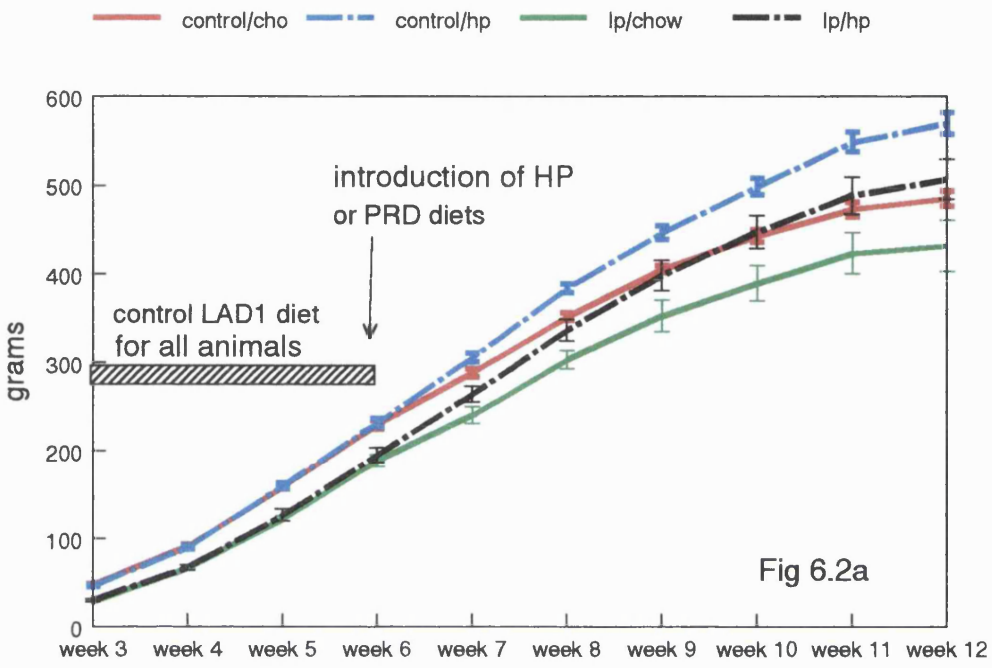
Body growth after weaning. (3 to 12 weeks of age).

Crossover (i.e. recuperated and postnatal low protein) animals were not studied in this experiment. All the animals were on a "normal" 21% protein diet between the ages of 3 and 6 weeks. Figure 6.2 (a= males; b=females) continues from figure 6.1 and shows the weight of the animals and figure 6.3 the weight of their food intake on a daily basis from the age of 21 days. Between the ages of 3 and 6 weeks, the control animals were significantly heavier than the animals whose mothers had been on a low protein diet during pregnancy and lactation and the two growth curves are parallel to each other with no attempt of the smaller animals to catch up, and inkeeping with the previous experiment. In fact, as can be seen from figure 6.3, the smaller animals eat significantly less up to the age of 12 weeks, even though they have been weaned onto the same diet as the control rats (compare the black with the blue line or the green with the red one). Thus if any hypothalamic programming occurs, it happens during the weaning period. After the age of 3 weeks, the food intake on a control diet appears tc be set, without a compensatory increase in food intake in smaller animals.

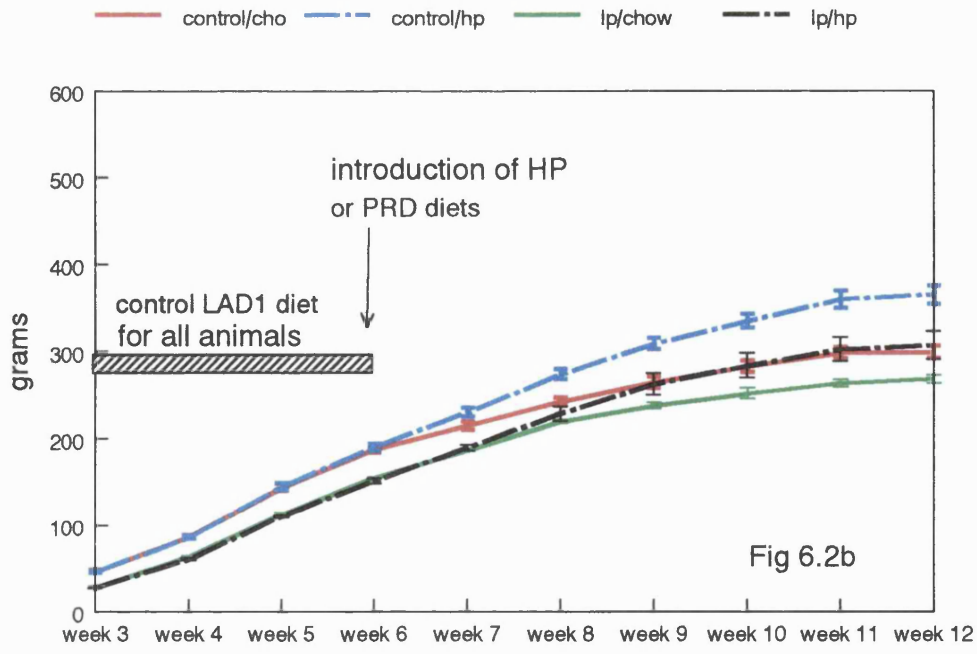
The effect of cafeteria feeding.

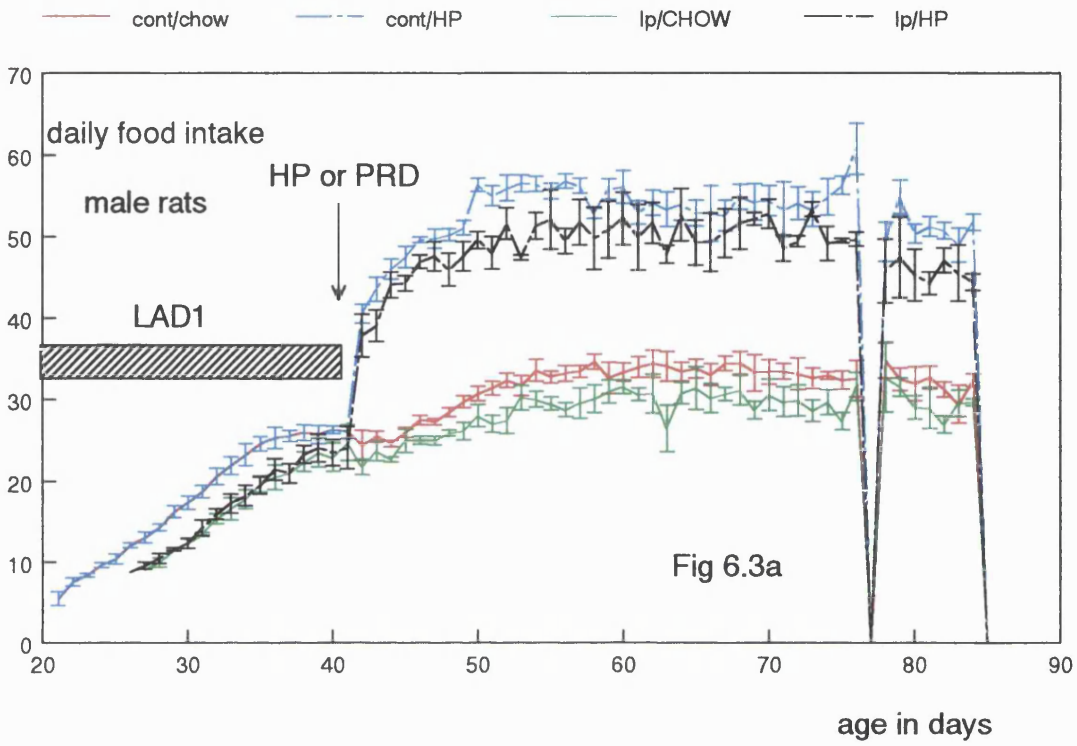
This can also be seen on figures 6.2 and 6.3. The animals were changed from control diet LAD1 to either another control diet (PRD) or the highly palatable diet at the age of 6 weeks and were maintained on this diet until the age of 12 weeks. Animals on the highly palatable diet ate almost twice as much, every day, as the control animals and also significantly increased their weight. Even at 12 weeks however, there remains a significant difference in animal weights depending on their early nutrition. Interestingly, the effect of the highly palatable diet appears to exactly compensate for the effects of early nutrition on body weight.

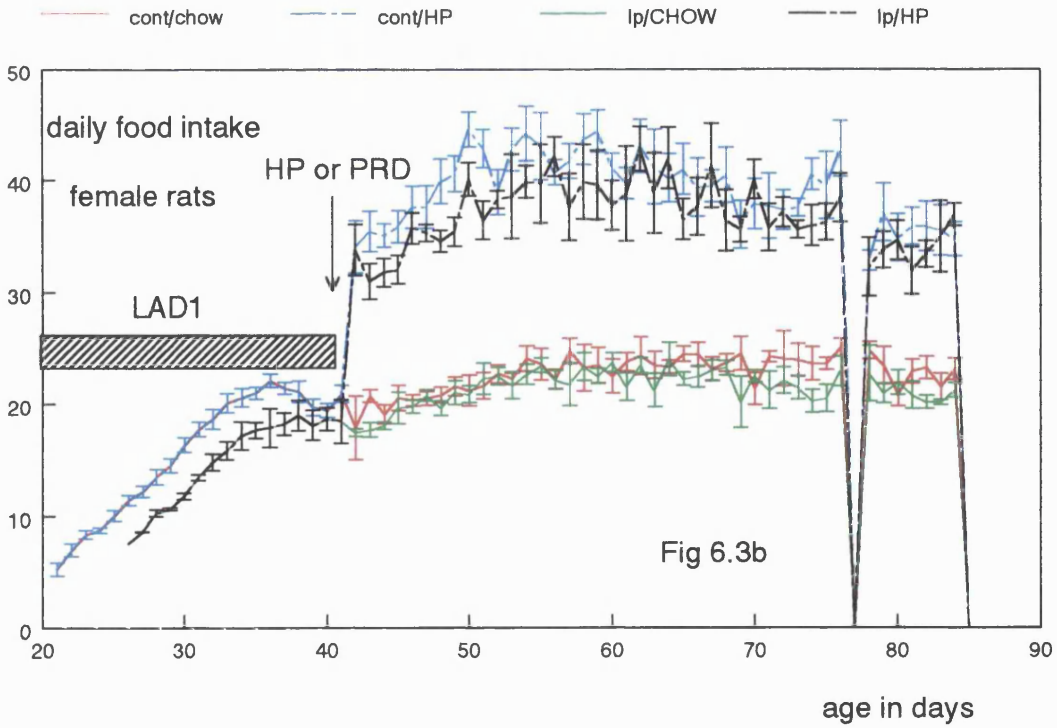
weight of male animals



weight of female animals




cont/chow
cont/HP
lp/CHOW
lp/HP
daily food intake
male rats
HP or PRD
LAD1
Fig 6.3a
age in days


cont/chow
cont/HP
lp/CHOW
lp/HP
daily food intake
female rats
HP or PRD
LAD1
Fig 6.3b
age in days

Hypothalamic NPY mRNA

Figure 6.4 shows that hypothalamic NPY mRNA is significantly increased at 21 days both in the low protein and the postnatal low protein groups. These differences disappear once the animals are weaned onto a normal diet for 3 weeks (figure 6.5). Cafeteria feeding for a further 6 weeks does not make any difference to hypothalamic NPY mRNA (figure 6.6).

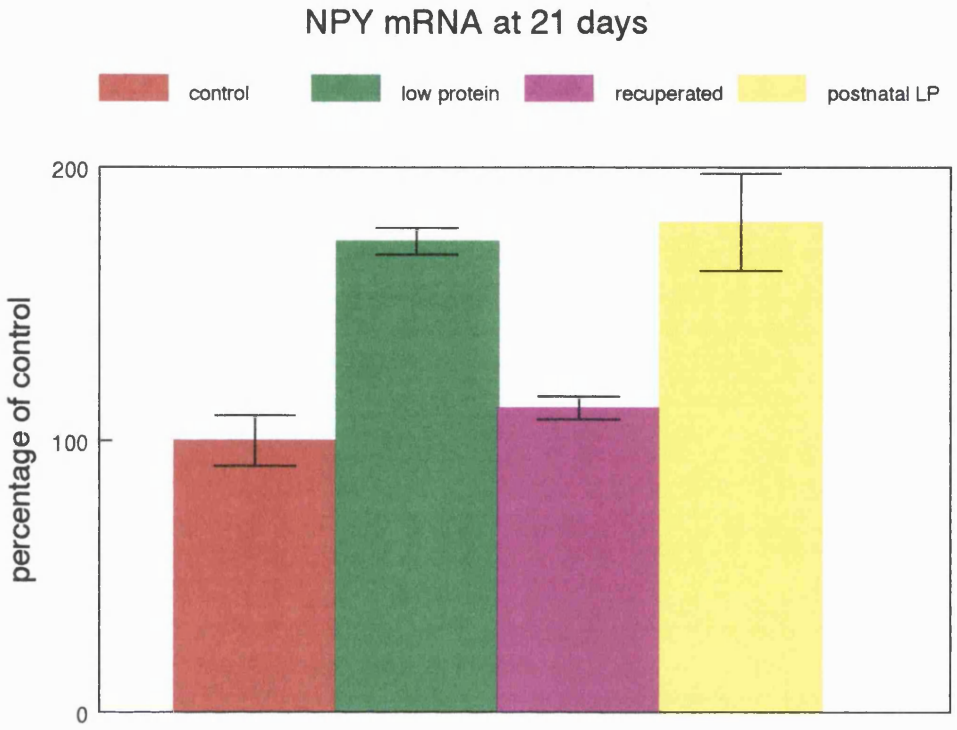


Figure 6.4

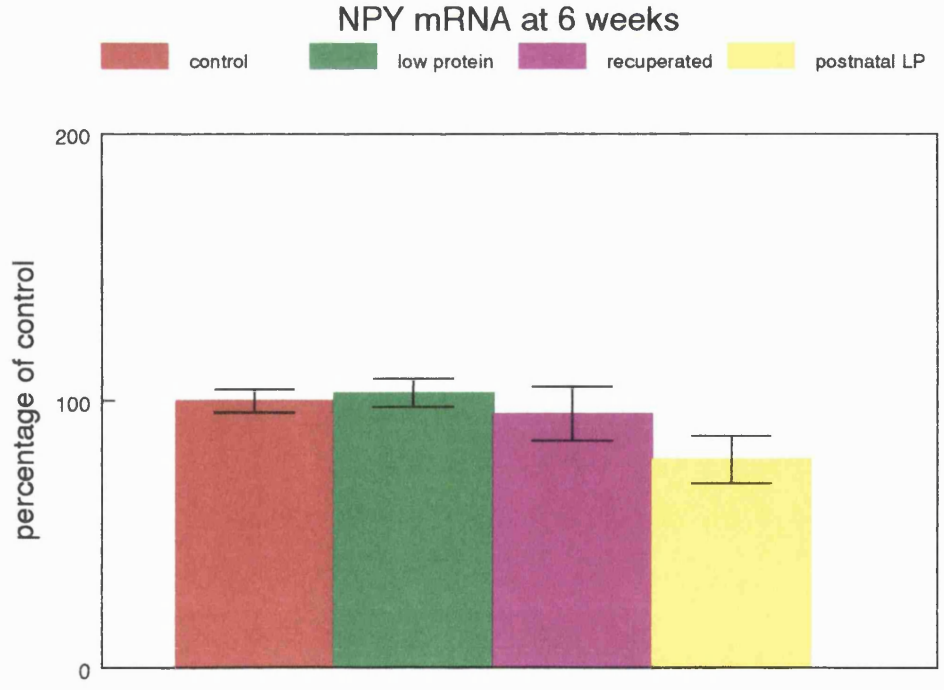


Figure 6.5

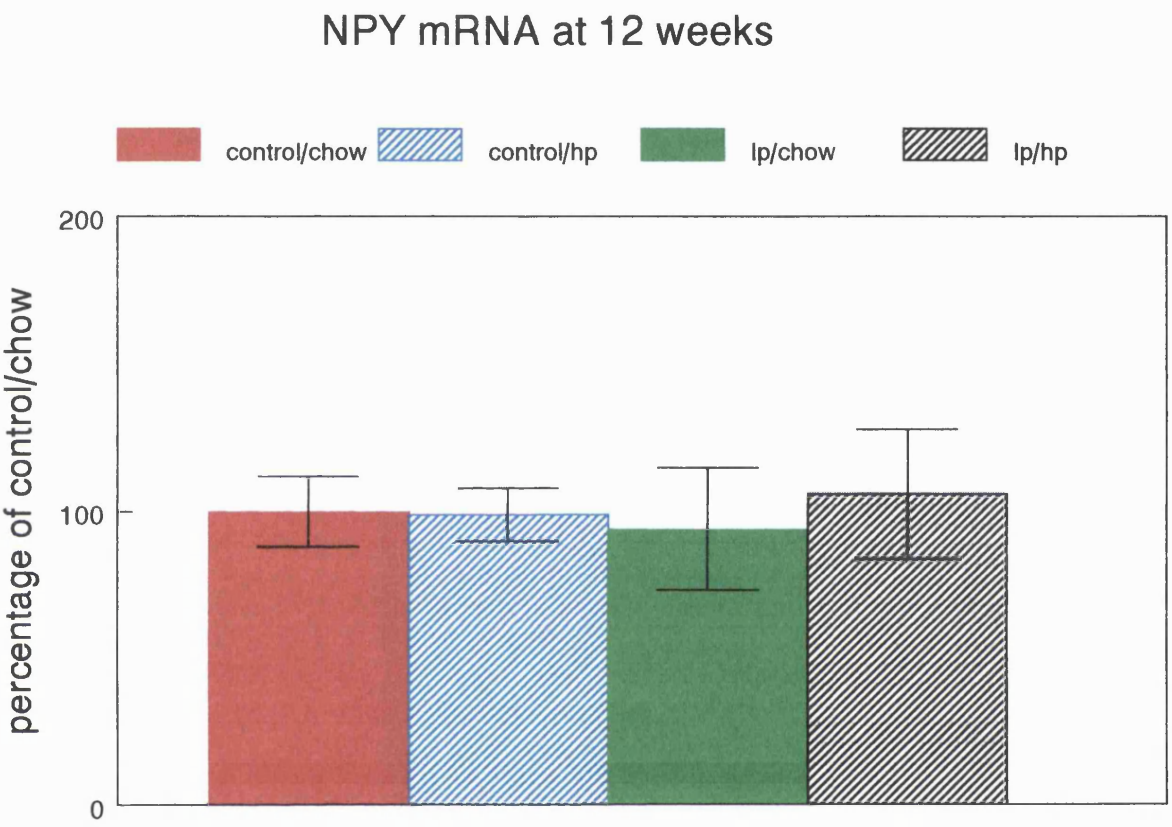

NPY mRNA at 12 weeks
control/chow
control/hp
lp/chow
lp/hp
200
100
0
percentage of control/chow

Figure 6.6

Discussion.

The mechanism whereby a growth trajectory can be set has not been elucidated. Two recently discovered satiety signals, leptin (Campfield et al. 1995) and GLP-1 (see chapter 7) may be peripherally altered and may thus influence the sensation of satiety and hunger in the long term. Both of these satiety factors, as well as neuropeptide Y have receptors in the hypothalamus. The hypothalamus is well known to be the site of integration of several peripheral metabolic signals (Hanson and Dallman, 1995). Alteration of hypothalamic sensitivity to a number of factors may occur, and as such may explain changes in programming. Neuropeptide Y is the most potent known stimulator of food intake (Stanley and Leibowitz, 1984; Levine and Morley, 1984; Stanley and Leibowitz, 1985). Following ICV or PVN injection, NPY causes a long lasting drive to feed. Tachyphylaxis does not occur following repeated dosing. Thus chronic changes in the regulation of feeding in the hypothalamus would be most likely to cause changes in hypothalamic NPY mRNA together with changes in receptor sensitivity or number and for this reason I chose to measure hypothalamic NPY mRNA as a marker of hypothalamic programming.

I showed that animals aged 21 days whose mother was on a low protein diet during lactation (whether the animal had had a normal or a low protein diet while in utero), had increased NPY mRNA whereas animals who had been on a normal diet during lactation had normal NPY mRNA levels. These results confirm that measurement of hypothalamic NPY mRNA was sensitive enough to detect changes caused by fasting. It is well known that acute and chronic starvation increase hypothalamic NPY mRNA levels (Sahu et al. 1988; Brady et al. 1990) and it is likely that the increased levels we found occur because

the lactating mother on a low protein diet is unable to make sufficient milk to feed all the pups adequately. Animals that have a low protein diet in utero but a normally lactating mother from birth up to 21 days of age have normal NPY mRNA levels.

The increased hypothalamic NPY mRNA associated with early malnutrition does not persist. By 86 days of age, despite a highly palatable diet for the 6 weeks before death, all groups had equal levels of NPY mRNA. This suggests that the changes seen at 21 days of age were not due to programming of the hypothalamus, but were simply a response to the acute starvation that occurs when a mother is not producing enough milk. If the hypothalamus is involved in appetite programming, the rodent model of early malnutrition does not show it through changes in NPY mRNA.

Chapter 7

Glucagon-like peptide-1 (7-36) amide, a central regulator of feeding.

The work in this chapter was carried out with the collaboration of the entire hypothalamic group in Professor Bloom's laboratory. Two major publications have arisen out of this work (Meeran et al. 1997; Turton et al. 1996). In this chapter, the abbreviation GLP-1 will be used for glucagon-like peptide-1 (7-36) amide.

Glucagon-like peptide -1 (7-36) amide (GLP-1), is a peptide of 30 amino-acids produced by post translational processing of preproglucagon in the central nervous system and gut (Kreymann et al. 1989). Its amino acid sequence is completely conserved in all mammals studied to date (Seino et al. 1986; Conlon, 1988), inferring a critical physiological role (Orskov, 1992). In the gut, it has important actions on carbohydrate metabolism (Wang et al. 1995).

Structure of GLP-1

Cloning and sequence analysis of cDNAs and DNA fragments from genomic libraries has led to an increase in the understanding of the glucagon-related peptides in recent years. GLP-1 is generated in intestinal L-cells and the central nervous system by a cell-specific proteolytic cleavage of the preproglucagon molecule (Mojsov et al. 1986). Preproglucagon mRNA from pancreas, brain and intestine are identical (Mojsov et al. 1986; Novak et al. 1987), but the pathway of post-translational processing of the primary transcript differs markedly in the pancreas compared with intestine and brain (Figure 7.1) (Yoshimoto et al. 1989; Conlon, 1988).

Preproglucagon processing in brain and pancreas

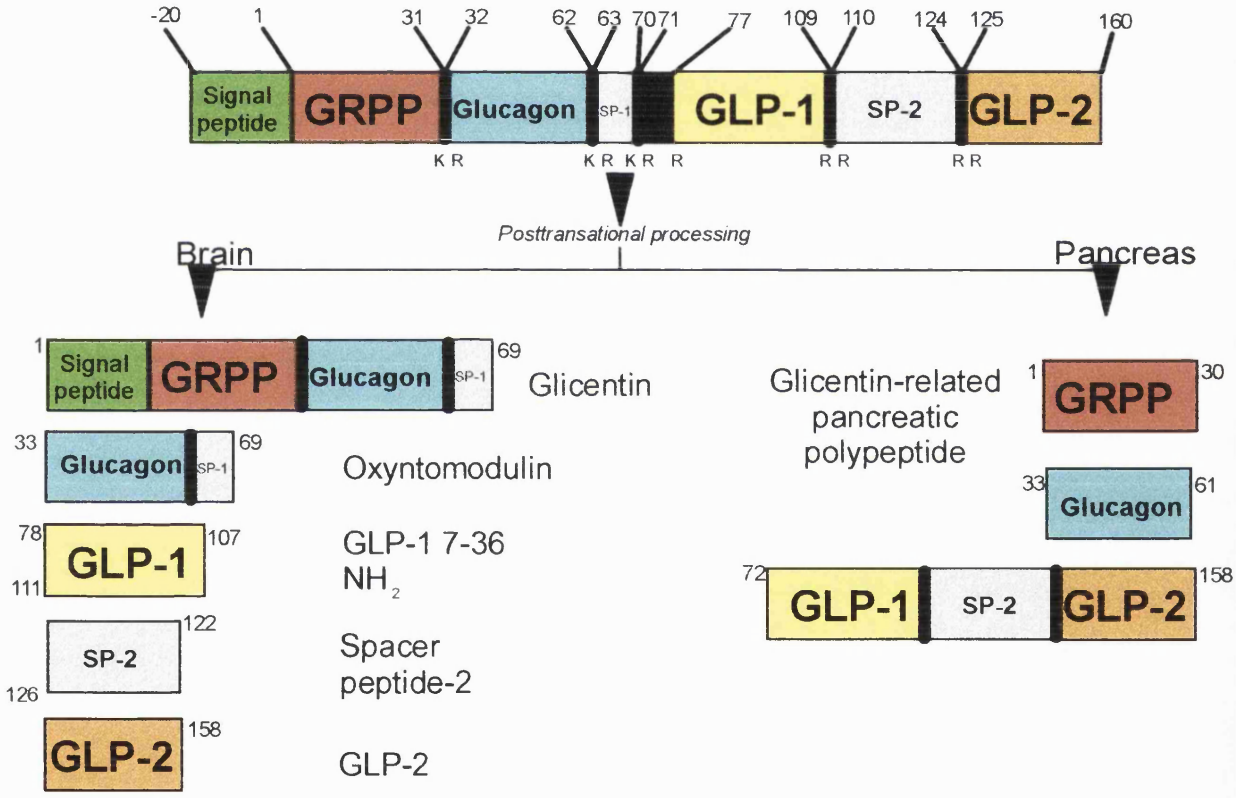


Figure 7.1

The preproglucagon sequence (180 amino acids) contains pairs of basic amino acids (Lysine="K" and Arginine="R" in figure 7.1), which are potential sites for cleavage by proteolytic enzymes involved in the maturation of peptide hormones (Steiner et al. 1992). The first 20 amino acids form the leader sequence. The glucagon sequence is present at position 33-61 of proglucagon. Two segments containing sequences homologous to glucagon are located at position 72-108 and 126-158 and are called GLP-1 and GLP-2, respectively. Purification of GLP-1 from pig and human intestine and its analysis by amino acid sequence and mass spectroscopy revealed that the naturally occurring peptide corresponded to proglucagon (78-107) amide or GLP-1 (7-36) amide (Orskov et al. 1989).

GLP-1 is synthesised in the intestinal L-cell and released into the circulation following a meal (Ebert and Creutzfeldt, 1987; Wang et al. 1995). Systemic administration of GLP-1 stimulates insulin release from pancreatic β-cells. GLP-1 is thus a physiological glucoincretin (ie a potent stimulant of insulin release in the presence of an elevated glucose) in rats (Wang et al. 1995) and humans (Kreymann et al. 1987; Ebert and Creutzfeldt, 1987).

Its recent discovery and distribution in the rat brain raise the possibility of GLP-1 having a central role (Kreymann et al. 1989). Highest concentrations were found in the hypothalamus. GLP-1 was released from hypothalamic tissue slices in a calcium dependent fashion by potassium induced depolarisation. Thus GLP-1 fulfils two of the criteria to be a neurotransmitter (Kreymann et al. 1989).

The GLP-1 receptor

The GLP-1 receptor has been cloned and characterised and is a G-protein coupled receptor with a molecular weight of 63,000 (Goke et al. 1989). Binding of GLP-1 to its receptor causes a concentration dependent increase in cAMP production (Goke and Conlon, 1988). Specific receptor antagonists are needed to elucidate the physiological role of any peptide. Exendin-3 (Eng et al. 1990) and exendin-4 (Eng et al. 1992) are bioactive peptides that have been isolated, purified, and sequenced from the venom of the Helodermatidae lizards (Figure 7.2 - *Heloderma suspectum*, Gila Monster). Interaction of these peptides with newly discovered exendin receptors, results in an increase in intracellular cAMP (Raufman et al. 1991; Eng et al. 1992). Interestingly, the exendins share a 50% sequence homology with GLP-1 (7-36)-amide (Raufman et al. 1992). Exendin-3 and exendin-4 have been shown to enhance insulin secretion when infused in dogs and are hence GLP-1 receptor agonists.

Recent findings have demonstrated that the truncated peptide exendin (9-39) amide (exendin (9-39)) is an antagonist at the GLP-1 receptor (Fehmann et al. 1994). Intravenous infusion of exendin (9-39) completely abolished the stimulation of insulin by GLP-1 at a ratio of 10:1 (Kolligs et al. 1995; Wang et al. 1995) and significantly increased blood glucose concentrations (Kolligs et al. 1995).

Figure 7.2. (Overleaf).

The Gila Monster, Heloderma suspectum, lives around the Gila river. Its bite is extremely tenacious. Its venom contains several peptides including exendin-3 and exendin-4, two potent GLP-1 receptor agonists. The truncated fragment of exendin 4, exendin (9-39) is a GLP-1 receptor antagonist.

Gila Monster

Autoradiography of GLP-1 binding sites in the brain, carried out by Miss Sarah A Beak, in this laboratory, shows dense specific binding of GLP-1 in the hypothalamus, particularly the paraventricular nucleus (PVN), the central nucleus of the amygdala and the anterodorsal thalamic nucleus. Addition of 5 nM exendin (9-39) blocks GLP-1 binding in these regions. This indicates that GLP-1 receptors present in the hypothalamus can be blocked by exendin (9-39). Exendin (9-39) was used as a tool, in the work described in this thesis, to assess the role of endogenous GLP-1 in the central nervous system where its physiological action had not been established.

Like NPY (see chapter 3), GLP-1 and its receptor are present in the hypothalamus, an area known to be important in the regulation of food intake and metabolism. For this reason, a potential role for GLP-1 in the control of feeding (this chapter) and in the control of glucose metabolism (chapter 9) was investigated. A possible interaction of GLP-1 with hypothalamic NPY was also investigated both by co-administration of NPY with GLP-1 and exendin (9-39), and by investigating the effects of ICV injection of GLP-1 on hypothalamic NPY mRNA expression (chapter 8). Increased NPY levels are found in the paraventricular nucleus of the hypothalamus of animals that are fasted (Sanacora et al. 1990; Sahu et al. 1988), animals treated with oral dexamethasone (Corder et al. 1988; Larsen et al. 1994) and in the obese hyperphagic Zucker (*fa/fa*) rat (Sanacora et al. 1990; McKibbin et al. 1991), an animal with a defect in the leptin receptor (Phillips et al. 1996). I have also investigated the effects of stimulation and blockade of central GLP-1 receptors in these animal models of endogenously raised hypothalamic NPY.

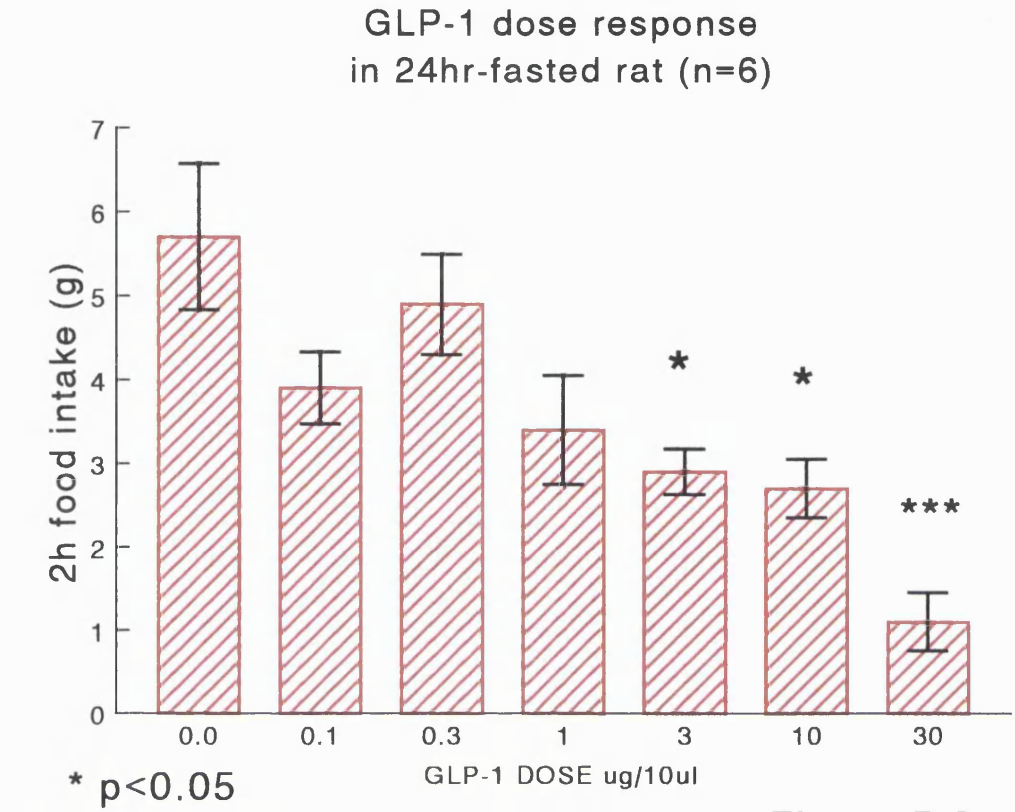
Experiments & Results:

Experiment 1.

40 rats were ICV cannulated as described in chapter 2. Following a 24 hour fast animals were injected with either saline or GLP-1 (0.3, 1, 3, 10 or 30 μg) into the third cerebral ventricle and two hour food intake was measured. ICV administration of GLP-1 reduces food intake in fasted rats, in a dose dependent manner (Figure 7.3).

Experiment 2.

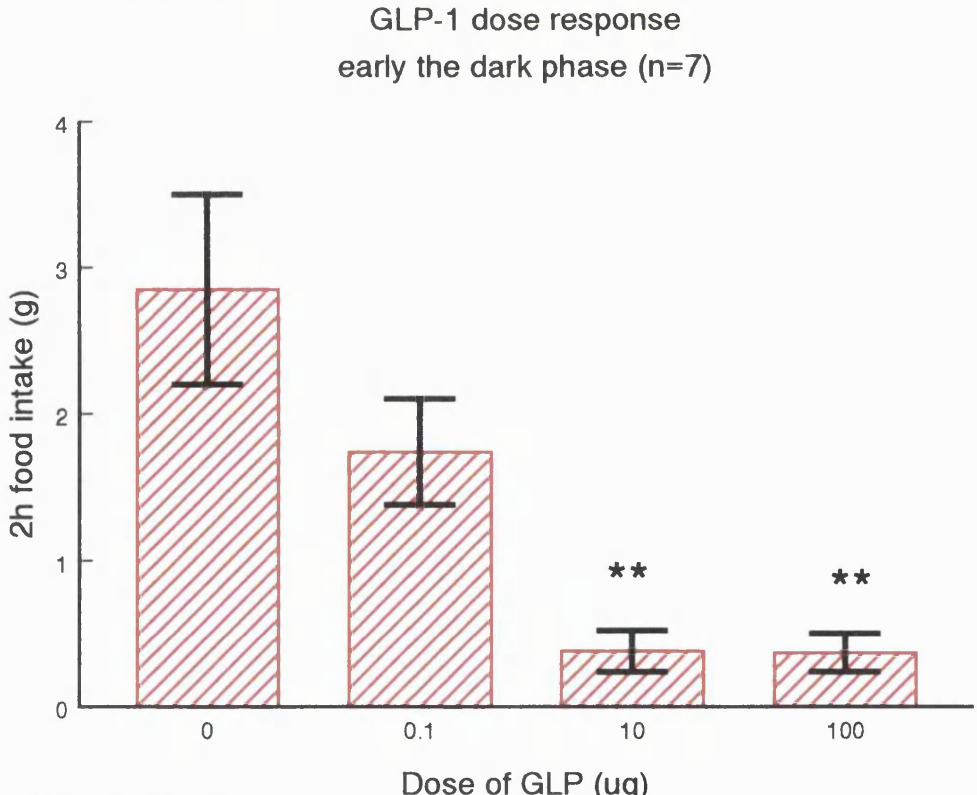
As rats feed maximally at the beginning of the dark phase (Jensen et al. 1983; Stewart et al. 1985), and fasting (experiment 1) is less physiological, the effect of ICV injection of GLP-1 in rats in the early dark phase was studied. ICV GLP-1 at the beginning of the dark (feeding) phase also results in a profound decrease in feeding (Figure 7.4).



* p<0.05

*** p<0.0005

Figure 7.3



** p<0.01

Figure 7.4

Experiment 3.

The circumventricular organs (eg the area postrema) are regions where the blood brain barrier is leaky. To eliminate the possibility that centrally administered GLP-1 was leaking out and acting peripherally to affect feeding for example by decreasing gastric emptying, GLP-1 was administered intraperitoneally (3a) and intravenously (3b) at high doses.

Experiment 3a.

Two groups of rats were injected with saline or GLP-1 intraperitoneally immediately after the lights were turned off, and when animals are expected to eat most. When administered intraperitoneally, GLP-1 (500 μg) had no effect on early dark phase feeding, suggesting that the action of GLP-1 on food intake is via central rather than peripheral mechanisms.

Experiment 3b.

It is possible that intraperitoneal GLP-1 was inactivated by first pass metabolism in the liver. For this reason, a feeding study was performed with intravenous GLP-1. A total of thirty Wistar rats had intravenous cannulae implanted and were housed in cages with a permanent tether connected to a swivel at the top of the cage (page 26) as described in chapter 2. Following a 24 hour fast, the animals were given an intravenous bolus of 1% glucose either alone or together with, 10 μg GLP-1 or 100 μg GLP-1. The injection volume was 0.5 ml in all cases. The animals were then given a preweighed amount of food and allowed to eat for 2 hours. The experiment was repeated three times so that all animals received each dose of GLP-1 in a crossover manner. Animals were allowed 72 hours between each study during which time they were fed *ad libitum*. In order to confirm that the GLP-1 was biologically active, a sample of blood was taken from each

rat before and 2 minutes after the intravenous injection for assay of insulin as described in chapter 2.

Both doses of intravenous GLP-1 caused a significant rise in insulin compared to glucose alone, confirming the incretin effect (Figure 7.5). Intravenous GLP-1 had no effect on 2 hour food intake at either dose (Figure 7.6). Thus the action of GLP-1 on food intake following ICV (Figures 7.3 and 7.4) injection is likely to occur through central rather than peripheral mechanisms.

Experiment 4.

In order to prove that the effect of GLP-1 on feeding was specific to GLP-1(7-36) amide, 10 μg of GLP-1 or 100 μg of several other fragments of GLP-1 including GLP-1 (8-36), GLP-1 (9-36), GLP-1 (11-36) and saline were administered ICV. 10μg GLP-1 (7-36) amide suppressed early dark phase feeding as previously, whereas larger doses of the other fragments had no effect on feeding compared to saline (Figure 7.7). This is good evidence that the effect of GLP-1(7-36) amide on feeding is specific to this peptide.

Experiment 5.

Before using exendin (9-39) to attempt blockade of endogenous GLP-1, it was necessary to demonstrate the ability of exendin to block exogenously administered GLP-1. Four groups of 24 hour fasted animals (12-14 in each group) were injected with saline, exendin alone, GLP-1 alone, or GLP-1 preceded by exendin. ICV administration of exendin (9-39) (100μg) blocks the inhibitory effect of GLP-1 (3μg) on food intake in fasted rats while exendin (9-39) alone does not affect food intake in the fasted state (Figure 7.8). This may be because endogenous GLP-1 levels are low when animals are fasting.

Legend to figures 7.5 and 7.6.

Intravenous injection of GLP-1 stimulated insulin release 2 minutes after administration, but had no effect on 2 hour feeding in 24 hour fasted rats.

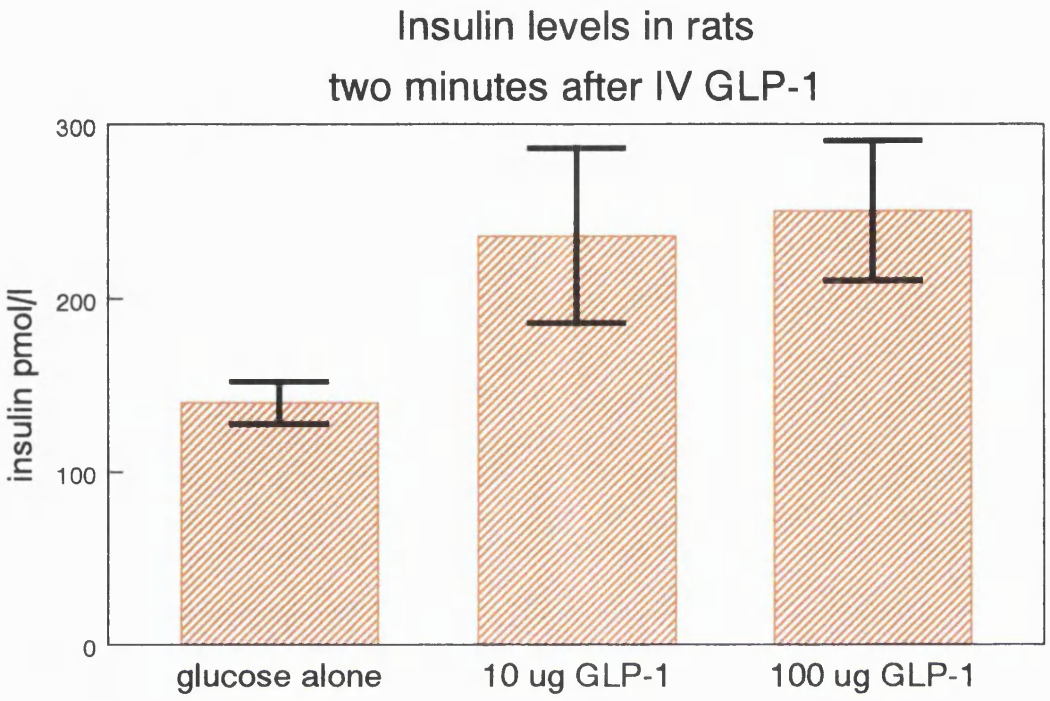


Figure 7.5

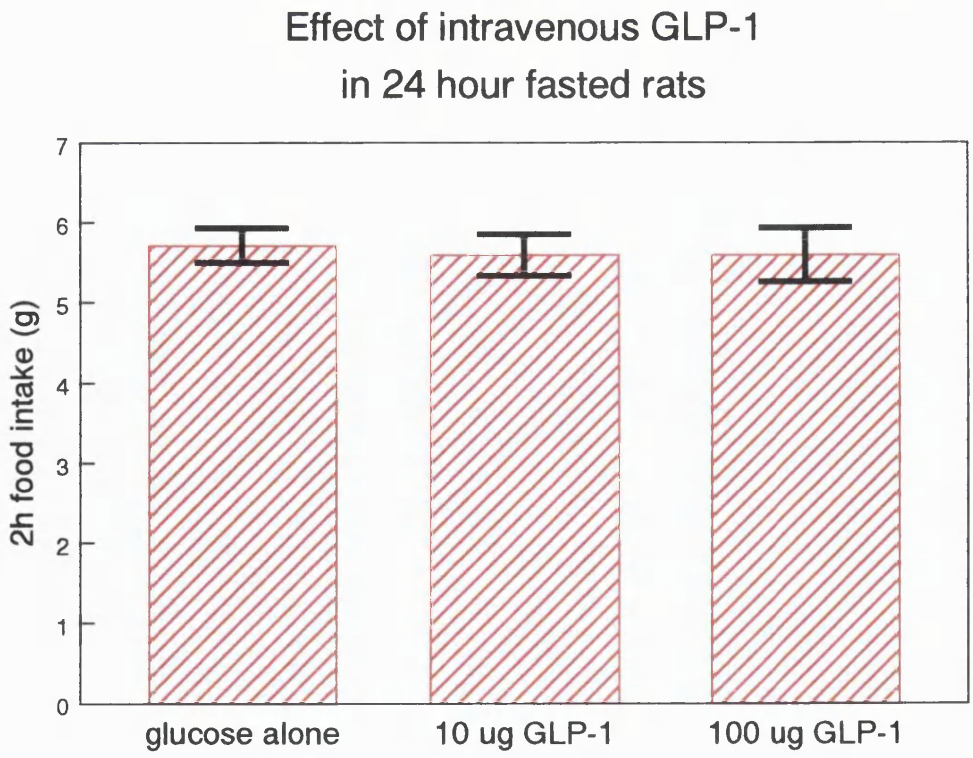


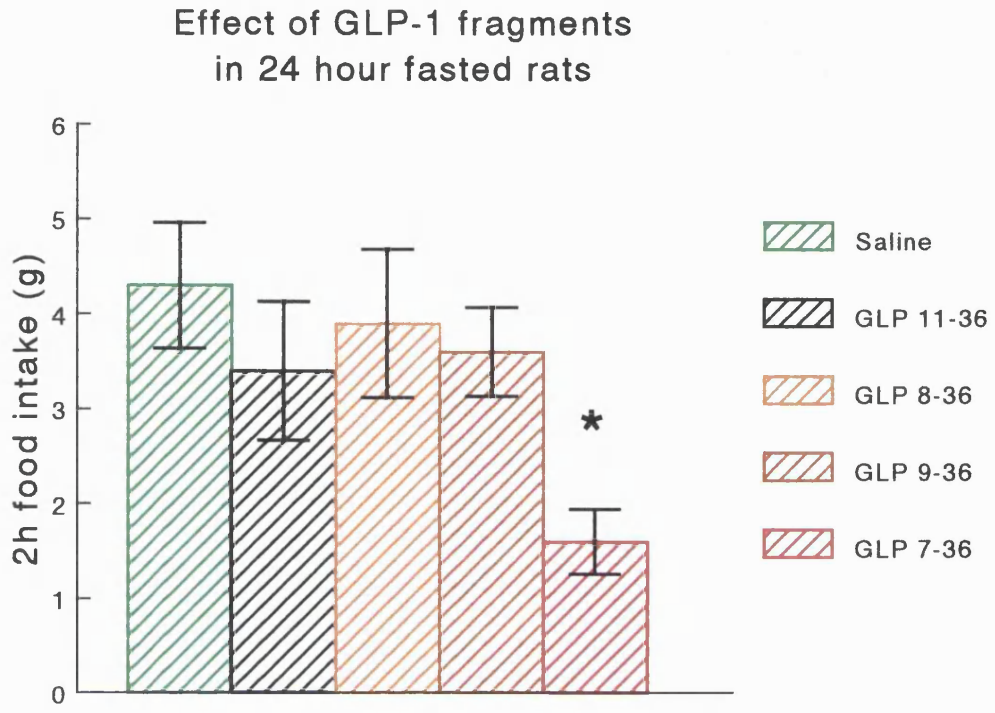
Figure 7.6

Legend to figure 7.7.

Various fragments of GLP-1 were administered by ICV injection to 24 hour fasted rats and the 2h food intake was measured. Only GLP-1 (7-36) amide (10 μg) suppressed feeding, all other fragments being inactive, even at a dose of 100 μg.

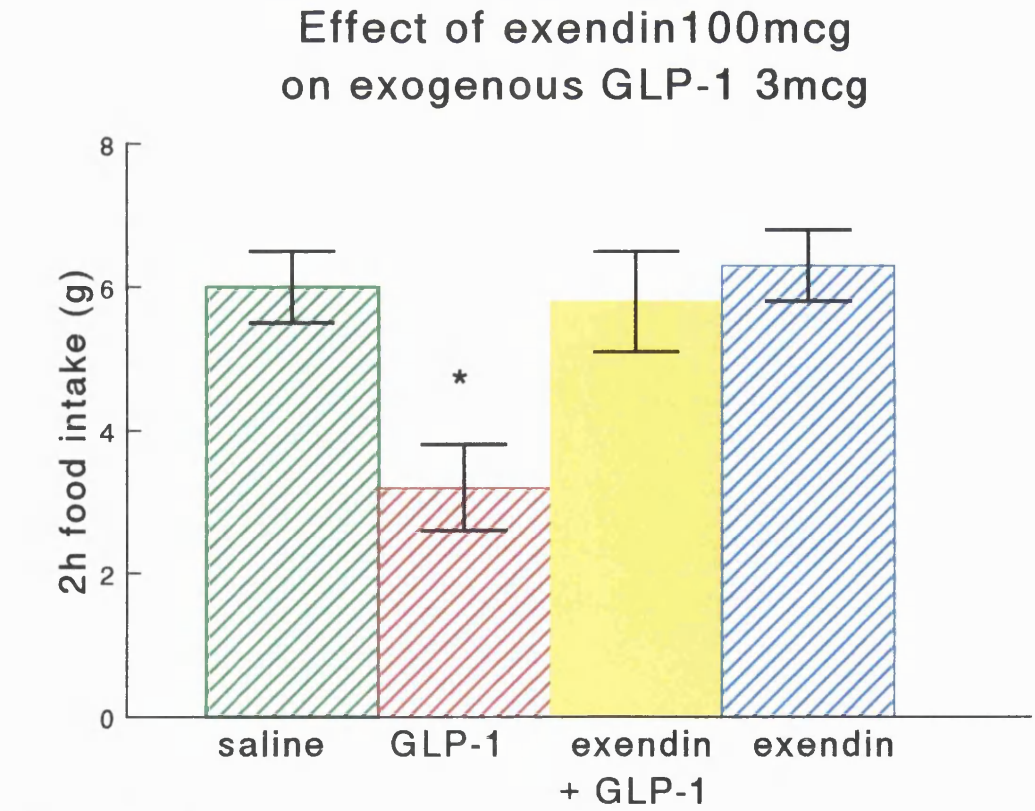
Legend to figure 7.8.

The effect of exendin (9-39) and GLP-1 (7-36) amide and the combination on 2h food intake in animals that have been fasted for 24 hours. Three micrograms of GLP-1 significantly suppressed feeding. This effect was blocked by co-administration of exendin (9-39).



* p< 0.05

Figure 7.7



* p<0.05

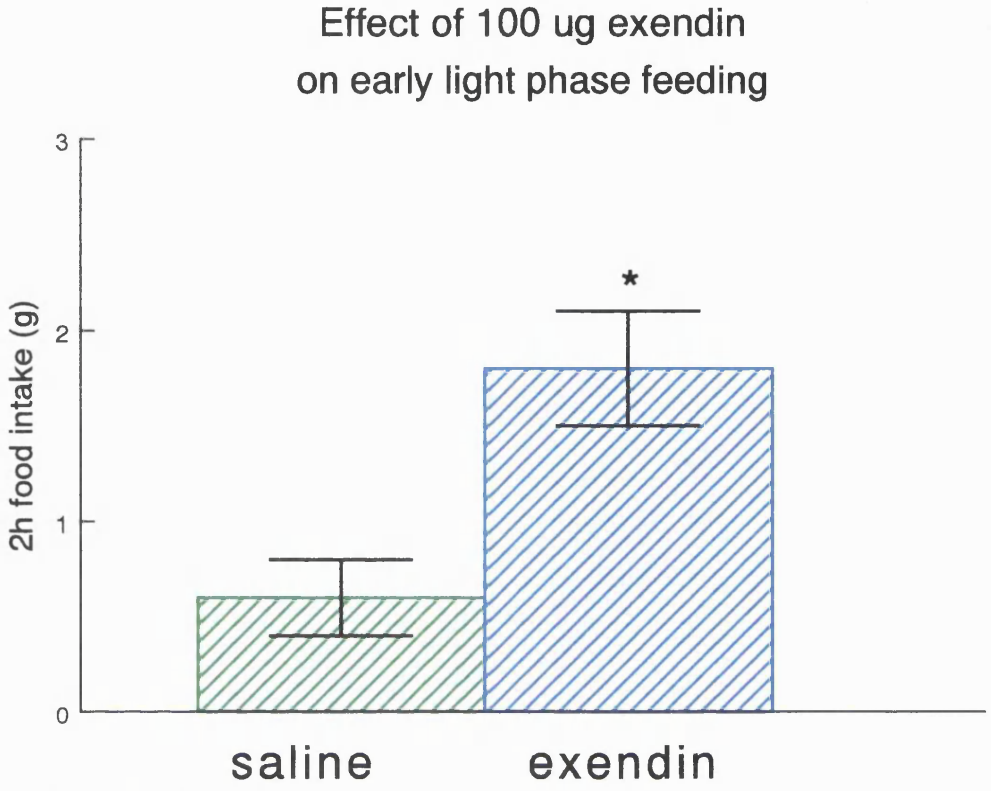
Figure 7.8

Experiment 6.

In order to determine whether endogenous GLP-1 has effects on feeding, and thus whether it is a *physiological* satiety factor, the effect of blockade of GLP-1 receptors in satiated animals (when endogenous satiety factors should be elevated) was assessed. Exendin (9-39) (100μg) was administered in the early light phase and was shown to treble food intake (Figure 7.9). The magnitude of the increase was only 1.2 g however.

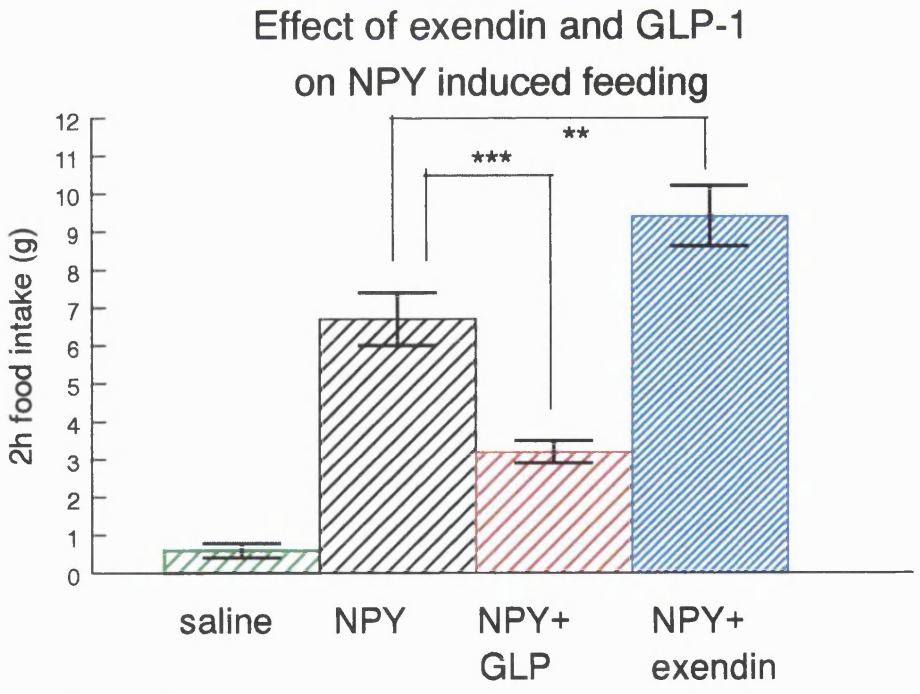
Experiment 7.

Exendin (9-39) had a marked effect in satiated animals (Figure 7.9), but not in fasted animals (Figure 7.8). This is either because endogenous GLP-1 levels are low when animals are fasting, or because the effect of exendin is masked when animals eat large amounts of food in 2 hours. To distinguish between these possibilities, exendin was administered to satiated animals that were stimulated to overeat by administration of NPY. In satiated animals, ICV administration of GLP-1(10μg) immediately prior to NPY (10μg) reduces the feeding response by over 50% (Figure 7.10). ICV administration of exendin (9-39) (100μg) immediately prior to NPY (10μg) significantly increases food intake compared to treatment with NPY (10μg) alone. The magnitude of the increase was 2.7g and surprisingly therefore, larger than when NPY was not used. I had expected the variability in feeding caused by NPY to possibly mask the effect of exendin (9-39). In fact the converse occurred. Thus the lack of effect of exendin (9-39) in fasting animals (Figure 7.8) is not due to the large amount of food consumed. These results strongly suggest that endogenous GLP-1 levels are low during fasting and high when animals are sated. This may be the reason that exendin (9-39) is most effective in sated animals.



* p<0.05

Figure 7.9



** p<0.01 ***p<0.001

Figure 7.10

Experiment 8.

NPY is the most potent stimulus to feeding yet discovered. Thirty micrograms (7.2 nmol) NPY is established as the maximal effect of NPY on feeding, 100 μg (24 nmol) being no more effective (see chapter 3). In order to determine whether exendin (9-39) could increase feeding over maximal NPY, NPY (7.2 nmol) was administered either alone or together with 30 nmol exendin (9-39) to sated animals at the beginning of the light phase and 2h food intake was measured. Animals co-administered exendin (9-39)(30 nmol) with the dose of NPY known to stimulate food intake maximally (7.2 nmol) ate significantly more (9.0±0.6g) than animals given this dose of NPY with saline (6.8±0.6g) ($p<0.02$).

Experiment 9.

The effects of stimulation and blockade of GLP-1 receptors in two models of appetite dysregulation, the dexamethasone treated (hyperinsulinaemic hyperglycaemic) rat and the genetically obese Zucker (*fa/fa*) rat have also been assessed.

9a) Twenty three Wistar rats were treated with dexamethasone by adding 10 mg dexamethasone to each 400 ml of drinking water. Twenty "control" animals were not given dexamethasone and studied in the same way. Water bottles were weighed daily. The rats drank on average, 40 ml water daily and hence had a daily dose of approximately 1mg dexamethasone. Animals were studied after they had received dexamethasone for three days. Glucose and insulin concentrations were measured in a subgroup (n=6) of the animals to confirm insulin resistance and hyperglycaemia. Seventeen dexamethasone and the twenty "control" animals were fasted for 24 hours and were given either GLP-1 (3 nmol) or saline ICV.

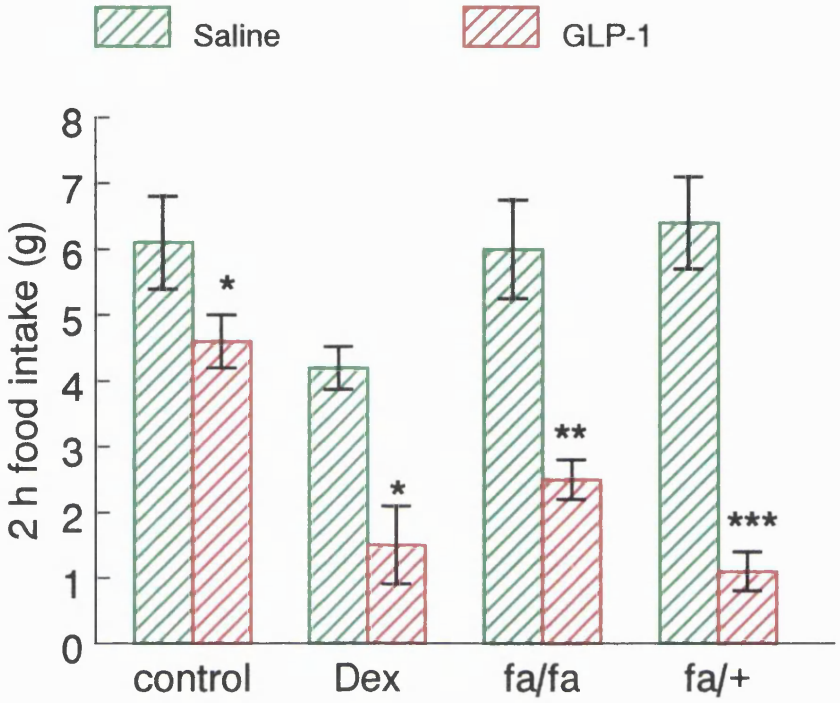
A further seventeen dexamethasone treated rats were studied at the beginning of the light phase when they were satiated and were given either exendin (9-39) (30nmol) or saline ICV. There was no difference in the water consumption and hence the dexamethasone intakes between any of the groups.

9b) Fifteen Zucker (*fa/fa*) rats and fifteen (*fa/+*) controls were cannulated and studied on two occasions. Initially they were given either exendin (9-39) (30nmol) or saline in the early light phase. Three days later they were re-randomised, fasted overnight and given either saline or GLP-1 (3 nmol) by ICV injection. After the experiments, the animals were *ad libitum* fed for 72 hours and then fasted for 24 hours before decapitation and collection of blood for measurement of plasma insulin and glucose to confirm the phenotype of the animals.

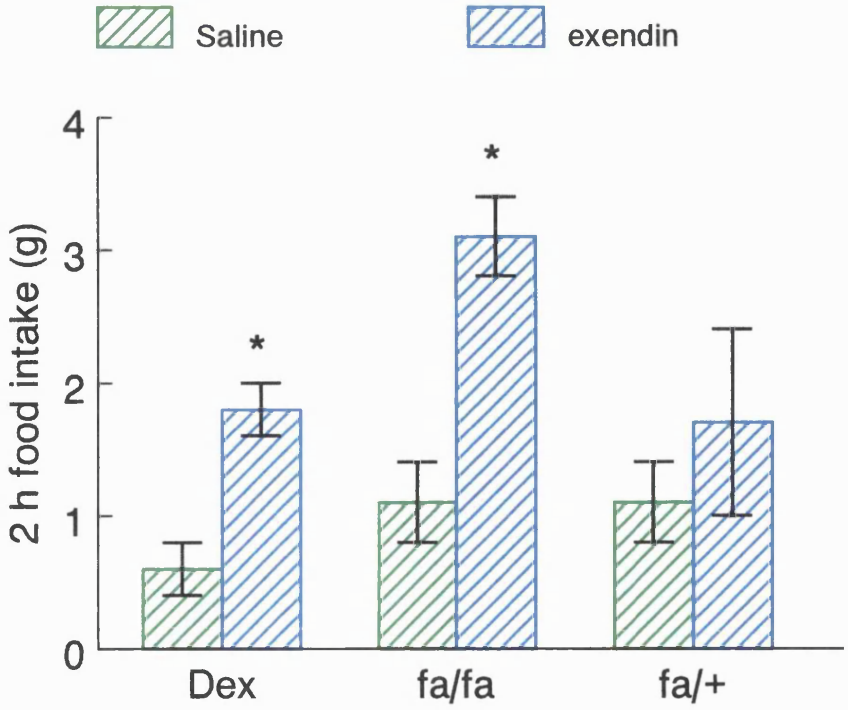
Dexamethasone treated rats were hyperglycaemic (fasting plasma glucose 10.1 ± 0.3 mmol/l) and hyperinsulinaemic (fasting plasma insulin 243.8 ± 35.3 pmol/l) and Zucker (*fa/fa*) rats were euglycaemic (fasting plasma glucose 5.7 ± 0.3 mmol/l) and hyperinsulinaemic (fasting plasma insulin 217.4 ± 32.6 pmol/l) compared to Wistar controls (fasting glucose 6.0 ± 0.2 mmol/l; fasting insulin 43.9 ± 3.5 pmol/l) and Zucker (*fa/+*) controls (fasting glucose 5.5 ± 0.6 mmol/l; fasting insulin 30.5 ± 3.8 pmol/l).

After a 24 hour fast, 3nmol GLP-1 suppressed 2h food intake by 65% (1.5 ± 0.6g vs 4.2 ± 0.3g) in dexamethasone treated Wistar rats and by 58% (2.5 ± 0.3g vs 6.0 ± 0.75g) in Zucker (*fa/fa*) rats compared to 85% (1.1 ± 0.3g vs 6.4 ± 0.7 g) in control Zucker (*fa/+*) rats and 25% (4.6 ± 0.4 vs 6.1 ± 0.7 g) in control Wistar rats (Figure 7.11).

In satiated rats, 30 nmol exendin (9-39) increased feeding approximately three fold in both Zucker (*fa/fa*) rats (1.1 ± 0.3g to 3.1 ± 0.3g) and in dexamethasone treated rats (0.6 ± 0.2g to 1.8 ± 0.2g) ($p<0.05$) but it had no significant effect on 2 hour feeding in control Zucker (*fa/+*) animals (1.1 ± 0.3 to 1.7 ± 0.7 g) (Figure 7.12).



* p<0.01 ** p<0.002 *** p<0.001 Figure 7.11



* p<0.05 Figure 7.12

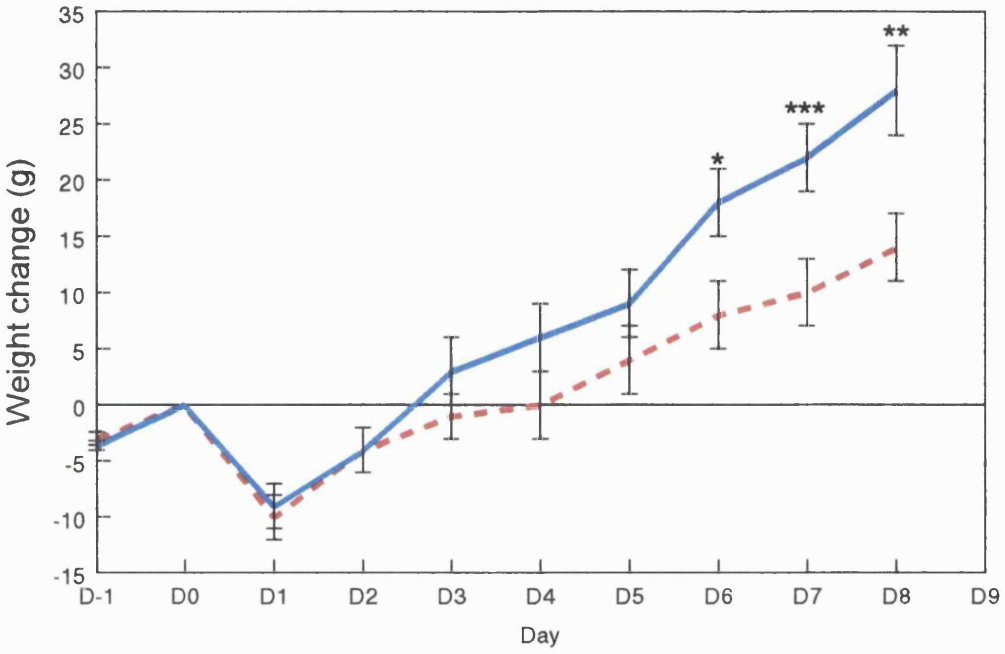
Experiment 10.

A further indication that GLP-1 may be a target for manipulation in conditions associated with abnormal feeding would be confirming that chronic blockade of central GLP-1 receptors is capable of causing a sustained increase in feeding and hence weight gain. To examine this 45 animals were divided into 2 groups: one group received 100μg exendin (9-39) with NPY 10μg and were compared to the second group of animals receiving NPY alone. Injections were given twice daily, at 9am (beginning of the light phase) and 5pm (end of the light phase), for eight days. Stimulated 2 hour food intake, 24 hour food intake and body weight were monitored. By the end of the study 20 animals in each group were still in the study. Body weight and food intake in the survivors only was assessed from the beginning of the study.

Animals given exendin (9-39) with NPY had a significantly greater increase in body weight over the eight day injection period (figure 7.13, $p<0.01$), a higher 24 hour food intake (Figure 7.14) and an increased 2 hour food intake following each injection (Figure 7.15) than animals given NPY with saline. When the 2 hour food intake over the eight days was integrated, and morning and evening injections were compared (figure 7.16), it was clear that NPY (2.4 nmol) induced feeding was similar in the morning (7.52 $\pm$ 0.33 g) and in the evening (7.38 $\pm$ 0.31 g), but that exendin (9-39) increased morning feeding by 43% (to 10.75 $\pm$ 0.43 g) and evening feeding by 27% (to 9.38 $\pm$ 0.44 g). Exendin (9-39) was significantly more effective when given in the early light phase (morning) than in the late light phase (evening) ($p<0.03$).

Legends to figures 7.13 and 7.14

The effect of twice daily injection of either 10 μg NPY alone (red line) or of a combination of 10 μg NPY with 100 μg exendin (9-39) on body weight (Fig 7.13) and 24 hour food intake (figure 7.14) over eight days.



* P<0.02 *** P<0.005 ** P<0.01

Figure 7.13

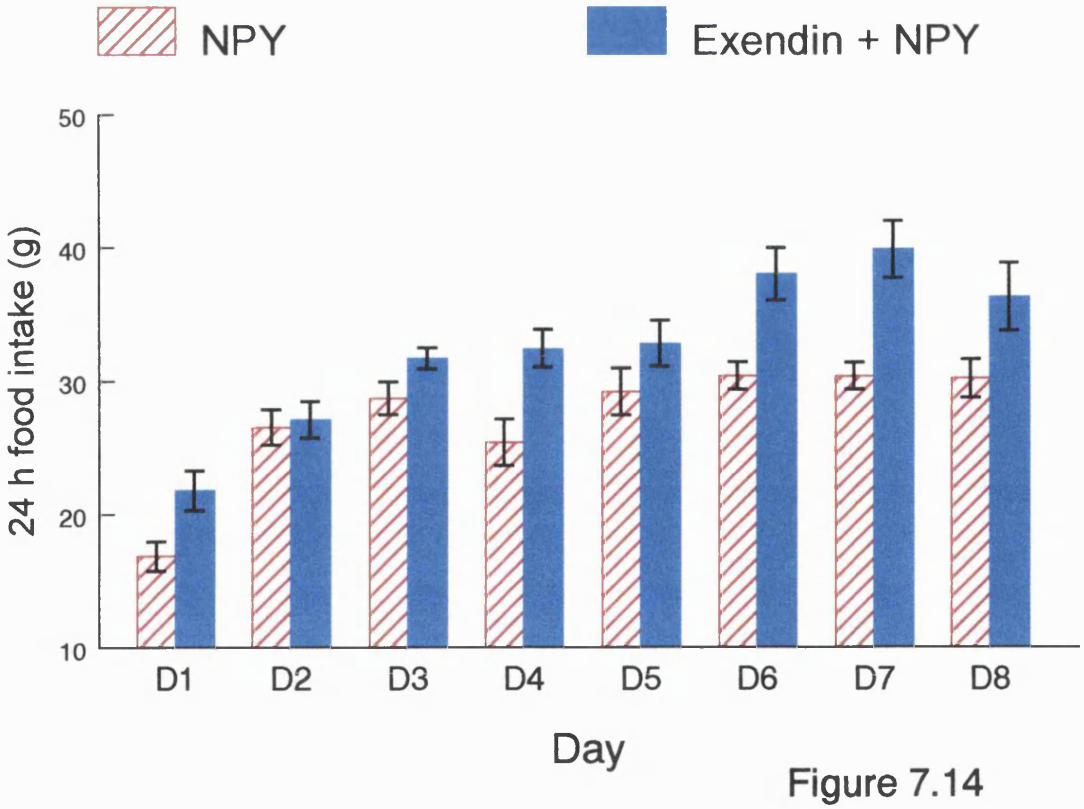


Figure 7.14

Legend to figure 7.15

Two hour food intake following each injection on each day, showing that the combination of exendin with NPY consistently and significantly stimulated feeding compared to NPY alone.

Legend to figure 7.16.

When the two hour food intake was integrated over eight days, and morning and evening injections were compared, it was clear that the effect of NPY on feeding was independent of time of day, whereas the effect of exendin (9-39) was greater in the morning.

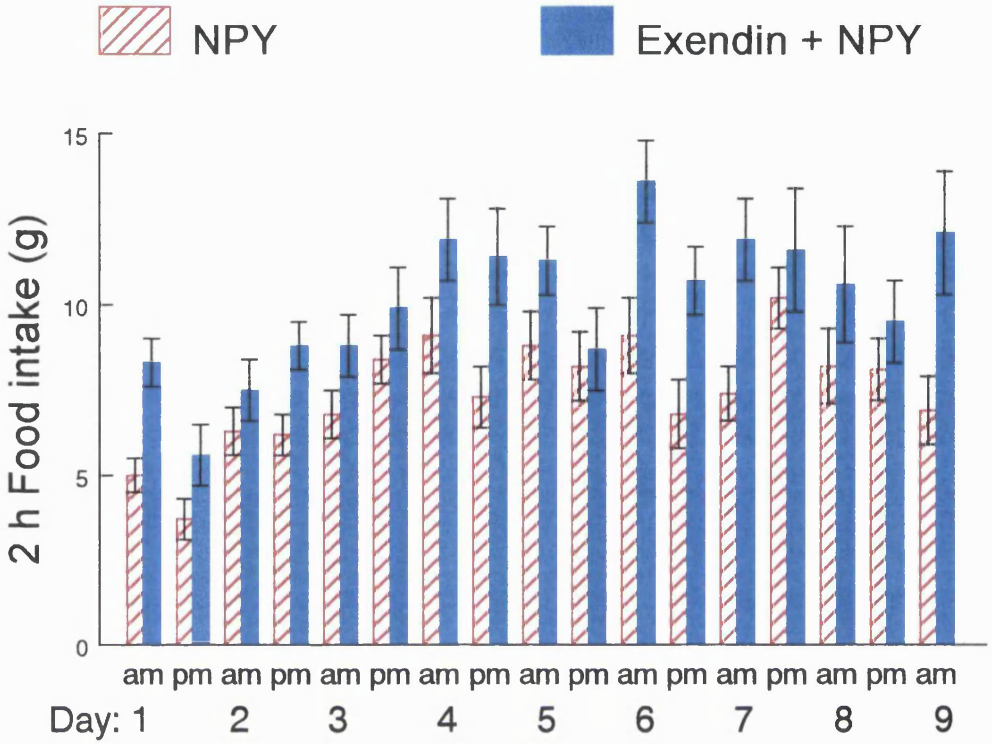


Figure 7.15

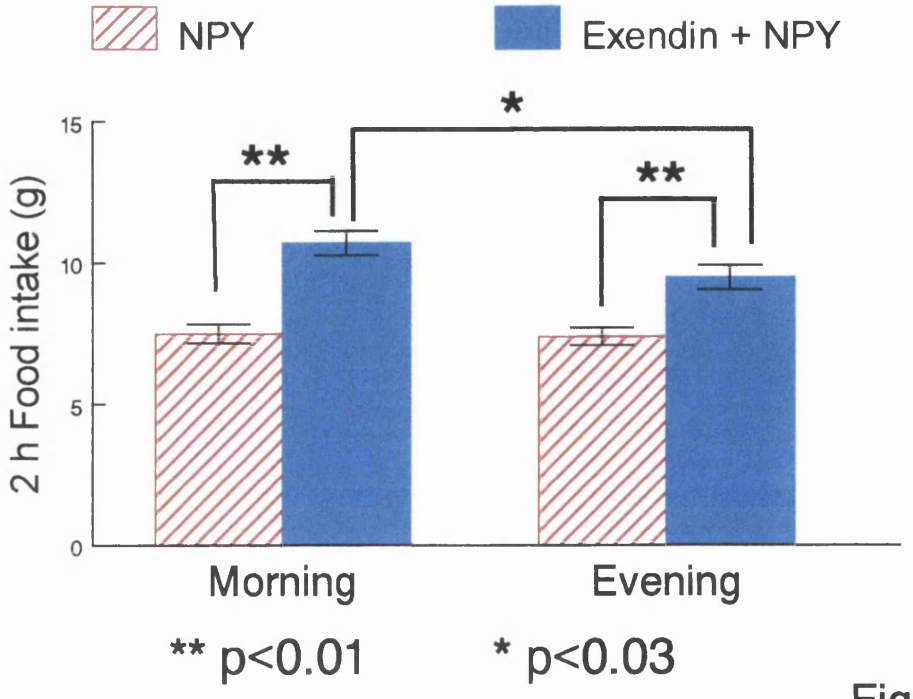


Figure 7.16

Discussion

The results of these studies provide strong evidence that endogenous GLP-1 is a physiological regulator of food intake and suggest that it may influence body weight. Endogenous peripheral GLP-1 has been shown to be important in the physiological regulation of circulating insulin and glucose (Wang et al. 1995). Exendin (9-39) has been shown to be a specific GLP-1 receptor antagonist in the periphery (Wang et al. 1995). GLP-1 binding is high in the paraventricular nucleus (PVN) of the hypothalamus, an area known to be involved in the regulation of food intake (Stanley and Leibowitz, 1984; Stanley and Leibowitz, 1985; Leibowitz et al. 1988). Radiolabelled GLP-1 and exendin-4 show specific binding within the hypothalamus, as determined by autoradiography (Goke et al. 1995). ^{125}IGLP-1 (17pM) binding in the PVN is inhibited in the presence of 5nM exendin (9-39) (S.A. Beak, personal communication, (Turton et al. 1996)). Exendin (9-39) is thus a GLP-1 receptor antagonist in the PVN.

NPY is the most potent stimulant of feeding yet described (Stanley and Leibowitz, 1984; Levine and Morley, 1984; Stanley and Leibowitz, 1985). I have shown that blockade of central GLP-1 receptors with exendin (9-39) causes a significant increase in the feeding induced by a dose of NPY reported to maximally stimulate feeding (chapter 3). This suggests that NPY and GLP-1 work through different pathways. The fact that exendin (9-39) is more effective on feeding in the morning (figure 7.16) may indicate that endogenous GLP-1 concentrations are higher at this time than in the evening. Exendin (9-39) did not have any effect on two hour food intake in fasted animals (figure 7.8), possibly because fasting causes a fall in endogenous hypothalamic GLP-1. Rats are known to consume most of their food at night (Jensen et al. 1983; Stewart et al. 1985).

Changes in the concentration of a central endogenous satiety factor such as GLP-1 may contribute to the diurnal rhythm in food intake exhibited by ad libitum fed rodents.

The body weight gain and sustained increase in food intake that follows the nine day administration of exendin (9-39) to block GLP-1 receptors indicates that escape from the effect of exendin (9-39) did not occur in the presence of NPY induced hyperphagia. Blockade of GLP-1 receptors also acutely stimulated feeding over and above the maximum feeding effect of NPY. These findings suggest that blockade of the action of GLP-1 in the brain is capable of overriding powerful short-term and long-term factors which control body weight and satiety.

Leptin, the protein product of the *ob* gene, has been postulated to be a circulating signal linking fat mass to the hypothalamic control of energy balance (Schwartz et al. 1996; Stephens et al. 1995; Campfield et al. 1995). The mechanism of the effect of leptin on feeding is still unknown. It has been suggested that part of its action occurs through inhibition of NPY biosynthesis and release (Schwartz et al. 1996; Stephens et al. 1995). Since leptin is effective in animals that do not express NPY (Erickson et al. 1996), other systems must be involved in certain circumstances. The effect of leptin on GLP-1 synthesis and release is unknown and requires further investigation. Leptin is known to inhibit feeding for over 24 hours (Campfield et al. 1995; Stephens et al. 1995) following ICV injection whereas the action of GLP-1 is much shorter than this. It is possible that leptin acts in part through stimulation of hypothalamic GLP-1 release and hence causes weight loss.

Both GLP-1 and exendin (9-39) were effective in the Zucker (*fa/fa*) rat which has a leptin receptor defect (Phillips et al. 1996). Both exogenous and endogenous GLP-1 are thus effective in these animals, suggesting that the obesity caused by a non-functional leptin receptor is not due to GLP-1 receptor down-regulation or a reduced post GLP-1 receptor action. This suggests that the action of GLP-1 is either distal to or independent of a functionally active leptin receptor.

Exogenous GLP-1 was particularly effective in the (*fa/+*) controls. Exendin (9-39) did not significantly increase 2 hour feeding in satiated (*fa/+*) controls. It is possible that endogenous GLP-1 levels are not as high in these (*fa/+*) controls and conversely, that GLP-1 levels are particularly high in Zucker (*fa/fa*) animals. If this finding is genuine, it would suggest that the obesity caused by a non-functional leptin receptor (Phillips et al. 1996) is not due to reduced levels of GLP-1 and hence that leptin does not directly increase central endogenous GLP-1 activity. However, it is important to note that the error bar in the (*fa/+*) group is particularly wide (figure 7.12). In addition, preliminary data suggests that the decreased feeding caused by ICV leptin can be reversed by ICV exendin (A.P. Goldstone, personal communication). Further studies are needed to determine whether leptin does indeed work through stimulation of GLP-1 release.

The fact that GLP-1 is effective in both Wistar rats treated with dexamethasone and in the Zucker (*fa/fa*) rat indicates that GLP-1 is active in instances when endogenous NPY levels are raised. While these models do not mimic all the metabolic aspects or behaviour of human type 2 diabetes, they are recognised models of severe insulin resistance which characterises the disease in man.

Continuous central infusion over eight days using a subcutaneously implanted osmotic minipump may have been preferable to repeated injections as used in experiment 10. Continuous infusion has been effective when peptides are stable (Catzeflis et al. 1993; Pierroz et al. 1996). In my experience, GLP-1 and exendin (9-39) are unstable at room temperature for more than four hours, and for this reason, these peptides have to be dissolved in saline immediately before each ICV injection. Repeated central injection of noradrenaline has been shown to increase daily food intake and body weight over five days (Leibowitz et al. 1984) and thus I used a similar protocol here.

Novel stresses decrease 24 hour food intake in rats. ICV cannulation and the stress of daily injection may reduce feeding. I minimised these effects by habituating the animals to the experimental procedures as far as possible with repeated daily handling and sham injections before the start of the study. There was a small fall in body weight after the first active ICV injection in experiment 10 although animals started to increase their body weight after subsequent injections.

Together, the effect of blockade of GLP-1 on NPY induced feeding and weight gain, along with the action of GLP-1 in the Zucker (*fa/fa*) rat and the dexamethasone treated rat indicate that GLP-1 receptors may be a therapeutic target in the management of feeding disorders. As with the NPY receptor (chapter 3), it is important to establish whether there are different GLP-1 receptors that mediate different effects of this peptide. In particular, the development of GLP-1 receptor agonists to target overeating may have effects on the pancreatic GLP-1 receptor and hence unwanted effects on insulin secretion. Although central and peripheral GLP-1 receptors resemble each other (Goke

et al. 1995), there is some evidence for heterogeneity of GLP-1 receptors. Rat adipose tissue has been shown to express GLP-1 receptors with high and low affinity (Valverde et al. 1993). In addition recent evidence presented at the American Diabetes Association meeting and The International Congress of Endocrinology in June 1996 suggests that targeted disruption of the GLP-1 receptor (a GLP-1 receptor "knockout mouse") significantly affects glucose metabolism (Scrocchi et al. 1996) but has no effect on food intake or weight gain (Scrocchi et al. 1996). The authors suggest that there may be a novel hypothalamic GLP-1 receptor that is highly related yet distinct from the pancreatic GLP-1 receptor (Scrocchi et al. 1996). Others have shown that muscle cells and pancreas express distinct GLP-1 receptors (Yang et al. 1996). Fragments of exendin 4 other than exendin (9-39) have also been shown to be antagonists to GLP-1 receptors stably transfected into CHO cells (Montrose-Rafizadeh et al. 1996). In particular the fragment exendin (5-39) was shown to be 10 times more potent than exendin (9-39) at inhibiting a GLP-1 induced rise in intracellular cAMP (Montrose-Rafizadeh et al. 1996). Preliminary studies with ICV exendin (5-39) suggest that this peptide does not block the effects of ICV GLP-1 on feeding whereas exendin (9-39) remains completely active (C.M.B. Edwards, personal communication). Other fragments and analogues of GLP-1 and the exendins may be useful in determining whether there are indeed further GLP-1 receptors. Indeed Eng (Eng, 1996) has generated chimaeric peptides of GLP-1 and exendin in order to determine which amino acids are important in agonist and antagonist activity. Such peptides should be studied for their effects on feeding and for their action on pancreatic insulin secretion in order to find selective agonists and antagonists for each receptor, in much the same way that fragments of NPY have been used to distinguish the NPY feeding receptor from the one that mediates ACTH release (chapter 3).

GLP-1 is as potent a physiological inhibitor of feeding as has yet been described. The studies in this chapter show that it is active in established models of hyperphagia and also when central NPY concentrations are raised. Future development of centrally acting GLP-1 agonists may ultimately lead to novel agents for the management of obesity.

Chapter 8

The effect of ICV GLP-1 on expression of hypothalamic mRNA to peptides involved in the control of feeding.

The mechanism of action of GLP-1 on feeding is unknown. As it has been shown it to be a potent physiological inhibitor of feeding, the effect of chronically administered ICV GLP-1 on NPY mRNA was investigated. NPY is the most potent stimulus to feeding known, and it was therefore possible that GLP-1 reduced feeding by reducing the synthesis of NPY. GLP-1 is unstable at 37°C, and therefore chronic ICV infusion is not possible. For this reason, newly dissolved GLP-1 was administered repeatedly. Animals were injected 48, 24, 12 and 4h before the hypothalamus was collected.

Method:

Animals were ICV cannulated and handled as described in chapter 2. Four groups of animals, with 12 animals in each group were used. In order to determine whether GLP-1 has any effect on NPY mRNA either in the fasted or the sated state, two groups were fasted for 72 hours while the other two groups were fed ad libitum, and GLP-1 was administered as in the table below.

Group 1: Fasted	saline
Group 2: Fasted	GLP-1
Group 3: Ad lib fed	saline
Group 4: Ad lib fed	GLP-1

Fasting is known to increase hypothalamic NPY mRNA (White and Kershaw, 1989; Sanacora et al. 1990; Sahu et al. 1988) so that the comparison of the fasted with the sated animals serve as an internal control.

The animals were administered with GLP-1 (50μg) or saline ICV 48, 24,12 and 4h before the hypothalamus was collected and total RNA extracted. Hypothalamic NPY mRNA was measured by northern blot analysis as described in detail in chapter 2. In addition, the northern blots were probed for galanin, neurotensin, substance P (pre-pro tachykinin A) and somatostatin mRNA. The filters were stripped between each reprobing. Finally the filters were probed with oligo dT to quantify total mRNA. The counts were corrected for total mRNA and the results are presented in figure 8.1.

Fasting increased NPY mRNA to 150 ± 21 % ($p<0.05$) compared to satiated animals (100 ± 18%). GLP-1 had no effect on NPY mRNA in either fasted or satiated animals. These results indicate that the inhibition of feeding seen after ICV GLP-1 is not mediated by changes in hypothalamic NPY mRNA. Fasting significantly increases NPY mRNA, but has no effect on hypothalamic galanin, neurotensin, substance P or somatostatin mRNA.

Although this study shows that the synthesis of NPY and the other peptides assessed is unchanged by ICV GLP, the effect of GLP-1 on secretion or storage of NPY has not been studied. Secretion of NPY can be measured either by microdialysis or by a push-pull technique, where small amounts of saline are injected into the PVN and withdrawn

and assayed for NPY. It is also possible that GLP-1 can affect NPY by altering the rate of NPY degradation.

Effect of fasting and chronic ICV GLP-1 on mRNA levels

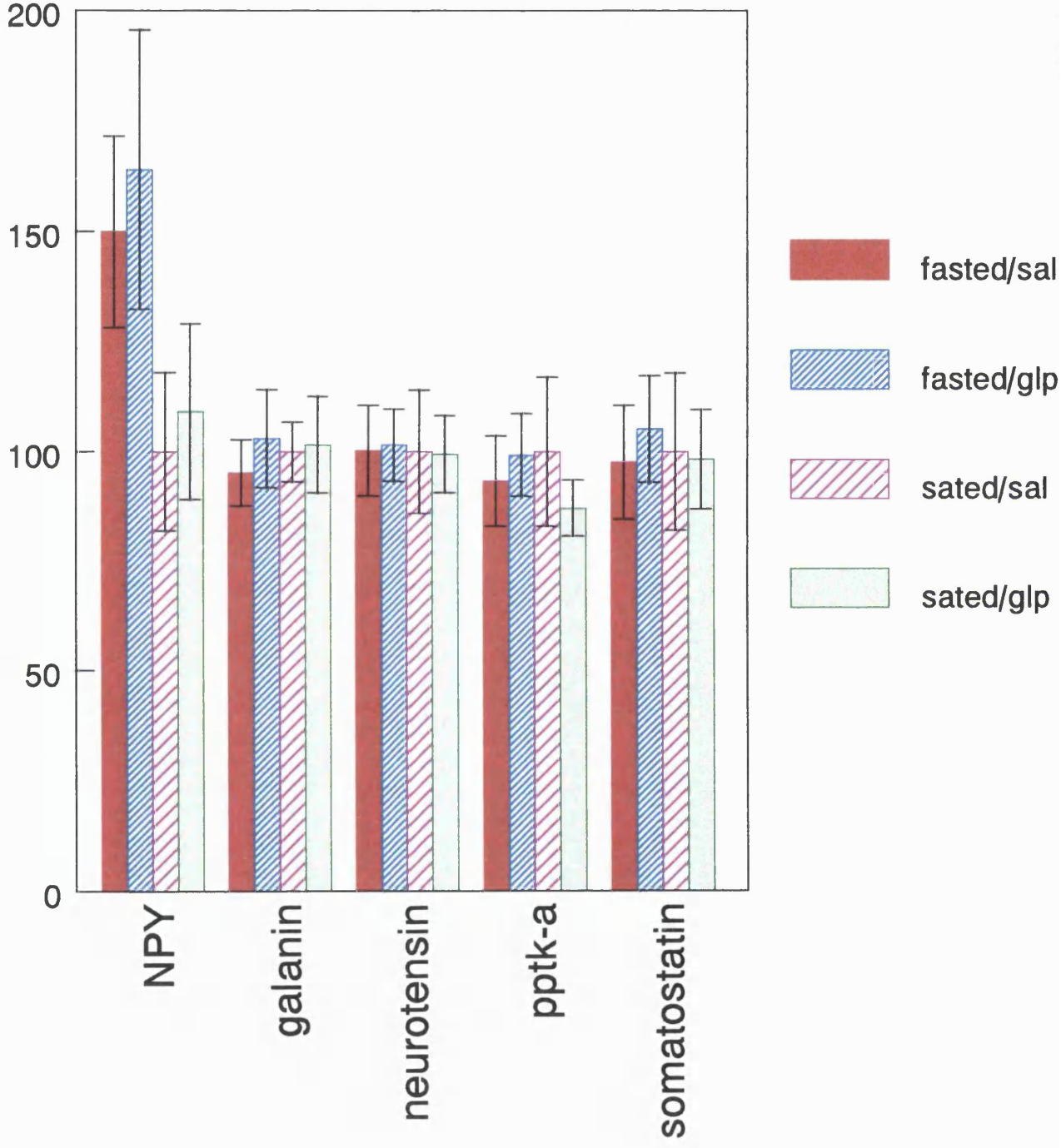


Figure 8.1

Nevertheless, chronic effects on feeding will be expected to affect the rate of synthesis of NPY, which is best measured by assessing mRNA levels. In-situ hybridisation is an alternative method of measuring mRNA which may be more sensitive when the mRNA is expressed only in single nuclei. Total hypothalamic NPY mRNA changes were measured as fasting is known to increase this (White and Kershaw, 1989; Sanacora et al. 1990; Sahu et al. 1988). In addition, it is difficult to accurately quantify mRNA levels when using in situ hybridisation.

The mechanism of action of GLP-1 in food intake remains unclear. It is unlikely to work through leptin, as the duration of action of leptin is longer than GLP-1. Further studies need to be carried out, in particular looking at secretion of NPY and GLP-1 from the hypothalamus using either push-pull cannulae or microdialysis.

Chapter 9

The effect of central injection on GLP-1 on peripheral glucose metabolism.

The work in this chapter was carried out in collaboration with Miss Melanie Heath, a PhD student working on the hypothalamic control of glucose metabolism.

The central nervous system has been known to have effects on peripheral glucose metabolism since Claude Bernard produced diabetes in dogs by puncturing the floor of the fourth ventricle with a trocar (Bernard, 1849). The mechanism of such control is still not clear but may include effects on the autonomic nervous system, the hypothalamo-pituitary adrenal axis (HPA), and also on other non-adrenergic non-cholinergic nerves.

Several peptides have been shown to have effects on glucose metabolism following ICV injection. Neuropeptide Y (NPY) affects many aspects of metabolism including feeding (Clark et al. 1984), changes in peripheral insulin sensitivity (Wilding et al. 1995; Zarjevski et al. 1993; Zarjevski et al. 1994) and changes in plasma ACTH and corticosterone (see chapter 3 and (Haas and George, 1987; Miura et al. 1992; Suda et al. 1993)). Bombesin (Brown et al. 1979), somatostatin (Spencer et al. 1991), corticotrophin releasing hormone (CRH) (Brown et al. 1982), beta-endorphin (Radosevich et al. 1989), glucagon (Marubashi et al. 1985) and growth hormone releasing hormone (GHRH) (Tannenbaum, 1984) all increase plasma glucose following central injection, but no peptide has previously been shown to decrease plasma glucose in rodents following ICV injection. ICV insulin prevents the rise in hypothalamic NPY after a fast (Schwartz et al. 1992). An ICV injection of insulin to dogs causes an acute rise in plasma glucose which did not occur in adrenalectomised animals (Agarwala et al. 1977). This effect did not occur in spinal cord transected-vagosympathectomised

animals, suggesting that the rise in glucose occurs through a neuronal reflex, possibly causing an acute rise in catecholamines.

Peripheral GLP-1 is a potent physiological gluco-incretin (Wang et al. 1995). This discovery was made because of the presence of the GLP-1 receptor antagonist exendin (9-39) (Goke et al. 1993; Thorens et al. 1993). Using the same antagonist, I have shown in chapter 7 that central GLP-1 is a physiological satiety factor and that chronic blockade of central GLP-1 receptors can cause weight gain.

The effects of central injection of GLP-1 on peripheral glucose metabolism have not been investigated. We injected both GLP-1 and exendin (9-39), intracerebroventricularly (ICV) and measured plasma glucose, insulin and glucagon in order to determine whether central GLP-1 has any role in the control of glucose metabolism.

Materials and methods.

Cannulation of animals, ICV injection, decapitation and blood collection were carried out as described in detail in chapter 2. After ICV injection, animals were decapitated and trunk blood was collected in plastic lithium heparin tubes containing 0.6 mg aprotinin. Plasma was separated by centrifugation, frozen immediately on dry ice and stored at -20°C until assay. Plasma glucose was measured using a YSI 2300 glucometer and plasma insulin and glucagon were measured by radioimmunoassay as described in chapter 2. A total of five separate studies were carried out, the first three in satiated rats and the last two in fasted rats.

Study 1: This was a time course study in order to determine the optimum time to detect changes in plasma glucose after ICV injection. Satiated rats were injected ICV with 3 nmol GLP-1, saline, [Pro^{34}]NPY or saline and decapitated at 10, 30 or 60 minutes post injection. The optimum effect of GLP-1 was seen at 10 minutes after ICV injection, and this time point was used for all subsequent studies.

Study 2: Dose response study.

Satiated rats were injected with 0.03, 0.1, 0.3, 1.0, 1.5, 3.0, or 6.0 nmol of GLP-1 or saline and decapitated 10 minutes later.

Study 3: In order to determine whether endogenous GLP-1 has a physiological role in the control of glucose metabolism, the potent GLP-1 receptor antagonist, exendin (9-39) was administered at a dose of 30 nmol to satiated animals. Animals received either saline, 3.0 nmol GLP-1 or 30 nmol exendin (9-39). This dose of exendin (9-39) has been shown previously to inhibit endogenous GLP-1 (see chapter 7). Animals were decapitated 10 minutes following ICV injection.

Study 4: The effect of GLP-1 in fasted animals was determined by ICV injection of 6.0 nmol GLP-1 or saline into animals that had been fasted for 24 hours.

Study 5:

Animals were treated with oral dexamethasone in order to render them insulin resistant. Dexamethasone 21 phosphate (Sigma) was added to the drinking water at a concentration of 25 mg/l. Average water consumption was 40 ml per day and each rat therefore received approximately 1 mg dexamethasone daily. Water bottles were weighed daily. Animals were given dexamethasone for three days before the study and were fasted for 24 hours prior to the study day, when they received an ICV injection of either saline or 6.0 nmol of GLP-1. There was no difference in the amount of water and hence dexamethasone consumed by each group of rats.

Results are shown as mean ± SEM. Comparisons between groups of data were made using an unpaired student's t-test.

Results.

Study 1.

An ICV injection of 3.0 nmol GLP-1 caused a significant fall in plasma glucose (figure 9.1) after 10 minutes compared to saline control (saline: 8.08 ± 0.3; GLP-1: 6.15 ± 0.4; $p<0.05$) with no significant change in insulin (figure 9.2) (saline: 181.3 ± 27.3; GLP-1: 110.0 ± 34.9), indicating that the fall in glucose was independent of insulin. There was no difference in glucose at any other time point. NPY and [Pro^{34}]NPY both caused a significant increase in insulin and served to confirm that the ICV injections were effective (Figure 9.2). Glucagon concentrations were not affected in this or any subsequent study.

Study 2.

Following ICV injection of GLP-1, a significant fall in blood glucose was noted with 1.5 nmol (7.26 ± 0.7; $p<0.005$), 3.0 nmol (7.32 ± 0.24; $p<0.01$) and 6.0 nmol (7.37 ± 0.11; $p<0.005$) compared to saline controls (8.22 ± 0.22) . Doses up to and including 1.0 nmol (8.16 ± 0.17) did not have any significant effect on plasma glucose. There was no significant change in plasma insulin levels at any of the doses of GLP-1 when compared to saline control.

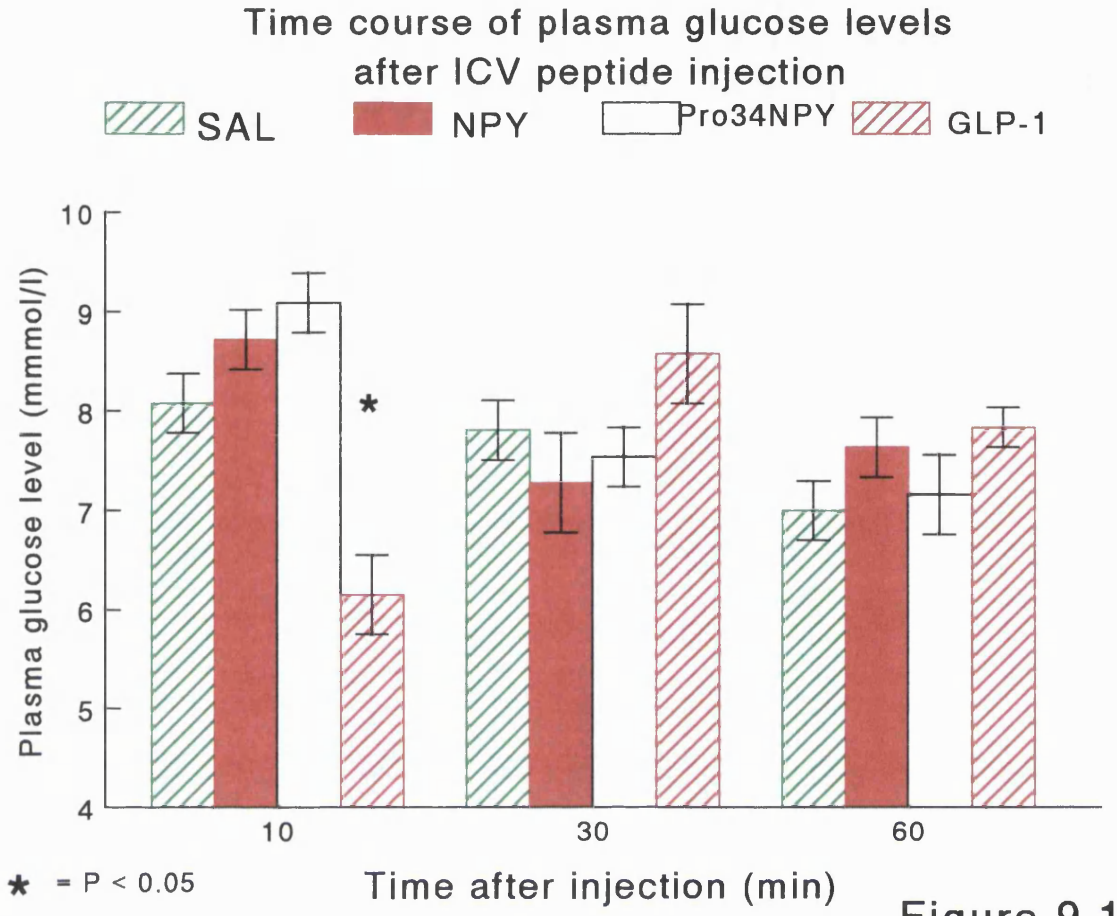


Figure 9.1

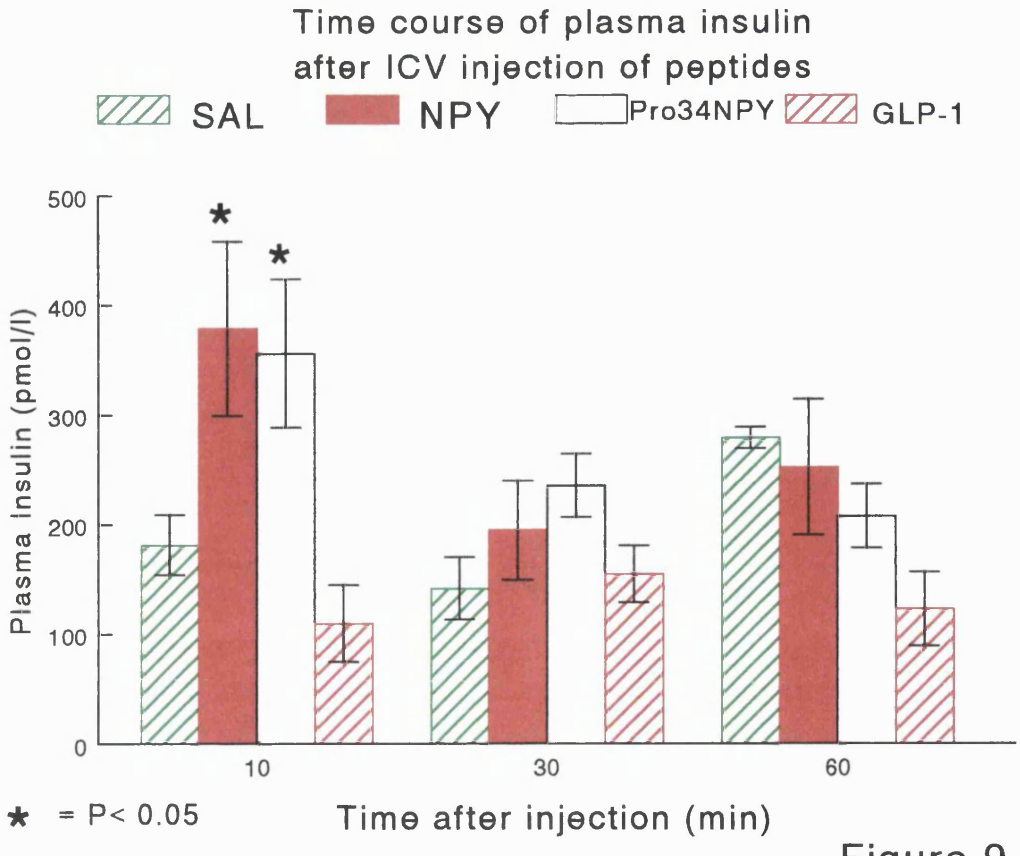


Figure 9.2

Study 3.

ICV administration of 3.0 nmol GLP-1 caused a significant fall in plasma glucose (saline: 8.27 ± 0.23; GLP-1: 7.01 ± 0.27; $p<0.01$) with no significant change in plasma insulin. The potent GLP-1 receptor antagonist exendin (9-39) caused a significant increase in plasma glucose (9.11 ± 0.28; $p<0.05$) with no significant change in plasma insulin, (Figure 9.3). The effect of exendin (9-39) on plasma glucose suggests blockade of endogenous GLP-1 levels and therefore a physiological response.

Study 4.

ICV administration of 6.0 nmol of GLP-1 into 24 hour fasted rats had no effect on plasma glucose levels (saline: 5.99 ± 0.23 mmol/l; GLP-1: 5.74 ± 0.21mmol/l) although there was a significant rise in plasma insulin (saline: 43.94 ± 3.51mmol/l; GLP-1: 83.96 ± 12.23mmol/l; $p<0.01$). (Figure 9.4).

Study 5.

Dexamethasone treatment resulted in hyperglycaemia and hyperinsulinaemia typical of a type 2 diabetic model. There was no significant change in plasma glucose levels following ICV administration of 6.0 nmol GLP-1 into 24 hour fasted animals when compared to saline control (saline: 10.1 ± 0.3 mmol/l; GLP-1: 10.4 ± 1.0mmol/l). There was a significant increase in plasma insulin concentrations (saline: 243.83 ± 35.23mmol/l; GLP-1: 390.48 ± 51.6mmol/l; $p<0.05$).

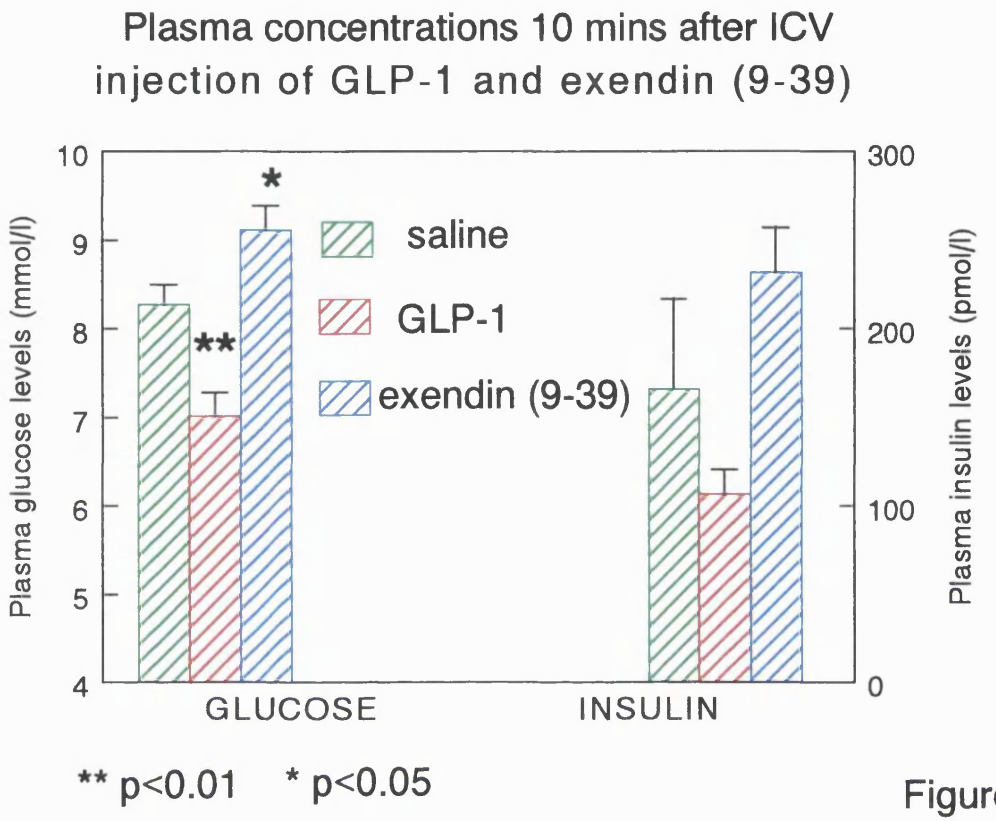


Figure 9.3

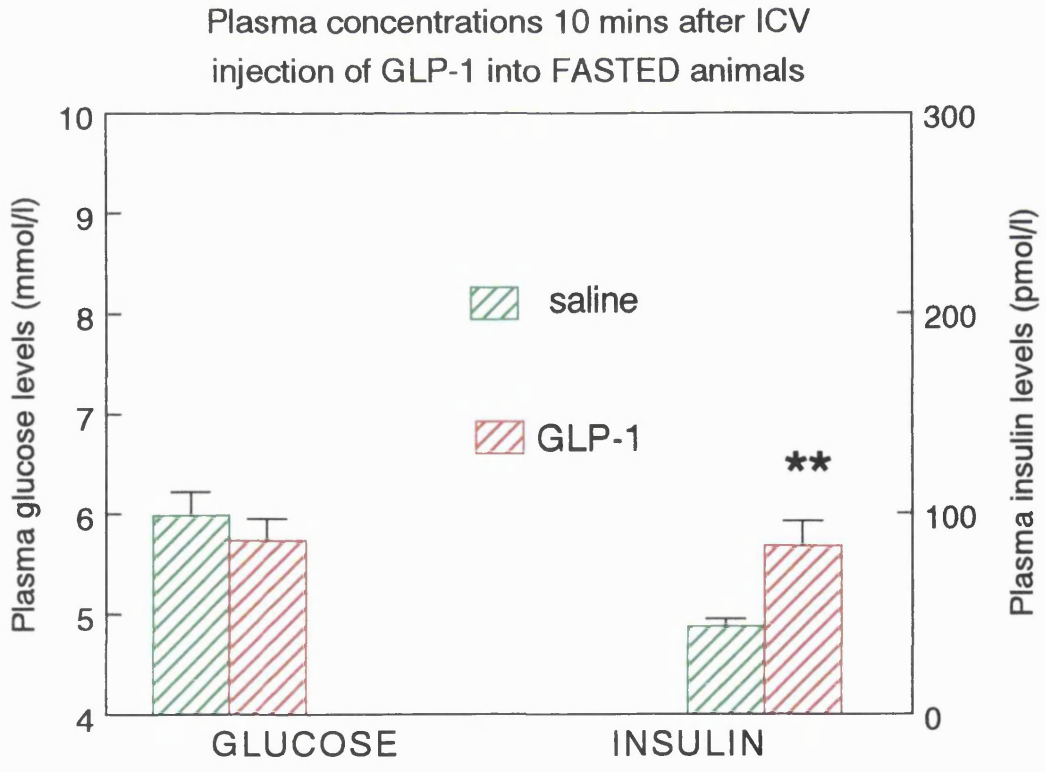


Figure 9.4

Discussion

The reduction in plasma glucose demonstrated after ICV administration of GLP-1 is the first example of a centrally administered peptide acutely reducing plasma glucose. Exendin (9-39), the potent GLP-1 receptor antagonist, when administered ICV, caused an increase in plasma glucose implying a potential physiological role for GLP-1. ICV GLP-1 while reducing plasma glucose, had no significant effect on plasma insulin or glucagon levels indicating that this effect on glucose is both insulin and glucagon independent. In fact there was a fall in plasma insulin that did not achieve statistical significance at the 5% level. GLP-1 caused a similar non-significant fall in insulin in the three satiated studies (studies 1, 2 and 3). It is therefore likely that ICV GLP-1 causes both a fall in glucose and a true resultant fall in insulin, suggesting that the action of ICV GLP-1 is to sensitise the animal to the action of insulin in some way. I have not assessed the mechanism whereby ICV GLP-1 causes this fall in plasma glucose and insulin at 10 minutes. Plasma glucose levels are influenced by several factors, including autonomic innervation of the liver (Pascoe et al. 1989; Lautt, 1979; Shimazu, 1971; Shimazu and Amakawa, 1968) and pancreas (Bloom and Edwards, 1981; Kaneto et al. 1975; Bloom, 1976) and circulating concentrations of several hormones including insulin, glucagon (Unson et al. 1989), adrenaline (Rousseau Migneron et al. 1975; Bugajski et al. 1978) and corticosterone (Billaudel and Sutter, 1982) and other corticosteroids (Bruno et al. 1994; Pagano et al. 1983). The hypothalamus can thus influence circulating glucose concentrations through several mechanisms (Porte, Jr. et al. 1975).

Sympathetic and parasympathetic control of the liver and pancreas is a well established mechanism for the control of glucose metabolism (Niijima, 1989; Pascoe et al. 1989). GLP-1 may be altering the balance between sympathetic and parasympathetic activation resulting in decreased hepatic glucose output and increased glucose disposal via skeletal muscle. The sympathetic nervous system is known to influence mesenteric artery diameter (Muramatsu et al. 1984; Muramatsu et al. 1989), and can hence alter rate of glucose absorption from the small intestine. The fall in plasma glucose that occurs in the satiated but not in the fasted state may indicate that the effect of GLP-1 relies either on high hepatic glycogen stores or a high gastrointestinal glucose absorption rate.

Administration of oral dexamethasone resulted in a hyperglycaemic hyperinsulinaemic model, typical of type 2 diabetes. In the fasted state GLP-1 caused an increase in plasma insulin but no reduction in glucose was seen. Dexamethasone is known to deplete hepatic glycogen stores and it is possible that the effect of ICV GLP-1 will be masked under such circumstances. It is also possible that the effect of ICV GLP-1 depends on a small intestine full of nutrients to have an effect on circulating glucose concentrations.

Specific GLP-1 receptor binding has been demonstrated in the hypothalamus, particularly in the paraventricular nucleus (PVN) (Sarah A. Beak, personal communication, (Turton et al. 1996)). Whether these receptors are the ones responsible for the hypoglycaemic effect of ICV GLP-1 remains to be seen. There is already some evidence for heterogeneity in GLP-1 receptors (see chapter 7, page 137). The effects of GLP-1 and exendin analogues on plasma glucose concentrations will be needed to determine the binding profile of the GLP-1 receptor that affects plasma glucose.

Type 2 diabetes is one of the fastest growing diseases in the western world affecting an estimated 6.6% of the population of the U.S.A. between 20 and 74 years of age (Harris et al. 1987). The risk of diabetes increases exponentially with increasing body weight (Perry et al. 1995). The ability of GLP-1 to reduce plasma glucose levels in the satiated state, together with the fact that GLP-1 decreases appetite (chapter 7), provides a potential model for work on type 2 diabetes.

Chapter 10.

Effect of adrenomedullin on food intake in the rat; a possible interaction with calcitonin gene-related peptide receptors.

The work in this chapter was carried out in collaboration with Gillian Taylor, a PhD student working on the calcitonin family of peptides. The work presented in this chapter has recently been published (see appendix) (Taylor et al. 1996).

Adrenomedullin is a recently discovered member of the calcitonin family of peptides, isolated from human phaeochromocytomas (Kitamura et al. 1993). Adrenomedullin shares structural homology and some effects with other members of the calcitonin family of peptides which include calcitonin, calcitonin-gene related peptide (CGRP) and islet amyloid polypeptide (IAPP). These peptides have an N-terminal six residue ring structure formed by an intramolecular disulphide bridge and a carboxy-terminal amidated residue. Immunoreactive adrenomedullin (Satoh et al. 1995; Sakata et al. 1994) and adrenomedullin mRNA have been detected in many human and rat tissues including heart, lung and brain (Ichiki et al. 1994; Kitamura et al. 1993). Immunoreactive adrenomedullin has been found in both human (chapter 11) and rat (Sakata et al. 1994) plasma at concentrations of 2-10 pmol/l .

Adrenomedullin, like CGRP, is a potent hypotensive peptide when administered peripherally in many species, including rat (Dewitt et al. 1994; Santiago et al. 1994; Nandha et al. 1996). Early experiments on the vasodilator effects of adrenomedullin in the isolated perfused mesenteric vascular bed (Nuki et al. 1993), and in the isolated rat heart (Entzeroth et al. 1994) showed marked inhibition by the specific $CGRP_1$ receptor antagonist, CGRP (8-37), indicating that in these vascular beds, the effect of adrenomedullin is via $CGRP_1$ receptors. However the hypotensive effect of CGRP but not, interestingly, of adrenomedullin is blocked in vivo by CGRP (8-37) (Nandha et al.

1996; Gardiner et al. 1995). This implies that the effect of intravenous adrenomedullin on systemic blood pressure does not occur through $CGRP_1$ receptors. The effects of intravenous adrenomedullin on systemic blood pressure in man is studied in chapter 11.

Unlike peripheral administration, intracerebroventricular (ICV) administration of adrenomedullin causes an increase in systemic blood pressure which is inhibited by administration of CGRP (8-37)(Takahashi et al. 1994). This implies that centrally administered adrenomedullin can have systemic effects through $CGRP_1$ receptors, which are widely distributed throughout the central nervous system including the hypothalamus (Tschopp et al. 1985; Skofitsch and Jacobowitz, 1985; Sexton et al. 1986). The calcitonin family of peptides potently inhibit food and water intake when administered centrally (Freed et al. 1979; Morley and Flood, 1991; Chance et al. 1991; Krahn et al. 1984). Salmon calcitonin is the most potent of these, a dose of 0.1 nmol administered by ICV injection completely abolishing 24 hour food and water intake (Twery et al. 1982). This effect is far more potent than GLP-1 (chapter 7) and leptin (Campfield et al. 1995), both now felt to be very important in the physiological regulation of food intake. There is no salmon calcitonin present in mammals and therefore another salmon calcitonin like peptide has been proposed to exist (Sexton and Hilton, 1992). As adrenomedullin is present in the brain, it may be the endogenous calcitonin family member which mediates inhibition on feeding. Recently specific adrenomedullin receptors have been demonstrated in the central nervous system (CNS) including the hypothalamus (Owji et al. 1995).

Adrenomedullin has been shown to inhibit the drinking response in water deprived rats in a dose dependent manner when administered ICV (Murphy and Samson, 1995). The effects of adrenomedullin on feeding have not been investigated. The purpose of the present study is to investigate the effects of adrenomedullin when given ICV on food intake and, by co-administration with CGRP (8-37), to determine to what extent any effect on feeding is mediated by $CGRP_1$ receptors.

Materials and methods.

Animals were cannulated ICV and prepared for ICV injection as in chapter 2.

Experiments

Study 1: The effect of increasing doses of adrenomedullin on fast induced feeding.

Animals (n=6 per group) were 24 hour fasted before injection in the early light phase. Rats received a 10μl ICV injection of adrenomedullin (0, 0.05. 0.17, 1.7, or 5.0 nmol) and 2 hour food intake was measured. Food intake between groups was compared using ANOVA with post-hoc Tukey's test.

Study 2: Antagonist studies.

To establish whether adrenomedullin was producing its effect on food intake via CGRP1 receptors in the hypothalamus, animals (n=6-9 per group) were ICV injected with 30 nmol CGRP (8-37) immediately prior to either saline or adrenomedullin (1.7 nmol). Food intake between groups was compared using ANOVA with post-hoc Tukey's test.

Results.

Study 1:

The food intake in animals after a 24 hour fast was inhibited in a dose dependent manner following ICV adrenomedullin. Two hour food intake was reduced by 57 % with 1.7 nmol and by 66% with 5.0 nmol adrenomedullin. At 120 minutes, $F_{4,23} = 5.141$, $p<0.01$ (figure 10.1)

Study 2:

Adrenomedullin (1.7 nmol) inhibited 2 hour fast induced food intake by 60% ($p<0.05$). This inhibition was reduced by 50% when CGRP (8-37) was injected ICV prior to adrenomedullin at a dose of 30 nmol (figure 10.2). (saline 6.1±0.7 g; adrenomedullin 2.5±0.5 g; both (CGRP and adrenomedullin) 4.3±0.6g; CGRP (8-37) 5.0±0.7g). When administered alone ICV CGRP (8-37) did not have any significant effect on fast induced food intake. No gross behavioural abnormalities were noted by the investigators other than a reduction in feeding.

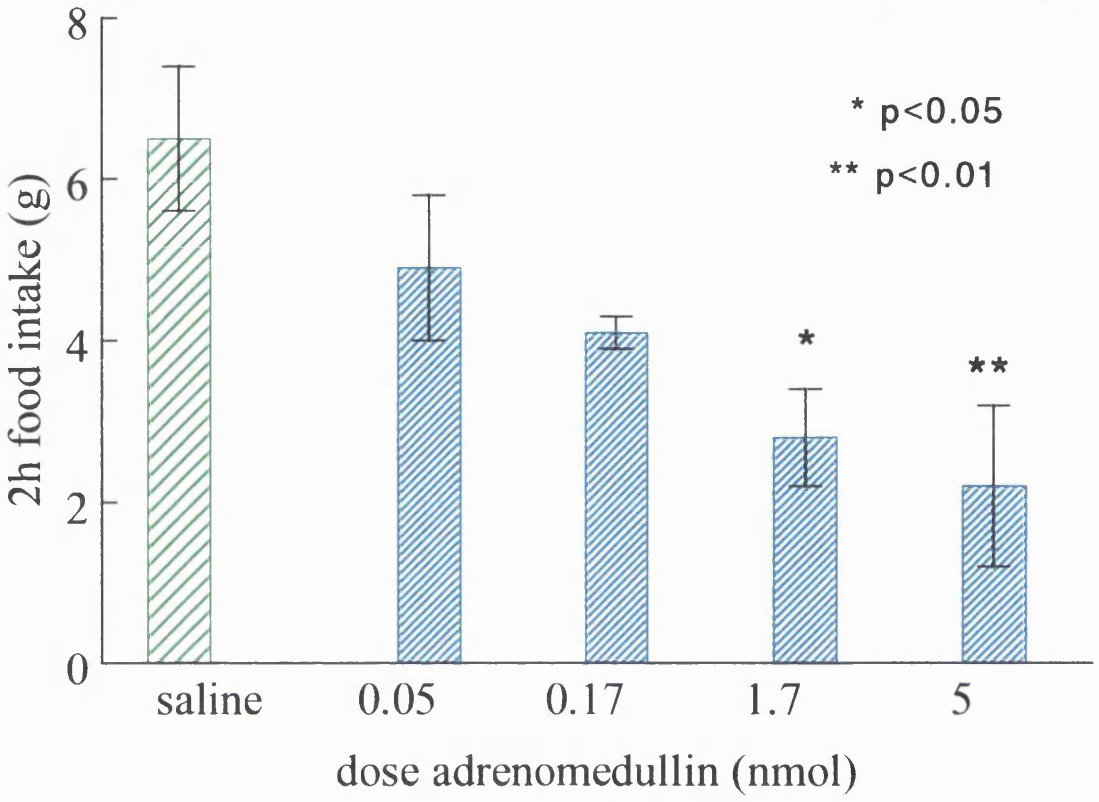


Figure 10.1

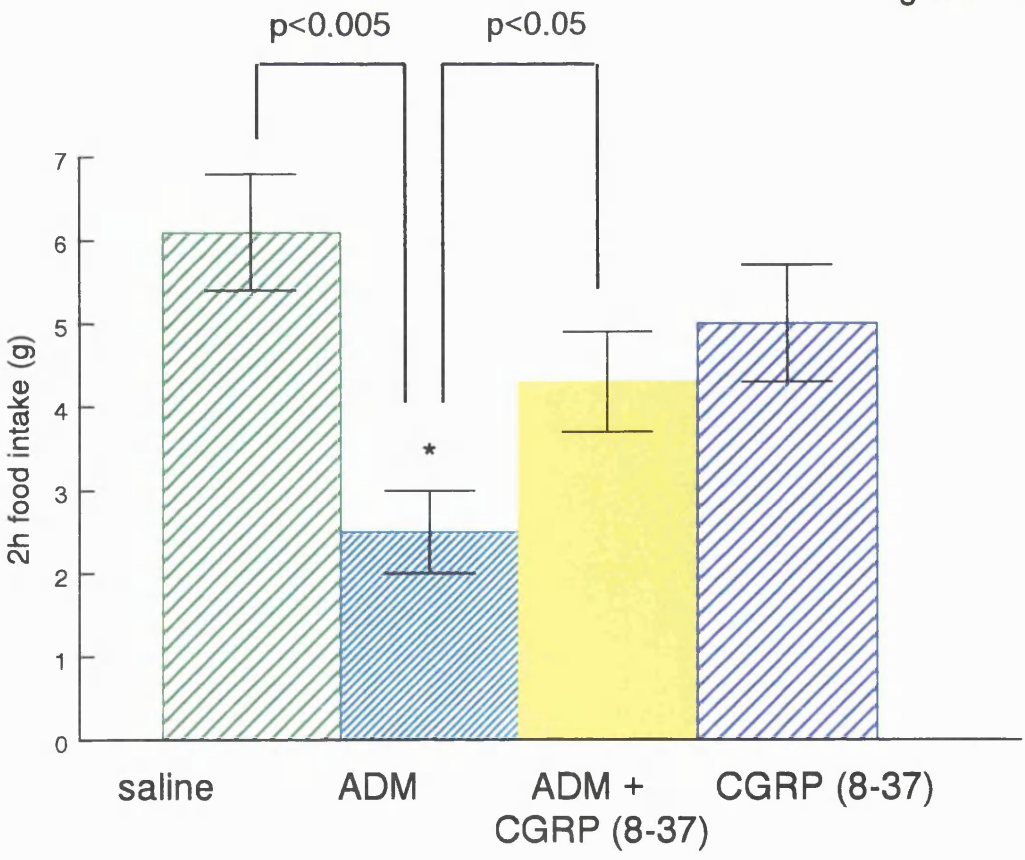


Figure 10.2

Discussion.

I have shown for the first time that adrenomedullin, like the other members of the calcitonin family, reduces feeding in a dose dependent manner when administered centrally. This effect is only partially blocked by the $CGRP_1$ receptor antagonist, CGRP (8-37) indicating that this effect of adrenomedullin is mediated at least in part through a central receptor other than the $CGRP_1$ receptor. Specific adrenomedullin receptors have previously been described in the brain including the hypothalamus (Owji et al. 1995). The hypothalamus has high affinity adrenomedullin binding sites (K_D = 0.54 nM) with a moderate to high concentration of sites (B_{max} = 214 fmol/mg protein) (G.M. Taylor, personal communication, (Taylor et al. 1996)). These sites did not bind either CGRP or CGRP(8-37).

Adrenomedullin can also bind to hypothalamic CGRP receptors with high affinity (K_i = 4.6 nM) although this is with 46 times less affinity than CGRP. It is this binding to CGRP receptors which appears to mediate the effects on feeding leaving the role of the specific adrenomedullin receptors open until a specific adrenomedullin receptor antagonist is developed. A CGRP receptor subtype, $CGRP_2$, which is not inhibited by CGRP (8-37) has been reported (Dennis et al. 1990). CGRP binds equipotently to $CGRP_1$ and $CGRP_2$ receptors but the CGRP induced reduction in feeding is inhibited by CGRP(8-37) (30). Therefore it is unlikely that activation of $CGRP_2$ receptors is involved in effects on feeding. It is possible that adrenomedullin has a physiological role in satiety and the control of food intake. Recently, adrenomedullin-immunoreactive neurons have been identified in the paraventricular and supraoptic nuclei of the rat (Ueta et al. 1995). These hypothalamic nuclei are known to be important in regulation of food intake but

further studies (e.g. immunoneutralisation studies) are required to define a physiological role for adrenomedullin.

Systemic blood pressure was increased by high dose (1-3 nmol/kg) ICV adrenomedullin (Takahashi et al. 1994). This hypertensive effect was completely abolished by 3 nmol/kg CGRP (8-37). The hypertensive effect of ICV adrenomedullin is thus entirely mediated by $CGRP_1$ receptors. Recently it has been reported that when adrenomedullin is ICV infused into conscious sheep at 100 μg/hour (approx. 4 nmol/kg), no significant changes in cardiovascular parameters were observed (Parkes and May, 1995). A dose of up to 0.18 nmol adrenomedullin had no effect on blood pressure when given ICV to rats (Murphy and Samson, 1995). This dose significantly reduced water intake, although the possibility that this effect was mediated via central $CGRP_1$ receptors was not investigated (Murphy and Samson, 1995). We have found that a similar dose (0.17 nmol) reduced feeding by approximately 40%, suggesting that the effect on feeding and drinking may be mediated by a similar mechanism. The fact that a much larger dose of ICV adrenomedullin is needed to cause hypertension than to decrease food or water intake in the rat may indicate that the receptors mediating hypertension are further from the ventricular system (Murphy and Samson, 1995; Takahashi et al. 1994). However, direct brain stem and hypothalamic injections of adrenomedullin are required before the potency of adrenomedullin on these systems can be properly compared.

Recently new information has emerged concerning the specific adrenomedullin receptor in the rat brain. A cDNA encoding the rat adrenomedullin receptor has been identified (Kapas et al. 1995) and shown to have binding properties similar to specific

adrenomedullin receptors in the hypothalamus (Owji et al. 1995). This cDNA was previously described as an orphan receptor present in a number of brain regions including the hypothalamus (Harrison et al. 1993). The molecular cloning of the specific adrenomedullin receptor may be useful in developing antagonists specific to this receptor and eventually unravelling its role in the hypothalamus.

Chapter 11.

Investigation of the role of circulating adrenomedullin in the regulation of blood pressure and pituitary function by intravenous infusion of the peptide in man.

Introduction.

The work in this chapter was performed with the help of Dr. Donal O'Shea and Mr. Paul Upton. The findings have recently been published (Meeran et al. 1997). As discussed in chapter 10, the physiological role of this recently isolated peptide is unknown. Adrenomedullin appears to be actively synthesised and secreted by vascular endothelial cells, both into the bloodstream and into the space between endothelial cells and vascular smooth muscle cells (Sugo et al. 1994). The major source of circulating adrenomedullin is probably the vascular endothelium (Sugo et al. 1994) rather than the adrenal gland (Nishikimi et al. 1994). Adrenomedullin may act in a paracrine fashion on vascular smooth muscle cells in regulating vascular tone, rather than as a circulating hormone. Adrenomedullin has also been proposed to be a paracrine regulator of the pituitary based on studies in dispersed rat anterior pituitary cells (Samson et al. 1995).

In animal studies, it has been shown to have pulmonary (Lippton et al. 1994; Dewitt et al. 1994) and systemic (Gardiner et al. 1995; Nandha et al. 1996) hypotensive properties, by acting as a capillary vasodilator. Work in this laboratory has demonstrated that the recently cloned adrenomedullin receptor (Kapas et al. 1995) is expressed in several rat tissues with particularly high levels in the lung (Owji et al. 1995). In animals with pharmacologically induced pulmonary hypertension, adrenomedullin reduces pulmonary pressures (Lippton et al. 1994; Dewitt et al. 1994). Ishimitsu *et al.* have found increased circulating plasma concentrations of adrenomedullin in patients with essential hypertension and proposed that it acts to protect the cardiovascular system from the effects of hypertension (Ishimitsu et al. 1994). Adrenomedullin has also been proposed

to be a paracrine regulator of the pituitary based on studies in isolated anterior pituitary cells (Samson et al. 1995).

The effects of adrenomedullin on humans have not been studied to date . The present study was designed to elucidate the pharmacokinetics of adrenomedullin, to ascertain whether it has a physiological role in the regulation of blood pressure as a circulating hormone and to determine its effects on pituitary hormone release. The gene expression of adrenomedullin is higher in endothelial cells than in other tissues, including the adrenal (Sugo et al. 1994), and for this reason we also studied several patients with diseases known to cause endothelial damage such as in patients with diabetic retinopathy or the vasculitides such as systemic lupus erythematosus (SLE) or Wegeners Granulomatosis. We also wanted to establish whether infusion of adrenomedullin is well tolerated in man.

Materials and methods

The safety of high dose administration was examined in rats by intravenous injection. The synthesised adrenomedullin was shown to bind to specific adrenomedullin receptors by displacing radiolabelled ligand, and was known to suppress feeding as in chapter 10.

Assay of adrenomedullin.

Adrenomedullin antiserum was raised in a rabbit against synthetic human adrenomedullin conjugated to bovine serum albumin (BSA) using carbodiimide (O'Shaughnessy, 1982) and used in the assay at a final dilution of 1:10000. The detection limit was 2 fmol/tube at 95% confidence limits and the assay did not cross react with synthetic CGRP, IAPP or calcitonin.

Radiolabelled ligand was prepared using synthetic adrenomedullin (22-52) fragment, using the iodogen method (Fraker and Speck, 1978) and was purified by reverse-phase HPLC. The specific activity of the tracer was 22.1 Bq/fmol. Assays were set up in 0.06M sodium phosphate (pH 7.2) containing 0.3% (w/v) BSA and incubated at 4°C for 3 days. Bound and free tracer were separated using dextran-coated charcoal.

Other assays.

Glucose was measured by the glucose oxidase method using a Yellow Springs YSI 2300 glucometer. Plasma LH, FSH, TSH, prolactin and cortisol were measured using an automated analyser. Plasma ACTH was determined using a two-site immunoradiometric assay (IRMA) kit as described in chapter 2.

Experiments

1. **Human infusions of adrenomedullin.**

A) To determine the effect on blood pressure and pituitary function in man.

Eight healthy male volunteers (aged 24-33 years) were studied. Informed consent was obtained from each subject, and all experiments had prior approval from the Royal Postgraduate Medical School Ethical Committee No: 94/4331. After an overnight fast, subjects were infused either with adrenomedullin or saline in random order into the antecubital vein of the left arm. There was an interval of at least seven days between each infusion. Infusions were commenced with a 30 minute run in period when saline only was infused. In order to minimise peptide adsorption to the infusion system, adrenomedullin was dissolved in 5 ml of the volunteers own plasma, which was then diluted to 50 ml with 0.9% saline. The infusion rate, initially 3.2 pmol/kg/min, was increased every 10 minutes up to a maximum of 13.4 pmol/kg/min which was maintained for 40 minutes. Subjects were supine throughout the study, except for measurement of a standing blood pressure during the period of maximum adrenomedullin infusion. An intravenous cannula was inserted into the antecubital vein of the right arm for blood sampling. After each sample, the line was flushed with 1ml 0.9% saline to keep the line patent. Pulse and blood pressure were recorded every 5 minutes. Blood was collected in heparin and trasylol at intervals from the cannula in the antecubital vein of the right arm and centrifuged immediately. Plasma was frozen on dry ice and stored at -20°C for assay of adrenomedullin, cortisol, glucose and the pituitary hormones prolactin, luteinising hormone (LH), follicle stimulating hormone (FSH), adrenocorticotrophic hormone (ACTH) and thyroid stimulating hormone (TSH).

B). **To determine the pharmacokinetics of adrenomedullin in man**

Pharmacokinetics were studied in seven further volunteers aged 24 to 29 years. A calculated loading dose of 20 pmol/kg/min adrenomedullin was infused over 10 minutes and an infusion of 6.5 pmol/kg/min was maintained for 60 minutes to achieve a steady state. At the end of this period, the infusion was discontinued and samples taken regularly to ascertain the half life of adrenomedullin in the circulation. The half life of adrenomedullin was calculated for each volunteer. The mean basal concentration of adrenomedullin was subtracted from the mean plateau concentration and from each subsequent sample. The post infusion values were normalised by expressing them as a percentage of the previous steady state concentration. These values were plotted for each volunteer and the half life derived from the resulting decay curve.

A sample of each volunteer's infusate was assayed for adrenomedullin to confirm the infusion rate. The metabolic clearance rate (MCR) of adrenomedullin was calculated for each volunteer from the steady state concentration and infusion rate at which this concentration was stable using equation (A) below. The volume of distribution was calculated from the half life and the steady state clearance using equation (B):

(A) $$\text{MCR (ml/kg/min)} = \frac{\text{steady state infusion rate (pmol/kg/min)}}{\text{Steady state plasma concentration (pmol/ml)}}$$

(B) $$\text{Volume of distribution} = \text{MCR} \times \text{half life} \times 1.44.$$

2. **Measurement of circulating adrenomedullin concentrations in various diseases.**

The study population consisted of eleven healthy volunteers, seventeen patients with background diabetic retinopathy (BDR), eight suffering from end stage renal failure on continuous ambulatory peritoneal dialysis (CAPD), six patients with systemic lupus erythematosus (SLE), twelve patients with Wegeners granulomatosis, five with chronic stable asthma and four with rheumatoid arthritis. All patients were attending the outpatient department at the Hammersmith Hospital and were being venesected for routine plasma electrolytes and creatinine. Blood was also collected from several other patients attending the outpatients clinic who were having their creatinine measured. Further patient details are given in table 11.3 on page 178.

Blood (10 ml) was collected in lithium heparin tubes containing trasylol (2000 IU) and centrifuged immediately at 4°C. Plasma was frozen on dry ice and stored at -20°C until extracted for assay of adrenomedullin. Plasma from patient samples was extracted using Sep-Pak cartridges according to the following method. Two ml of plasma was mixed with an equivalent volume of 4% (v/v) acetic acid and loaded onto a Sep-Pak C18 cartridge (Waters) which had been pre-equilibrated with 4% (v/v) acetic acid. After washing with 10 ml 4% acetic acid and 5 ml 10% acetonitrile containing 0.1% trifluoroacetic acid (TFA), the adsorbed materials were eluted with 2ml of 60% acetonitrile containing 0.1% TFA, dried in a vacuum rotary evaporator (Savant), resuspended in radioimmunoassay (RIA) buffer and submitted directly to assay.

Samples from volunteers receiving adrenomedullin infusions were assayed directly without extraction since plasma concentrations during infusion were well above the detection limit for the assay.

Results.

1. Adrenomedullin infusions.

There was no change in pulse or blood pressure when adrenomedullin was infused at 3.2 pmol/kg/min achieving circulating concentrations of 52 pmol/l. Higher dose adrenomedullin infusions (13.4 ± 0.5 pmol/kg/min) were well tolerated with no adverse effects apart from facial flushing. At this infusion rate the plasma adrenomedullin concentration was 448 ± 58 pmol/kg and the diastolic blood pressure was significantly reduced, with a reflex tachycardia, although there was no change in systolic blood pressure. The fall in diastolic blood pressure was not however associated with any postural hypotension. Indeed standing diastolic blood pressure at maximum infusion rate was significantly higher than supine diastolic blood pressure. (Table and Figure 11.1).

Table 11.1

infusion rate	systolic BP	diastolic BP	pulse
baseline (0)	114 ± 3	69 ± 2	57 ± 3
3.2 pmol/kg/min	115 ± 3	68 ± 2	60 ± 3
6.45 pmol/kg/min	113 ± 3	60 ± 2	66 ± 4
13.4 pmol/kg/min	115 ± 2	53 ± 2	81 ± 3
standing with 13.4 pmol/kg/min infusion	114 ± 4	62 ± 2	95 ± 5

Plasma prolactin was significantly increased (372 ± 64 IU/l) when adrenomedullin was infused at 13.4 pmol/kg/min compared to baseline (197 ± 46 IU/l) and control (204 ± 30 IU/l) infusions (Table and figure 11.2). There was no change in plasma concentrations of ACTH, cortisol, LH, FSH or TSH during either infusion.

Table 11.2.

	adrenomedullin	prolactin	ACTH	cortisol	LH	FSH	TSH	glucose
	pmol/l	IU/l	ng/l	nmol/l	IU/l	IU/l	IU/l	mmol/l
baseline (t=0)	8.1 ± 0.7	197 ± 46	7.2 ± 1.2	330 ± 56	2.6 ± 0.4	3.0 ± 0.3	1.4 ± 0.2	4.5 ± 0.1
6.7 pmol (t= +30)	116 ± 28.3	190 ± 38	5.4 ± 1.1	217 ± 32	1.9 ± 0.3	2.7 ± 0.3	1.2 ± 0.2	4.5 ± 0.1
13.4 pmol/kg/min (t= +70) [peak infusion rate]	448 ± 57.6	372* ± 64	7.6 ± 1.7	233 ± 56	2.3 ± 0.7	2.7 ± 0.3	1.1 ± 0.2	4.8 ± 0.2
recovery (t= +110)	150 ± 21.0	275* ± 38	6.0 ± 1.1	223 ±108	2.0 ± 0.6	2.6 ± 0.4	1.0 ± 0.1	4.8 ± 0.1

* $p < 0.01$ compared to values during saline infusions in the same individuals (data not shown).
A similar decline in cortisol occurred with time during the saline infusion, presumably as the volunteers progressively relaxed.
There was no rise in plasma prolactin during the saline infusion.
All values are mean ± SEM.

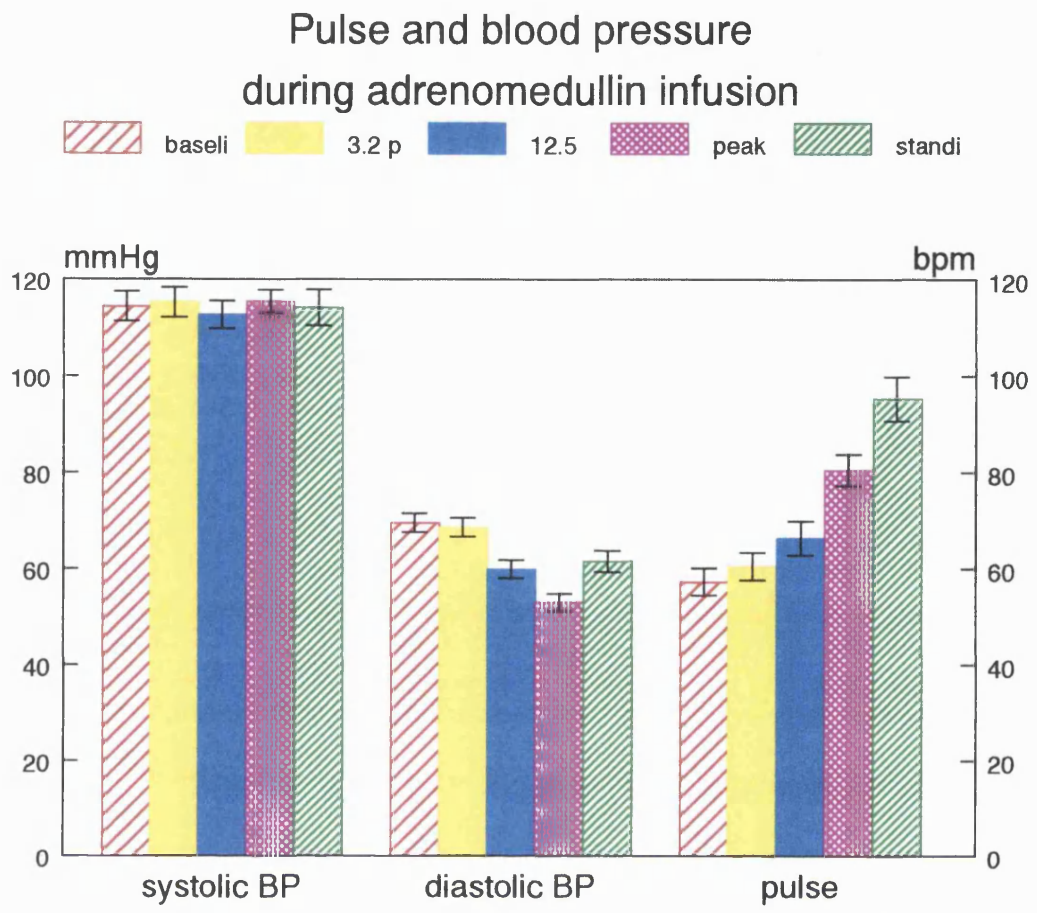


Figure 11.1

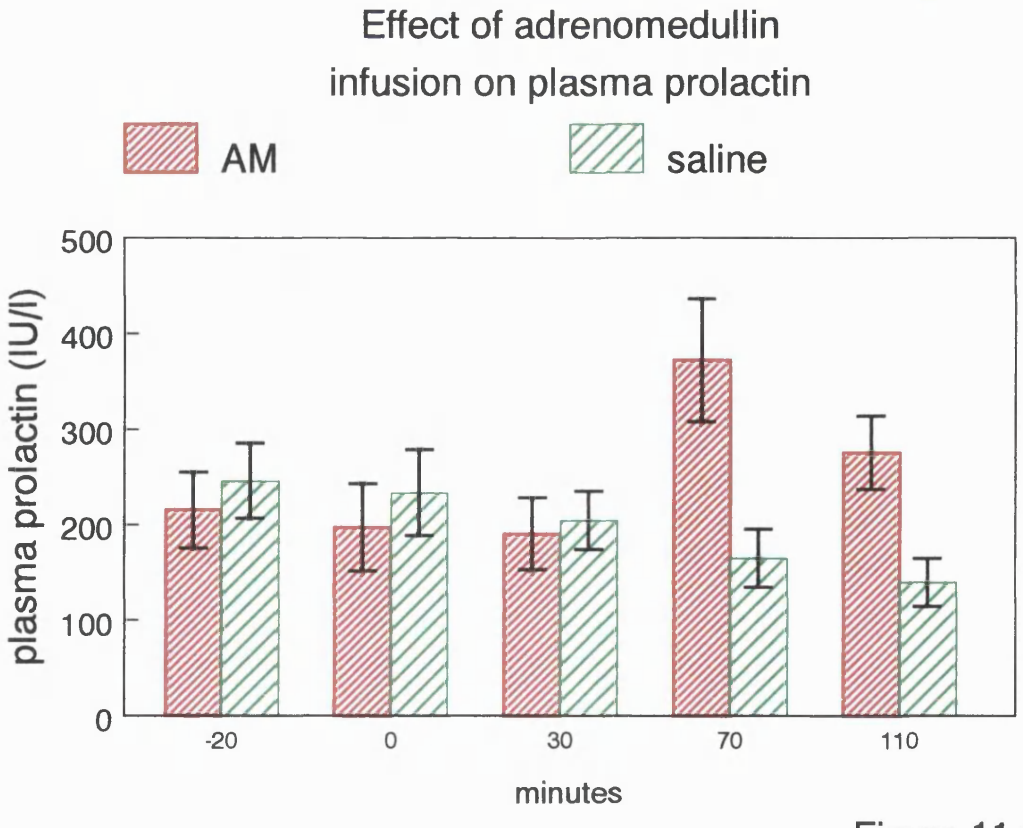


Figure 11.2

1B) Pharmacokinetics.

During the second set of infusions, a steady state of 258 ± 33.8 pmol/l circulating adrenomedullin was achieved within ten minutes (figure 3) with a steady state infusion rate of 6.45 ± 0.36 pmol/kg/min. A decline in the plasma concentration of adrenomedullin was observed when the infusion was discontinued. From this rate of decline, the plasma half life of adrenomedullin in man was calculated as 22 ± 1.6 minutes. The mean Metabolic Clearance Rate (MCR) was 27.4 ± 3.6 ml/kg/min and the apparent distribution space was 880 ± 150 ml/kg. The results for each volunteer are given in table 11. 3.

Table 11.3.

volunteer	steady state	infusion rate	MCR	half life	Vd
	(pmol/l)	pmol/kg/min	ml/kg/min	minutes	l/kg
1	285 ± 11	6.25	21.9	20	0.63
2	221 ± 16	4.75	21.5	20	0.61
3	387 ± 18	7.13	18.4	20	0.53
4	224 ± 5	7.00	31.2	25	1.125
5	348 ± 16	7.50	21.5	28	0.87
6	122 ± 5	5.63	46.2	25	1.66
7	220 ± 11	6.88	31.2	16	0.72
mean	258	6.44	27.4	22	0.88
SEM	33.8	0.36	3.6	1.6	0.15

2. **Basal circulating concentrations of adrenomedullin.**

Demographic data on the patients and volunteers as well as mean circulating adrenomedullin concentration is given in table 11.4. Age is expressed as mean (range). Adrenomedullin concentration is expressed as mean ± SEM and also as a percentage compared to controls (100%).

Table 11.4.

patient group	n(male)	age (range)	adrenomedullin (pmol/l)	(percent of control)
controls	11 (2)	40 (25-58)	8.1 ± 0.7	100 %
Asthma	5 (4)	53 (34-62)	9.3 ± 0.3	115 %
Rheumatoid arthritis	4 (0)	63 (32-86)	8.2 ± 0.2	101 %
Retinopathy (creatinine <100)	13 (10)	54 (26-80)	8.1 ± 0.3	100 %
Retinopathy (creatinine >150)	4 (2)	67 (51-83)	13.3 ± 1.1	164 % *
CAPD patients	8 (3)	52 (34-68)	13.9 ± 1.5	172 % **
SLE (creatinine <100)	2 (0)	45 (41-49)	6.5 ± 0.4	80 %
SLE (creatinine >150)	4 (0)	51 (37-61)	12.9 ± 3.4	159 %
Wegeners (creatinine <100)	7 (1)	52 (20-70)	10.7 ± 0.7	132 %
Wegeners (creatinine >150)	5 (3)	62 (54-71)	16.0 ± 1.4	198 % **
All renal failure	21(8)	57 (34-83)	14.1 ± 0.9	174 % **

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

In addition, very high plasma concentrations were found in individual patients with tumours, one with a lymphoma (17.2 pmol/l) and one with a hypernephroma (28.1 pmol/l), both with normal renal function. Apart from these two patients, there was a good correlation ($r=0.86$) between circulating plasma adrenomedullin and plasma creatinine (figure 11.4).

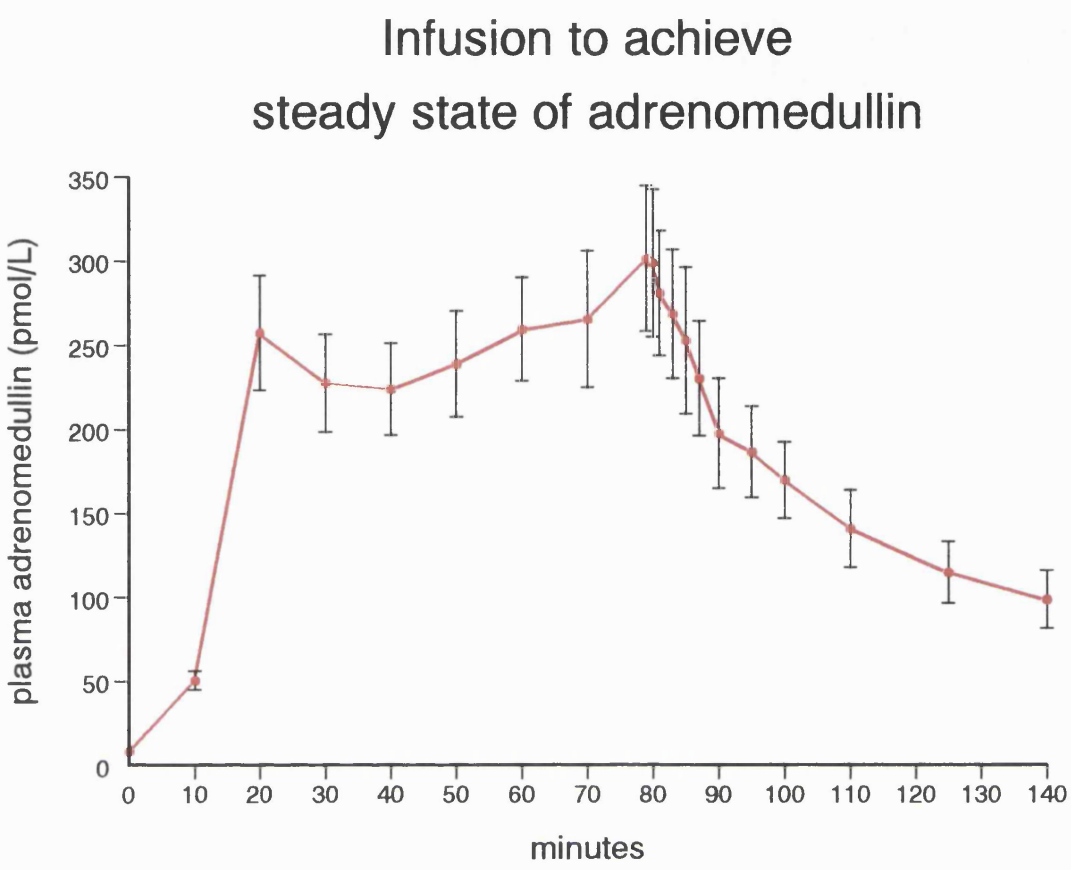


Figure 11.3

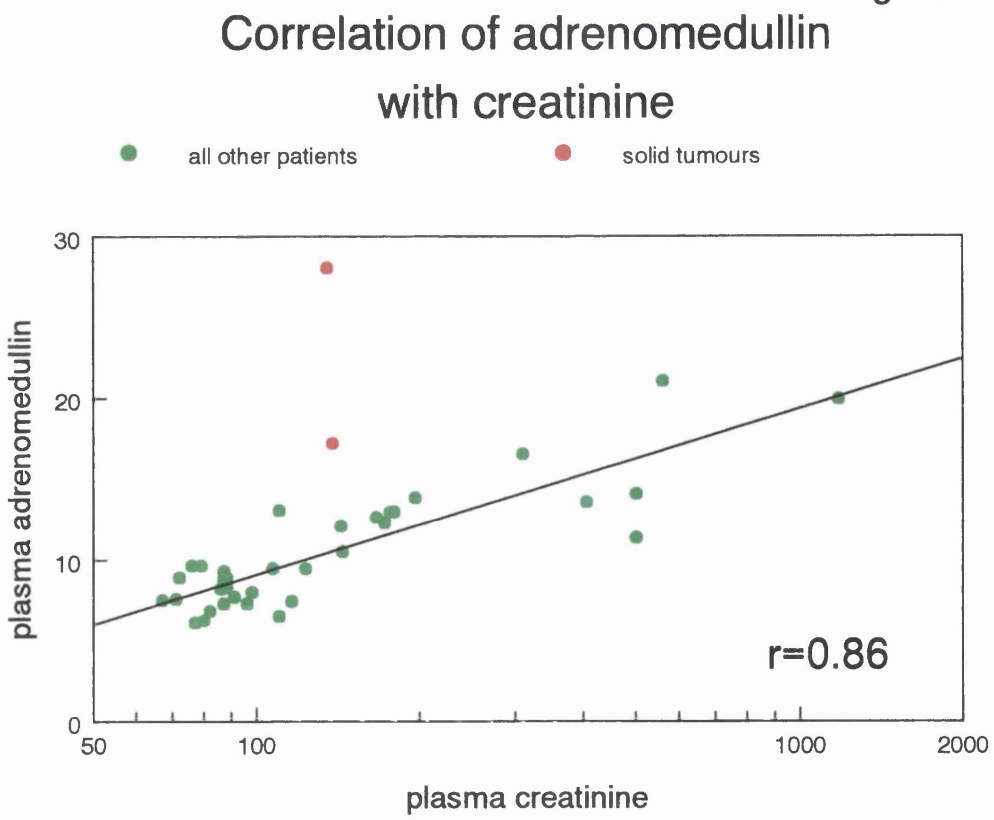


Figure 11.4

Discussion.

Adrenomedullin has not previously been infused into humans and was well tolerated in our volunteers. Although our first infusion was not a dose response study, we found that more than quadrupling the circulating concentration had no effect on blood pressure. Even during high dose adrenomedullin infusion, a plasma concentration of 448 pmol/l was not associated with any change in systolic blood pressure and only minor changes in diastolic blood pressure. All biological effects of adrenomedullin disappeared within one half life of the infusion being discontinued, confirming that high plasma levels (10 fold higher than normal) have no effect on systemic blood pressure. We have previously shown that this adrenomedullin is fully biologically active (Taylor et al. 1996).

These results strongly suggest that circulating adrenomedullin plays no role in the control of systemic blood pressure in man. The proposal that circulating adrenomedullin is involved in a defence mechanism preserving the integrity of the cardiovascular system in hypertension (Ishimitsu et al. 1994) is not supported by our findings. Adrenomedullin is more likely to have a paracrine role in the control of vascular tone. Endothelial cells actively secrete adrenomedullin (Sugo et al. 1994) which can directly stimulate vascular smooth muscle cells through specific adrenomedullin receptors (Ishizaka et al. 1994; Eguchi et al. 1994; Eguchi et al. 1994). The proximity of vascular smooth muscle cells to endothelial cells means that the concentration of adrenomedullin around these cells is likely to be much higher than the basal concentrations that we have found in the plasma, and may be similar to those achieved by our maximal infusion rate. Adrenomedullin has been shown to inhibit endothelin production (Kohno et al. 1995) and to be an antimigration factor (Horio et al. 1995) in vascular smooth muscle cells.

Cultured vascular smooth muscle cells express adrenomedullin mRNA at a 3-4 fold higher concentration than that in adrenal gland by northern blot analysis (Sugo et al. 1994). In addition, adrenomedullin mRNA appears to be under hormonal regulation in endothelial and vascular smooth muscle cells (Minamino et al. 1995; Imai et al. 1995), suggesting that adrenomedullin has an important function in these cell types. The possibility that some tumours may secrete adrenomedullin is raised by our finding of increased circulating adrenomedullin concentrations in two patients with malignancy, a hypernephroma and a lymphoma. Pulmonary tumours have been shown to secrete adrenomedullin (Martinez et al. 1995).

The half life and apparent volume of distribution of adrenomedullin are larger than those of other related peptides. Rat CGRP was found to have a plasma half life of 6.9 minutes and a MCR of 11.3 ml/kg/min in man (Kraenzlin et al. 1985). Human calcitonin has a plasma half life of 10.1 minutes and an MCR of 8.4 ml/kg/min (Huwyler et al. 1979). Amylin has a half life of 11.8 minutes, an MCR of 5.7 ml/kg/min and an apparent distribution space of 94 ml/kg (Bretherton Watt et al. 1990). The very high apparent distribution space for adrenomedullin suggests that adrenomedullin is extensively tissue bound, possibly to the receptors found both on the endothelium (Nandha et al. 1996; Shimekake et al. 1995) and in vascular smooth muscle cells (Eguchi et al. 1994; Eguchi et al. 1994).

Plasma adrenomedullin was reported to be raised by 26% in patients with hypertension without organ damage (Ishimitsu et al. 1994) and by between 78% and 214% in patients with renal impairment. We found a similar increase in patients with renal impairment, and

a significant correlation between plasma adrenomedullin and degree of renal impairment as previously described (Kohno et al. 1996). Circulating adrenomedullin concentrations were normal in our patients with normal renal function, even when they had diseases known to affect the endothelium, including Wegener's granulomatosis, systemic lupus erythematosus or background diabetic retinopathy. It is likely that adrenomedullin is metabolised or cleared, at least in part by the kidney, and that this metabolism is impaired in patients with renal failure. The small increase in plasma adrenomedullin reported in patients with hypertension (Ishimitsu et al. 1994) may indicate early renal impairment. Creatinine clearance was not checked in their study (Ishimitsu et al. 1994).

Adrenomedullin has been proposed to act through $CGRP_1$ receptors in the isolated perfused mesenteric vascular bed of rats (Nuki et al. 1993), although the effect on blood pressure in the intact animal is known not to act through these receptors (Gardiner et al. 1995; Nandha et al. 1996). The change in diastolic blood pressure during our human infusions did not have a postural component and was associated with facial cutaneous flushing, suggesting that the effect may have occurred distal to the arterioles at the capillary level. Intravenous CGRP caused a similar facial flushing when infused at between 0.96 and 1.92 pmol/kg/min (achieved plasma concentration of 184 ± 9 pmol/l) in humans (Kraenzlin et al. 1985).

Samson *et al.* have proposed a paracrine role for adrenomedullin in the pituitary based on their studies demonstrating that adrenomedullin inhibits ACTH release from dispersed rat anterior pituitary cells (Samson et al. 1995). We found that high dose adrenomedullin infusion was associated with increased release of prolactin but no change in other

pituitary hormones including ACTH. This would suggest a possible role for adrenomedullin as a regulator of lactotroph function. A paracrine rather than an endocrine effect is likely since the high dose intravenous adrenomedullin may achieve local concentrations around the pituitary cells, consistent with those of a paracrine peptide. Adrenomedullin is known to be synthesised in the anterior pituitary (Washimine et al. 1995).

A possible role for adrenomedullin in the management of primary pulmonary hypertension has been suggested following studies of its effects in animals models of this disease (Lippton et al. 1994; Dewitt et al. 1994). Under conditions of resting (low) pulmonary vasomotor tone, intralobar arterial injection of adrenomedullin had little effect on baseline lobar arterial or systemic blood pressure. In contrast, when pulmonary vasomotor tone was actively increased by intralobar arterial infusion of the thromboxane A2 mimic U-46619, adrenomedullin decreased lobar arterial pressure in a dose dependent manner with minimal effects on systemic blood pressure. There is at present no pulmonary vasodilator available that does not cause systemic hypotension. Angiotensin converting enzyme inhibitors, calcium antagonists, CGRP, prostacyclin, nitric oxide and oxygen have been used with minimal success. We have shown that high dose adrenomedullin is well tolerated in human volunteers, and that adrenomedullin has a minimal effect on systemic blood pressure. Some patients with raised pulmonary pressure might respond to infusion of adrenomedullin with an improved pulmonary pressure and consequent improvement in ventilation-perfusion matching. Adrenomedullin is known to be metabolised or cleared in the lung (Nishikimi et al. 1994) since concentrations are higher in the pulmonary artery than in the aorta. This may be

because the lung has a large number of adrenomedullin receptors.

In conclusion we found that adrenomedullin could lower blood pressure in man, as predicted from animal and tissue experiments (Nandha et al. 1996). The circulating concentrations required to achieve this effect however, were well above those found in plasma taken from patients with a variety of conditions. Thus we conclude that adrenomedullin is likely to influence vascular tone mainly through paracrine mechanisms.

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Chapter 3.

O'Shea D, Morgan DG, Meeran K, Edwards CMB, Turton MD, Choi SJ, Heath MM, Gunn I, Taylor GM, Howard JK, Bloom CI, Small CJ, Haddo O, Ma JJ, Callinan W, Smith DM, Ghatei M, Bloom SR. 1997. Neuropeptide Y induced feeding in the rat is mediated by a novel receptor. Endocrinology In Press:

Abstract

There are now six recognised neuropeptide Y (NPY) receptor subtypes (Y1-Y4 and two recently cloned distinct receptors labelled Y5), of which Y1 and one of the Y5's have been suggested could mediate the effect of NPY on feeding. The fragments NPY(2-36) and NPY(3-36), which bind Y1 only poorly, were injected intracerebroventricularly (ICV) and found to have similar dose-response relationships to NPY in the stimulation of feeding. However NPY(13-36), which stimulates both Y2 and Y5 caused no increase in food intake, even at high doses. Maximal stimulation with the classical Y1 agonist [Pro34]-NPY produced only 50% of the maximum effect of NPY itself despite fully inhibiting adenylyl cyclase activity *in vitro* in a Y1 system. The novel fragment [Pro34]-NPY(3-36) is as effective at stimulating food intake as the classical Y1 analogue [Pro34]-NPY, but bound to the Y1 receptor with only 1/20th of the affinity of NPY, and failed to inhibit adenylyl cyclase through this receptor. [Pro34]-NPY(3-36) is therefore a relatively appetite-selective ligand. Co-administration of high dose NPY(13-36) and [Pro34]NPY did not enhance feeding compared with [Pro34]-NPY alone. In addition, the NPY Y1 receptor antagonist BIBP-3226 which does not bind Y2, Y4 or Y5 receptors significantly reduced NPY induced feeding. These results indicate that the feeding effect of ICV NPY involves a novel receptor and that it is functionally distinct from the recognised receptor subtypes.

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Abstract
Cobalt Protoporphyrin (CoPP) reduces food intake and body weight following intracerebroventricular (ICV) administration in rats. We injected 0.2 μmol CoPP per kg body weight ICV and monitored body weight and daily food intake for 7 days. The body weight and 24 hour food intake of CoPP treated animals was significantly lower than that of vehicle treated animals in all studies ($p<0.05$) from day 2. The 2 hour feeding response (CoPP vs vehicle treated) to 10 μg neuropeptide Y (NPY) (4.0 vs 7.1g; $p<0.05$), the 1 hour feeding response to 10 μg galanin (1.3 vs 3.2g; $p<0.05$) and 30 μg norepinephrine (0.6 vs 1.9 g; $p<0.05$) in CoPP treated animals were all reduced compared to the vehicle treated group. In addition there was no change in hypothalamic NPY mRNA in CoPP treated animals. ICV CoPP decreases sensitivity to exogenous NPY, galanin and norepinephrine. The effect of CoPP is not specific to NPY as previously described.

Chapter 6.

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Meeran K, O'Shea D, Edwards CMB, Heath MM, Choi SJ, Taylor GM, Gunn I, Turton MD, Rossi M, Small CJ, Goldstone AP, Ghatei M, Bloom SR. 1997. **Blockade of Hypothalamic Glucagon-Like Peptide-1 Receptors augments Neuropeptide Y induced weight gain and food intake in the rat.**
J. Clin. Invest. In Press:
Notes : Abstract:
Central glucagon-like peptide-1 (7-36) amide (GLP-1) has been reported to be a physiological satiety factor in the rat. Here we demonstrate that endogenous GLP-1 may have an effect on body weight. Blockade of central GLP-1 receptors, with the specific GLP-1 receptor antagonist exendin (9-39), augmented the weight gain induced by neuropeptide Y, the most potent stimulant of feeding known. Animals receiving twice daily injections of NPY with exendin (9-39) gained 28 ± 4 g compared with 14 ± 3g in those receiving NPY with saline over 8 days ($p<0.01$). Blockade of GLP-1 receptors resulted in a consistent and significant increase in 2 hour and 24 hour food intakes. There was no tolerance to the effect of blockade of central GLP-1 receptors.
The effect of GLP-1 receptor blockade in the Zucker *(fa/fa)* rat indicated that endogenous GLP-1 was also active in animals known to have a leptin receptor defect. In satiated Zucker rats, 30 nmol exendin (9-39) increased 2 hour feeding almost three fold (1.1 ± 0.3 to 3.1 ± 0.3g). After a 24 hour fast, 3nmol GLP-1 suppressed 2h food intake by 58% (2.5 ± 0.3 vs 6.0 ± 0.75). Similar results were found in animals treated with oral dexamethasone to increase endogenous hypothalamic NPY.
These findings suggest that GLP-1 is a potent physiological regulator of feeding and that disturbance of this system could be associated with obesity.

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Karim Meeran, Melanie M. Heath, Caroline J. Small, Mohammad A. Ghatei and Steven R. Bloom. (1997)
Central injection of glucagon-like peptide-1 causes an acute fall in plasma glucose.
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Chapter 10.

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Notes : Abstract: The central effect of adrenomedullin on feeding was investigated in fasted rats. Following intracerebroventricular (ICV) administration, adrenomedullin decreased 2 hour food intake in a dose dependent manner. A dose of 1.7 nmol adrenomedullin decreased 2 hour food intake by 45 %. Adrenomedullin shares sequence homology with calcitonin gene-related peptide (CGRP), a central anorectic agent and binding sites for both are present in the hypothalamus. Adrenomedullin competed for 125I-adrenomedullin and 125I-CGRP binding sites in hypothalamic membranes. The Kd for the 125I-adrenomedullin binding site was 0.54 ± 0.07 nM with a Bmax of 214 ± 27 fmol/mg membrane protein (n = 3). CGRP and the CGRP receptor antagonist CGRP (8-37), at concentrations up to 1 μM, did not compete at these sites. The Kd for the CGRP binding site was 0.10 ± 0.02 nM with a Bmax of 250 ± 31 fmol/mg and the K i values for adrenomedullin and CGRP (8-37), were 4.60 ± 2.10 and 4.00 ± 1.60 nM respectively (n = 3). Thus adrenomedullin showed high affinity binding at both adrenomedullin and CGRP binding sites. To establish whether adrenomedullin reduces feeding via CGRP receptors, we co-administered adrenomedullin (1.7 nmol) and CGRP (8-37) (30 nmol). The reduction in 2 hour food intake induced by adrenomedullin was 70% inhibited by CGRP (8-37). These results show that adrenomedullin decreases food intake in the rat, and that this effect is mediated at least in part via CGRP receptors.

Chapter 11.

Meeran K, O'Shea D, Upton P, Small CJ, Ghatei M, Byfield P, Bloom SR. 1997. Circulating adrenomedullin does not regulate systemic blood pressure but increases plasma prolactin after intravenous infusion in man: A Pharmacokinetic study.
J. Clin. Endocrinol. Metab. In Press:

Notes : Abstract:Adrenomedullin has been proposed to be a circulating hormone regulating systemic and pulmonary blood pressure. A potential therapeutic role in management of pulmonary hypertension has been suggested based on animal studies but the pharmacokinetics and pharmacodynamics in man have not been studied. We have infused adrenomedullin into volunteers at 3.2 pmol/kg/min which more than quadrupled (52 pmol/l) normal circulating concentrations. At this dose no change in heart rate or blood pressure was noted. When infused at 13.4 pmol/kg/min to achieve a concentration over 40 times normal circulating levels (448 pmol/l) there was a significant fall in diastolic blood pressure from 69 ± 2 mmHg to 53 ± 2 mmHg and a significant increase in pulse rate from 57 ± 3 bpm to 95 ± 4 bpm. Circulating prolactin concentrations rose from 197 ± 46 IU/l to 372 ± 64 IU/l (mean ± SEM, $p<0.01$). No effect was seen on ACTH, TSH, FSH, LH or cortisol. When the infusion was discontinued, baseline pulse and blood pressure were re-established after 20 minutes. Adrenomedullin has a metabolic clearance rate of 27.4 ± 3.6 ml/kg/min with a circulating half life of 22 ± 1.6 minutes and an apparent volume of distribution of 880 ± 150 ml/kg. Column chromatography of plasma taken during infusion and decay of adrenomedullin showed no evidence of the production of additional molecular forms. These results are consistent with a peptide which is markedly tissue bound.

Plasma adrenomedullin concentrations were found to be increased in patients with renal impairment (14.1 ± 0.9 pmol/l), compared to those in healthy volunteers (8.1 ± 0.7 pmol/l) with a good correlation ($r=0.86$) between circulating adrenomedullin and plasma creatinine.

The circulating concentration of adrenomedullin necessary to affect blood pressure greatly exceeds that observed in healthy volunteers and in patients with a range of pathological conditions. Thus adrenomedullin may be a paracrine regulator of vascular smooth muscle in man.

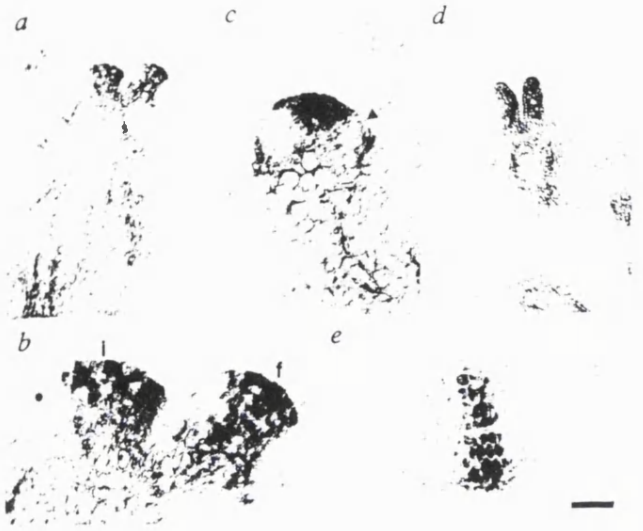

FIG. 4 Expression of *STM* during inflorescence and floral development. *a*, Inflorescence showing *STM* expression in central portions of inflorescence meristem and in central portion of stage 2 floral buds. *b*, Higher magnification of *a*. *STM* expression is downregulated in stage 1 floral buds (asterisk). Note lack of *STM* expression at position of incipient sepal primordia. i, Inflorescence meristem; f, stage 2 floral meristem. *c*, A stage 3 flower. *STM* expression is lacking at position of incipient medial stamen primordia (arrow). *d*, Stage 7–8 floral bud showing expression of *STM* in central portion of developing gyneocium. e, Transverse section through stage 7–8 gyneocium. Two ridges of expression are observed. Ovules will develop off the flanks of these ridges. Scale bar, 77 μm in *a* and *d* and 25 μm in *b*–*e*. Stages of flower development are as defined in ref. 20.

whereas *KN1* mRNA is not detectable in the maize L1 layer, *STM* mRNA is detectable in the *Arabidopsis* L1 layer.) It has been hypothesized that *KN1* functions to keep cells in an undifferentiated, undetermined, state[6,7]. This is similar to the proposed role for *STM* in SAM maintenance and suggests that these genes may be orthologous to one another. □

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A role for glucagon-like peptide-1 in the central regulation of feeding

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The sequence of glucagon-like peptide-1 (7–36) amide (GLP-1) is completely conserved in all mammalian species studied, implying that it plays a critical physiological role[1]. We have shown that GLP-1 and its specific receptors are present in the hypothalamus[2,3]. No physiological role for central GLP-1 has been established. We report here that intracerebroventricular (ICV) GLP-1 powerfully inhibits feeding in fasted rats. ICV injection of the specific GLP-1-receptor antagonist, exendin (9-39)[4], blocked the inhibitory effect of GLP-1 on food intake. Exendin (9-39) alone had no influence on fast-induced feeding but more than doubled food intake in satiated rats, and augmented the feeding response to the appetite stimulant, neuropeptide Y. Induction of c-*fos* is a marker of neuronal activation[5]. Following ICV GLP-1 injection, c-*fos* appeared exclusively in the paraventricular nucleus of the hypothalamus and central nucleus of the amygdala, and this was inhibited by prior administration of exendin (9-39). Both of these regions of the brain are of primary importance in the regulation of feeding[6]. These findings suggest that central GLP-1 is a new physiological mediator of satiety.

We report that ICV administration of GLP-1 reduces food intake in fasted rats, with greater effect at higher doses (Fig. 1*b*). ICV injection of GLP-1 in rats at the beginning of the dark (feeding) phase also results in a profound decrease in feeding (Fig. 1*a*). When administered intraperitoneally up to a dose of 500 μg, GLP-1 did not affect early dark-phase feeding (data not shown), suggesting that the action of GLP-1 on food intake is through central rather than peripheral mechanisms. A reduction in locomotor activity is a well defined part of the satiety sequence and follows nutrient ingestion[7]. In a subgroup of the animals given ICV GLP-1 at the beginning of the dark phase, locomotor activity was monitored by the frequency of line-crossing[8]. A significant reduction in activity was seen after ICV administration of GLP-1 (10 μg; 41 ± 7% of control activity, $P < 0.05$: 100 μg; 32 ± 9%, $P < 0.01$, $n = 8$ per group) compared to controls. Following ingestion of a palatable meal[9], the reduction in activity was similar to that observed following ICV injection of GLP-1 (10 μg) (palatable meal; 54 ± 19% of control activity, $P < 0.05$, $n = 6$). Although not assessed formally, the behaviour of the GLP-1-treated animals could not be distinguished, by observation, from those fed a palatable meal[10]. Fragments of GLP-1 are inactive peripherally[11]. To establish the specificity of GLP-1 on feeding,

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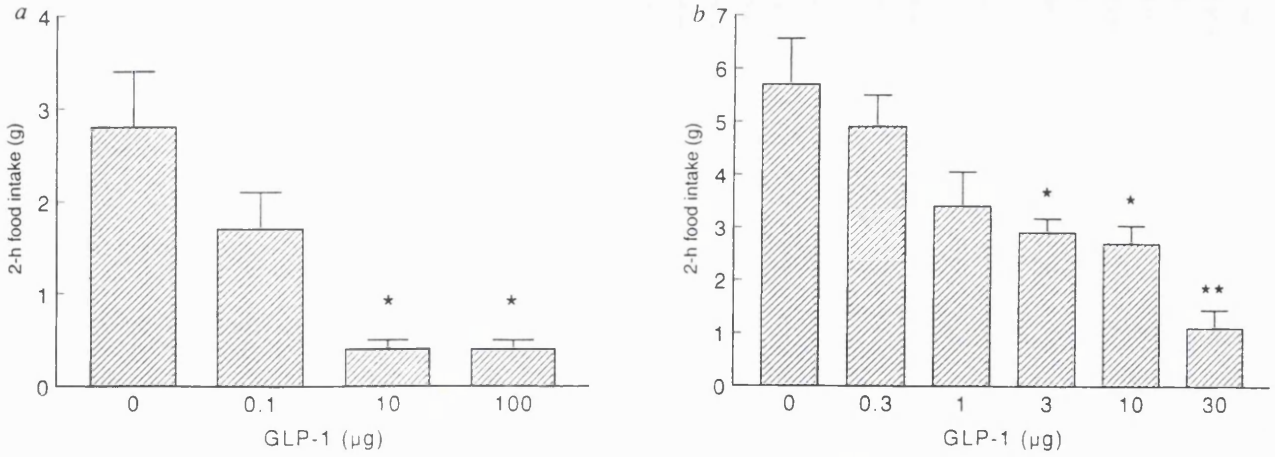

FIG. 1 a, Reduction in 2-h food intake in rats (n = 7–8 per group) injected ICV with GLP-1 or 0.9% saline (10 μl) at the onset of the dark phase ($F(3,27)$ = 10.6, $P < 0.001$), (GLP-1 versus control, $^*P < 0.001$). *b*, reduction in 2-h food intake in 24-h fasted rats (n = 6–7 per group) injected ICV with GLP-1 or 0.9% saline (10 μl) ($F(5,34) = 8.0$, $P < 0.001$), (GLP-1 versus control, $^*P < 0.05$, $^{**}P < 0.01$).
METHODS. Adult male rats (200–250 g) were maintained in individual cages under controlled conditions of temperature (21–23 °C) and light (13-h light, 11-h dark) with *ad libitum* access to chow and water. Rats were anaesthetized and stainless steel guide cannulae implanted into the third cerebral ventricle as previously described[17]. After a 5-day recovery period, rats (<10%) exhibiting a negative drinking response to ICV angiotensin II (AII) (150 ng 3 μl^{-1}) were excluded. Responders were sham injected before the study, and weighed and handled daily. Substances were diss 0.9% saline and administered ICV as previously described[17]. Fo injection, rats were returned to cages containing pre-weighed cho observed. At the end of the study period, chow was reweighed and total intake in grams (g) for each rat calculated. All injections were given at 72 h apart, between 08:30 and 11:00. Cannula placement was verifi the end of the study by a drinking response to ICV AII, followed b injection of dye, removal of the brain and visual examination of co slices. Those exhibiting a negative drinking response or absence of d the ventricular system were excluded. Results are shown as mean ± s Comparisons between groups of data were made using *t*-tests or A with post-hoc Tukey's test.

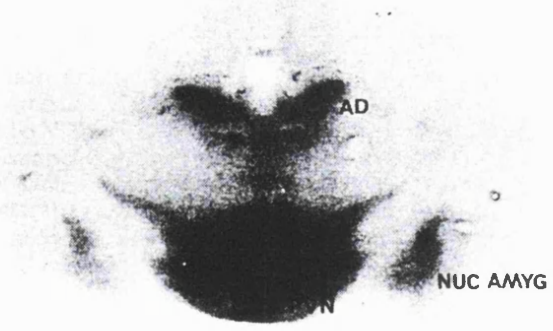

FIG. 2 Receptor autoradiography of GLP-1 binding sites in rat brain. Autoradiograph of a coronal section of rat brain incubated with 17 pM mono-^{125}I-GLP-1 alone, indicating total binding. No binding was observed in the presence of 200 nM unlabelled GLP-1 (nonspecific binding, not shown). High-density binding sites are visible in the hypothalamus (in particular the PVN), central nucleus of the amygdala (NUC AMYG) and anterodorsal thalamic nucleus (AD). No specific binding of GLP-1 was observed in the presence of 5 nM exendin (9-39) (not shown).
METHODS. ^{125}I-GLP-1 labelled by the chloramine-T method[3] was purified by reverse-phase HPLC (Waters C_{18} Novapak, Millipore). The specific activity of the label, determined by radioimmunoassay, was 60 Bq fmol^{-1}. Receptor autoradiography was carried out in a similar manner to that previously described[22]. Coronal brain sections (15 μm) were pre-incubated in 25 mM HEPES assay buffer, pH 7.4, containing 2 mM $MgCl_2$, 0.1% bacitracin, 0.05% Tween 20 and 1% BSA, for 20 min. Excess liquid was drained from the slides, which were then incubated in assay buffer containing ^{125}I-labelled GLP-1 (1,000 Bq ml^{-1}) for 90 min at room temperature. Non-specific binding was determined in the presence of 200 nM unlabelled peptide. The slides were washed four times for 30 s in ice-cold assay buffer, rinsed in ice-cold distilled water and dried overnight at 4 °C under vacuum. Autoradiographs of the slides were developed after 10 days exposure at −80 °C.

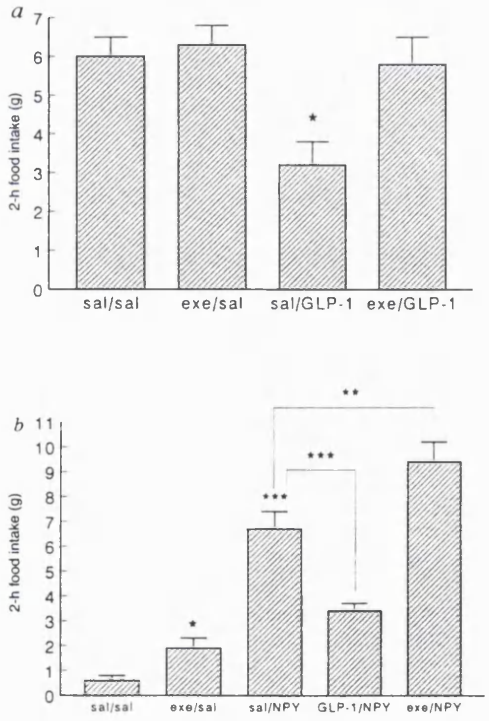

FIG. 3 Two-hour food intake in *a*, 24-h fasted rats (n = 12–14 per gro injected ICV with 0.9% saline (sal) (5 μl) or exendin (9-39) (exe) (100 5μl^{-1}), immediately followed by 0.9% saline (5 μl) or GLP-1 (3 μg 5 μl ($F(3,48) = 6.2$, $P < 0.005$; GLP-1 versus control, $^*P < 0.05$). *b*, Rats *ad libitum* (n = 6–9 per group) injected ICV at the start of the light pha with 0.9% saline (5 μl) (sal) or NPY (10 μg 5 μl^{-1}), immediately followed either 0.9% saline (5 μl), GLP-1 (10 μg 5 μl^{-1}) or exendin (9-39) (e (100 μg 5 μl^{-1}). Both exendin (9-39) and NPY significantly increased fo intake ($^*P < 0.05$ and $^{***}P < 0.001$, respectively, compared to contr GLP-1 significantly reduced ($^{***}P < 0.001$) whereas exendin (9-39) s nificantly increased ($^{**}P < 0.01$) the feeding response to NPY. Methods for Fig. 1.

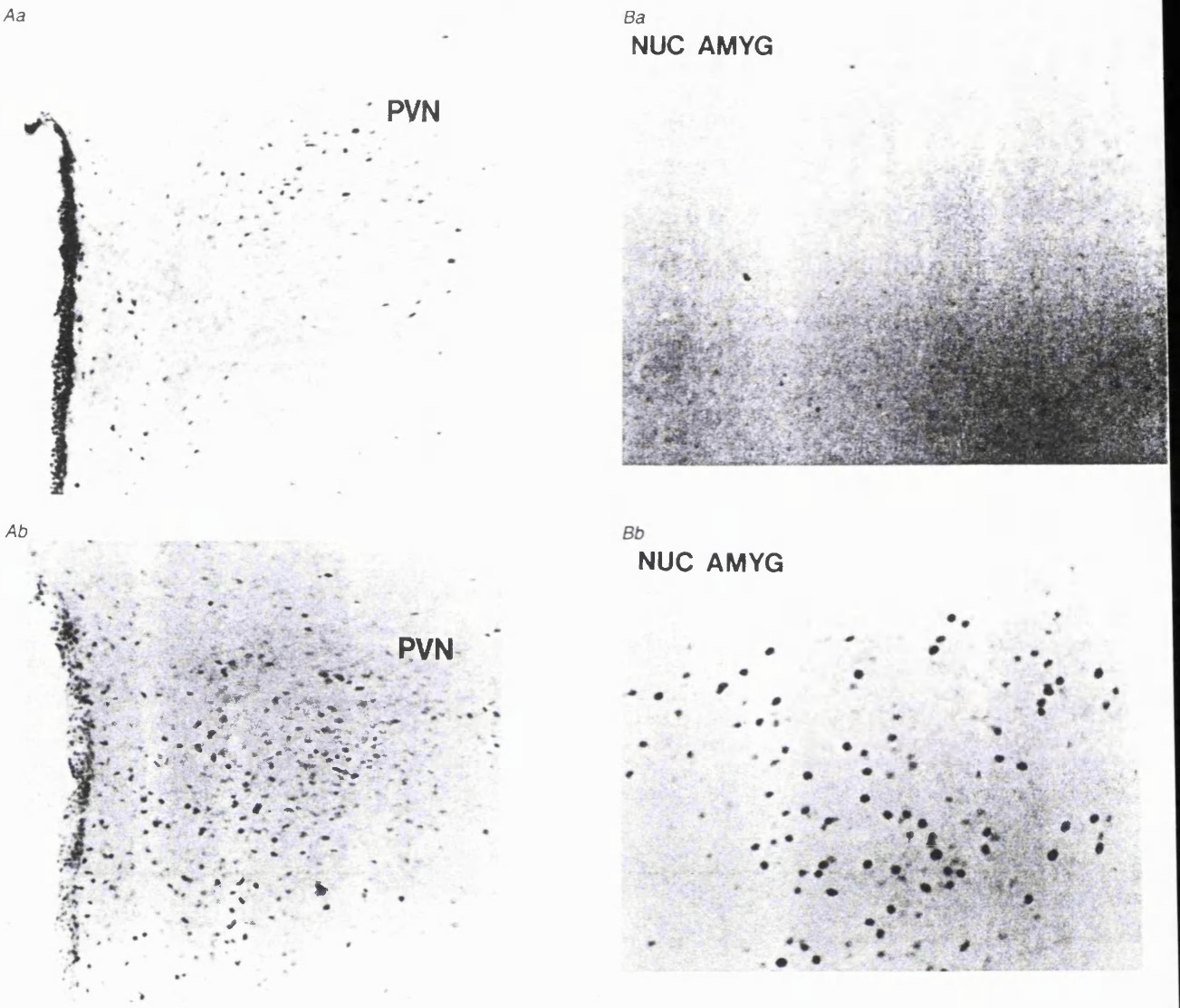

FIG. 4 Activation of c-*fos* following ICV injection of: *Aa*, 0.9% saline (10 μl) (control), demonstrating standard basal expression in the PVN (21 ± 2.2 positive cells per 10^4 μm^2, identical to that observed following ICV exendin (9-39) (100 μg 10 μl^{-1}) (not shown); and *b*, GLP-1 (10 μg 10 μl^{-1}). Significant activation is observed in the PVN (57 ± 2.3 positive cells per 10^4 μm^2, $P < 0.001$ compared to control). *Ba*, 0.9% saline (10 μl) demonstrating standard basal expression in the central nucleus of the amygdala (4 ± 0.5 positive cells per 10^4 μm^2); and *b*, GLP-1 (10 μg 10 μl^{-1}). Significant activation is observed in the central nucleus of the amygdala (NUC AMYG) (13 ± 0.5 positive cells per 10^4 μm^2; $P < 0.001$ compared to control).

METHODS. Adult male rats (200–250 g) were maintained in individual cages under controlled conditions of temperature (21–23 C) and light (13-h light, 11-h dark) with *ad libitum* access to chow and water. Rats were anaesthetized and stainless steel guide cannulae were implanted into the lateral cerebral ventricle as previously described[23]. Rats were handled 10 min each day following surgery, to familiarize them with infus procedures and to minimize stress, which can readily activate c-*fo* Substances were dissolved in 0.9% saline and administered ICV previously described[23]. Following injection, rats were returned to t cages for 1 h with no access to food. Rats were anaesthetized and perfus transcardially with 0.1 M PBS followed by 4% paraformaldehyde soluti The brains were removed and stored in 20% sucrose solution before be processed for detection of c-*fos* as previously described[23]. Coronal secti (40 μm) were washed and incubated with antisera and processed using Vectastain ABC kit (Vector Laboratories, USA). Sections were immerse 0.1% diaminobenzidinetetrahydrochloride solution and mounted on slid Expression of c-*fos* was then quantified as previously described[23]. Resu are expressed as mean ± s.e.m. of positive cells per 10^4 μm^2. Comparis between groups of data were made using *t*-tests.

GLP-1 (8–36), (9–36) and (11–36) amide were therefore tested by ICV injection in fasted rats. At doses of up to 100 μg, these peptides had no effect on food intake or locomotor activity compared to controls (data not shown).

Exendin (9-39) has sequence homology to GLP-1 (ref. 12). It is a highly selective antagonist at GLP-1 receptors *in vitro*[1]. Furthermore, we have shown that exendin (9-39) specifically abolishes GLP-1-induced insulin secretion in the rat at a ratio of 15 : 1 (ref. 13), and *in vitro* in a clonal β-cell line at a ratio of 10 : 1 (ref. 14), indicating blockade of β-cell GLP-1 receptors. We therefore used a dose of 100 μg of exendin (9-39) to antagonize a dose of 3 μg GLP-1 *in vivo*. Autoradiography of GLP-1 binding sites in the brain shows dense specific binding of GLP-1 in the hypothalamus, especially the paraventricular nucleus (PVN); the central nucleus of the amygdala and the anterodorsal thalamic nucleus (Fig. 2). Addition of 5 n exendin (9-39) blocks GLP-1 binding in these regions (not shown

ICV administration of exendin (9-39) (100 μg) blocks t *inhibitory effect of GLP-1* (3 μg) on food intake in fasted r (Fig. 3*a*), but exendin (9-39) alone does not affect food intake this circumstance (Fig. 3*a*). When administered by ICV injecti to rats fed *ad libitum* at the start of the light phase (satiate exendin (9-39) (100 μg) more than doubled food intake (Fig. 3*b* The profound effect of exendin (9-39) in satiated animals witho effect in fasted animals suggests a role for endogenous GLP-1 as central satiety factor[15].

Neuropeptide Y (NPY) is the most powerful stimulant feeding known[16,17]. In animals fed *ad libitum*, at the start of t light phase, ICV administration of GLP-1 (10 μg) immediate

before NPY (10 μg) greatly reduced food intake (Fig. 3*b*). Furthermore, ICV administration of exendin (9-39) (100 μg) immediately before NPY (10 μg) significantly increased food intake, compared to treatment with NPY (10 μg) alone (Fig. 3*b*). Hypothalamic NPY messenger RNA was assessed, as previously described[18], following repeated ICV administration of 0.9% saline or GLP-1 (100 μg) in both 72-h fasted and *ad libitum* fed rats. ICV injections were given during this 72-h period at 48, 24, 12 and 4 h before being killed. No change in NPY mRNA was found following ICV GLP-1 injection (data not shown). This suggests that GLP-1 does not act by altering hypothalamic NPY synthesis. The increase in food intake following blockade of GLP-1 receptors by exendin (9-39) and the augmented NPY response with coadministration of exendin (9-39) supports a physiological role for central GLP-1 in the regulation of feeding.

To establish the neuronal population(s) activated by central GLP-1, expression of the immediate early gene c-*fos*, a well established marker of neuronal activation[5], was mapped following ICV injection of GLP-1. Administration of exendin (9-39) (data not shown) or 0.9% saline (Fig. 4*Aa*, *Ba*) did not activate c-*fos* in any region of the brain. In contrast, rats injected with GLP-1 exhibit dense expression of c-Fos in the PVN (Fig. 4*Ab*) and central nucleus of the amygdala (Fig. 4*Bb*), but not the remainder of the hypothalamus or anterodorsal thalamic nucleus. The induction of c-*fos* following ICV GLP-1 injection was inhibited by prior administration of exendin (9-39) (data not shown).

In conclusion, central GLP-1 is a new physiological regulator of feeding in the rat. Many neuropeptides have been proposed as central satiety factors[6], but few have an established physiological role. GLP-1 may be the most potent inhibitor of feeding yet identified in the rat. Its role in man remains to be determined. The interaction of GLP-1 with other established regulators of food intake, such as leptin (Ob protein)[19-21] and NPY, also need investigation. Understanding the function of these centrally active regulators should ultimately lead to new and more effective agents for the management of appetite disorders.

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Rapid colour-specific detection of motion in human vision

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THE human visual system is much better at analysing the motion of luminance (black and white) patterns than it is at analysing the motion of colour patterns[1-4], especially if the pattern is presented very briefly[5] or moves rapidly[6]. We report here that observers reliably distinguish the direction of motion of a colour pattern presented for only 17 milliseconds, provided that the contrast is several times the threshold value (the contrast needed to detect the presence of the pattern). A control experiment, in which a static luminance 'mask' is added to the moving colour pattern, proves that discrimination of the direction of motion of these brief stimuli is colour-specific. The mask drastically impairs discrimination of the direction of motion of a luminance pattern, but it has little effect on a colour pattern. We conclude that the human visual system contains colour-specific motion-detection mechanisms that are capable of analysing very brief signals.

The prevailing view is that the visual system's primary analyses of colour and movement occur in parallel in different areas of the brain[7-9]. An important observation supporting this view is that the motion of many patterns defined solely by colour is undetectable[1] or hard to see[4]. The colour of such a pattern must be analysed before its motion, so if motion and colour are analysed in parallel, motion analysis must inevitably be compromised.

To demonstrate that colour contributes directly to the analysis of motion, it is important to show that motion discrimination is not secondary to analysis of position[10] or to the production of eye movements that track the stimulus. The only way to be sure of this is to use a very brief presentation so that the stimulus disappears before an eye movement can be initiated. Previous demonstrations that colour contributes distinctively to motion analysis have always used long presentations[11,12]. Thus, even though direction discrimination thresholds for colour stimuli may be lower (in contrast) than those of luminance stimuli[6], we cannot yet exclude the possibility that the motion of such stimuli is analysed by conscious monitoring of changes in position[10].

Figure 1 shows that the motion of colour gratings can be discriminated reliably even when the grating is only present for 17 ms, about 5 times less than the time taken to initiate an eye movement[13]. Discrimination of such brief stimuli could only be mediated by a mechanism whose primary function is the analysis of motion.

We must ensure that the motion of colour gratings shown in Fig. 1 depends on a mechanism selective for colour and is not caused by inadvertent stimulation of mechanisms sensitive to luminance patterns[14]. Accordingly, we retested motion discrimination using a red–green colour grating to which a static luminance mask had been added. The test depends on the assumption that if the colour grating's motion is detected by a luminance sensitive mechanism, then we can treat it as equivalent to the luminance grating that would support the same direction discrimination performance. The exact position of the luminance grating relative to the colour grating (its phase) is unknown because it depends on the nature of the hypothetical artefact that causes the colour grating to stimulate a luminance-sensitive mechanism.

The basis of the masking test is shown in Fig. 2, which illustrates the effect of static masks added to a moving luminance grating in different phases. In the absence of the mask (lower left-han

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BRAIN RESEARCH

Research report

Weight loss in rats treated with intracerebroventricular cobalt protoporphyrin is not specific to the neuropeptide Y system

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Abstract

Cobalt protoporphyrin (CoPP) reduces food intake and body weight following intracerebroventricular (i.c.v.) administration in rats. We injected 0.2 μmol CoPP per kg body weight i.c.v. and monitored body weight and daily food intake for 7 days. The body weight and 24 h food intake of CoPP-treated animals was significantly lower than that of vehicle-treated animals in all studies ($P < 0.01$) from day 2 to day 7. The 2 h feeding response (CoPP vs. vehicle-treated) to 10 μg neuropeptide Y (NPY) (4.0 vs. 7.1 g; $P < 0.05$), the 1 h feeding response to 10 μg galanin (1.3 vs. 3.2 g; $P < 0.05$) and 30 μg norepinephrine (0.6 vs. 1.9 g; $P < 0.05$) in CoPP-treated animals were all reduced compared to the vehicle-treated group. In addition there was no change in hypothalamic NPY mRNA in CoPP-treated animals. I.c.v. CoPP decreases sensitivity to exogenous NPY, galanin and norepinephrine. The effect of CoPP is not specific to NPY as previously described.

Keywords: Cobalt protoporphyrin; Intracerebroventricular injection; Feeding; Neuropeptide Y; Hypothalamus

1. Introduction

Cobalt protoporphyrin (CoPP), a synthetic metalloprotoporphyrin, has been reported to cause a dramatic and prolonged reduction in food intake. It induces a significant weight loss in normal and genetically obese rats, [7,8,10,11,13–15] and beagle dogs [12] when administered subcutaneously or i.c.v., but its mechanism of action is unknown. The weight loss caused by CoPP has two stages [7]. There is an initial 2 to 3 days of severe hypophagia and weight loss, followed by a gradual return to normal food intake. The difference in body weight persists for as long as 300 days after i.c.v. injection [13], implying that animals defend a new body weight following CoPP injection. The likely site of action of CoPP on body weight and food intake is the hypothalamus. When CoPP is given i.c.v., only 1% of the systemic dose is required for an equivalent effect on food intake and body weight [10]. Following i.c.v. injection, a higher content of cobalt is found in the hypothalamus compared with other brain areas [13]. In addition direct microinjection of CoPP (20 nmol/kg) into the medial, but not the lateral, nuclei of the hypothalamus causes reduced food intake and body weight in rats [15]. No evidence of structural hypothalamic change is found either macro- or microscopically after CoPP injection [10].

Neuropeptide Y (NPY) is a 36-amino-acid peptide and a profound stimulant of feeding following i.c.v. administration [3]. There is a 12–20 min delay before feeding commences and this effect is evident for at least 2 h [4,5,23]. NPY stimulates carbohydrate feeding preferentially [19,26]. Galanin is a 29-amino-acid peptide which stimulates feeding 7 min after i.c.v. administration [24]. The effect is over by 40 min. Galanin stimulates fat intake preferentially [25]. Norepinephrine stimulates food intake by binding to α_2-adrenoreceptors [30]. Its effect starts within three min and lasts up to 1 h [1], with a marked preference for carbohydrate feeding [29]. The differences in onset and duration of action of these compounds, and the differences in food preference that they cause suggests that they stimulate feeding by independent mechanisms.

Previous studies performed by Galbraith et al. indicate that a dose of between 0.1 and 0.4 μmol/kg is effective in reducing body weight [13]. We chose a dose of 0.2 μmol/kg for our feeding studies and a maximum dose of 0.4 μmol/kg in order to determine whether any change in NPY mRNA would occur. This is the dose used by Galbraith et al. in previous studies looking at the effect of CoPP on NPY mRNA [7]. For the stimulants of feeding,

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we chose doses that are known to give a near maximal feeding response in the early light phase [1,24].

In elucidating the mechanism of action of i.c.v. CoPP on food intake, only NPY-induced feeding has previously been studied. Based on these studies, a specific effect on central NPY has been proposed as a mechanism of action for CoPP [7,28]. To test this hypothesis, we assessed the effect of i.c.v.-administered norepinephrine and galanin, in addition to NPY on food intake in CoPP-treated animals.

2. Materials and methods

2.1. Animals and surgery

Adult male Wistar rats (200–250 g) were maintained in individual cages under controlled temperature (21–23°C) and light (11 h of light, 13 h of darkness), with ad libitum access to food and water.

Rats were anaesthetised with xylazine (20 mg/kg) (Rompun, Bayer, Suffolk, UK) and ketamine (100 mg/kg) (Ketalar, Parke Davis, Pontypool, Gwent, UK). Permanent 22-gauge stainless steel cannulae (Plastics One, Roanoke, VA) were stereotactically placed 0.8 mm posterior to the bregma in the midline and implanted 6.5 mm below the outer surface of the skull into the third cerebral ventricle. After surgery, a small wire plug was inserted into each cannula to prevent blockage. All animals were allowed at least 7 days to recover before being used in the study. Human angiotensin II (Sigma, Poole, Dorset, UK) (150 ng per rat) was injected i.c.v. to confirm the correct position of the cannula and only animals which showed a sustained drinking response within 2 min were used for the study. Cannula placement was verified at the end of the study by a second drinking response to i.c.v. angiotensin II, followed by i.c.v. injection of dye, removal of the brain, and visual examination of coronal slices.

2.2. Experimental protocol

NPY and galanin were synthesised in an Applied Biosystems 431A peptide synthesiser and purified using HPLC. Norepinephrine bitartrate was purchased from Sigma. Substances were administered through a 28-gauge stainless steel injector, placed in and projecting 1 mm below the tip of the cannula. The injector was connected by a polyethylene tubing to a Hamilton syringe (Reno, NV) attached to a syringe pump set to dispense 10 μl solution/min. CoPP (Porphyrin Products, Logan, UT) is insoluble at the concentration we required for i.c.v. injection in all aqueous solutions including sodium hydroxide, which has been used for subcutaneous [9,21,22] and in vitro [2] studies. For this reason, it was dissolved in 70% ethanol and the pH adjusted to 7.5 with 1 M NaOH. The final volume was made up to 6 ml and the solution was filtered through a 0.2 μm millipore filter. The filtered solution was diluted 1:1000 and the optical absorbance was measured at 417 nm to confirm the concentration of the dissolved compound before administration. All other compounds were dissolved in 0.9% saline.

CoPP or vehicle (70% ethanol filtered, pH 7.5) was administered i.c.v. and 24 h food intake and body weight were monitored daily for 2 days prior to treatment and until day 7 following injection. The animals were handled daily and observed for any obvious behavioural abnormalities.

In the first two experiments, 1 h or 2 h food intake in response to saline, norepinephrine, galanin or NPY injection was carried out. Each animal received one of the three compounds (including saline) in succession in random order, 7, 10 and 13 days after i.c.v. CoPP injection. Further details of each study are given below. In experiment III, the effects on NPY mRNA were studied following i.c.v. CoPP. All injections were carried out at the beginning of the light phase, when we would expect all animals to be fully satiated.

2.2.1. Experiment I

CoPP (0.2 μmol/kg) was injected i.c.v. Porcine NPY (10 μg in 10 μl), porcine galanin (10 μg in 10 μl) or saline (10 μl) was injected i.c.v. on days 7, 10 and 13 by crossover design (CoPP $n = 14$, vehicle $n = 11$). After i.c.v. injection, premeasured food pellets were given in the metal hopper with water available ad libitum and 1 h and 2 h food intakes were measured.

2.2.2. Experiment II

CoPP (0.2 μmol/kg) was injected i.c.v. NPY (10 μg in 10 μl), norepinephrine (30 μg in 10 μl) or saline (10 μl) was injected i.c.v. on days 7, 10 and 13 by crossover design (CoPP $n = 9$, vehicle $n = 6$). The 1 h and 2 h food intakes were measured as in Experiment I.

2.2.3. Experiment III

CoPP (0.4 μmol/kg) was injected i.c.v. and the hypothalami were collected in liquid nitrogen nine days later (CoPP $n = 9$, vehicle $n = 12$). Hypothalamic NPY mRNA was measured and quantified by Northern blot assay as previously described [31].

2.3. Statistical analysis

All results are given as mean ± standard error of the mean (S.E.M.). Comparison between CoPP and vehicle-treated animals was made using an unpaired Student's t-test using the method of summary measures as given by Matthews et al. [20]. Significance was taken as $P < 0.05$.

3. Results

CoPP-treated animals had reduced locomotor activity for up to 24 h after i.c.v. CoPP injection. No other obvious differences in behaviour were noted.

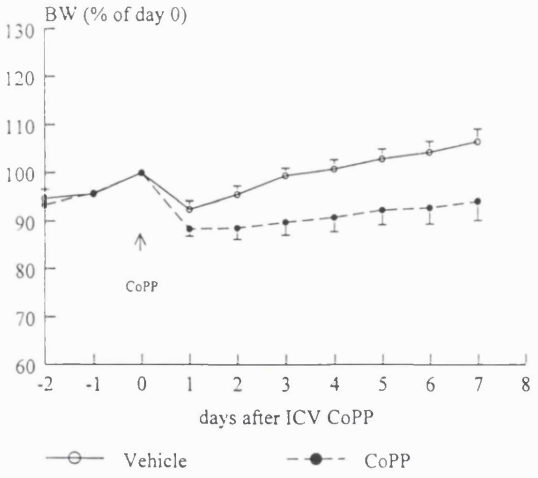

Fig. 1. Effect of CoPP on body weight. Male Wistar rats were injected i.c.v. with CoPP (0.2 μmol/ kg) ($n = 14$) or vehicle ($n = 11$) at day 0 (↑). Body weight was expressed as percentage of body weight at day 0. Data are mean ± S.E.M. The body weight was significantly reduced from day 2 to day 7 by the method of summary measures ($P < 0.01$) (CoPP- vs. vehicle-treated group).

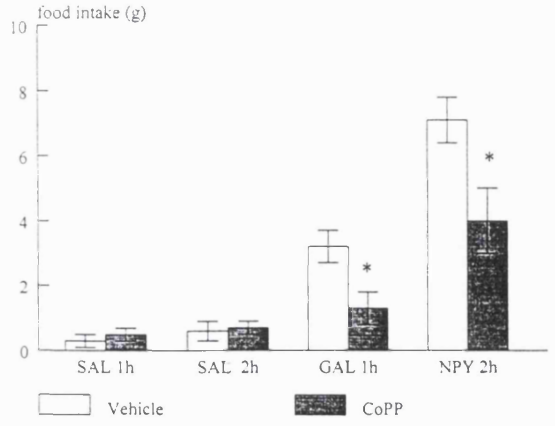

Fig. 3. Effect of CoPP on food intake following i.c.v. injection of NPY or galanin. Male Wistar rats were injected i.c.v. with CoPP (0.2 μmol/kg) ($n = 14$) or vehicle ($n = 11$) at day 0. At day 7, 10 and 13 after i.c.v. CoPP, the animals were injected with NPY (10 μg in 10 μl), galanin (GAL, 10 μg in 10 μl) or saline (SAL, 10 μl) and 1 h (1h) and 2 h (2h) food intakes were measured. Data are mean ± S.E.M. * $P < 0.05$ (CoPP- vs. vehicle-treated group).

3.1. Experiment I

The body weight and food intake of CoPP-treated animals was significantly reduced compared to controls from day 2 to day 7 following treatment ($P < 0.01$) (Fig. 1, Fig. 2). The 2 h feeding response to i.c.v. NPY in CoPP-treated animals was 4.0 ± 1.0 g compared to 7.1 ± 0.7 g ($P < 0.05$) in vehicle-treated animals (Fig. 3). The 1 h feeding response to i.c.v. galanin was 1.3 ± 0.5 g in CoPP-treated animals compared to 3.2 ± 0.5 g ($P < 0.05$) in vehicle-treated animals (Fig. 3).

3.2. Experiment II

The effect of CoPP on body weight and food intake in this experiment was similar to that in Expt. I. The 2 h feeding response to i.c.v. NPY in CoPP-treated animals was 4.6 ± 0.9 g compared to 10.1 ± 2.0 g in vehicle-treated animals ($P < 0.05$) (Fig. 4). The 1 h feeding response to

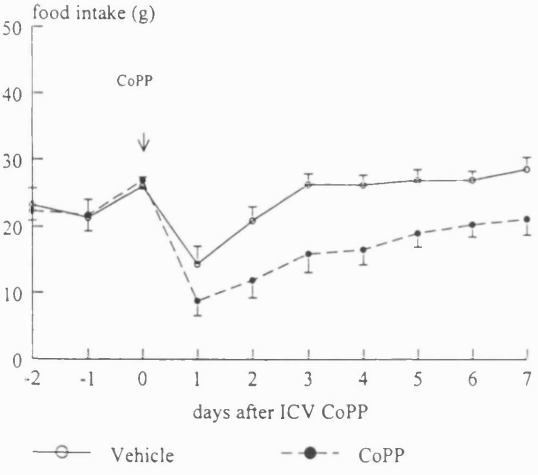

Fig. 2. Effect of CoPP on 24 h food intake. Male Wistar rats were injected i.c.v. with CoPP (0.2 μmol/kg) ($n = 14$) or vehicle ($n = 11$) at day 0 (↓). 24 h food intake was measured for two days prior to and 7 days following injection. Data are mean ± S.E.M. The food intake was significantly reduced from day 2 to day 7 by summary measures ($P < 0.01$) (CoPP- vs. vehicle-treated group).

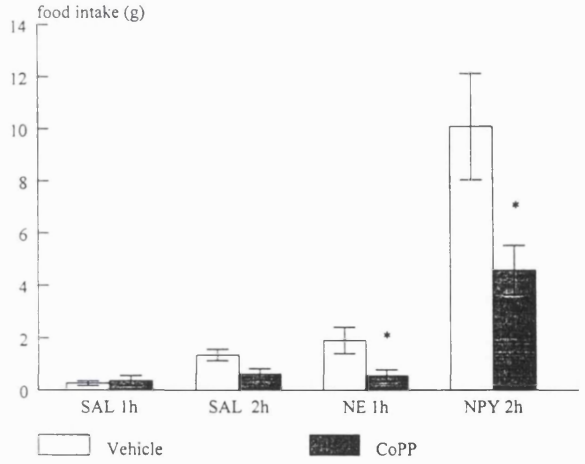

Fig. 4. Effect of CoPP on food intake following i.c.v. injection of NPY or norepinephrine. Male Wistar rats were injected i.c.v. with CoPP (0.2 μmol/kg) ($n = 9$) or vehicle ($n = 6$) at day 0. At day 7, 10 and 13 after i.c.v. CoPP, the animals were injected with NPY (10 μg in 10 μl), norepinephrine (NE, 30 μg in 10 μl) or saline (SAL, 10 μl) and 1 h (1h) and 2 h (2h) food intakes were measured. Data are mean ± S.E.M. * $P < 0.05$ (CoPP- vs. vehicle-treated group).

i.c.v. norepinephrine in CoPP-treated animals was 0.6 ± 0.2 g compared to 1.9 ± 0.5 g in vehicle-treated animals ($P < 0.05$) (Fig. 4).

3.3. Experiment III

The effect of CoPP on body weight and food intake in this experiment was similar to that in Expt. I. Hypothalamic NPY mRNA measured at day 9 was 104.5 ± 19.9% in CoPP-treated animals compared to 100 ± 8.8% in vehicle-treated animals.

4. Discussion

In this study we confirmed that following i.c.v. CoPP, the body weight and food intake of rats remain decreased for 7 days as previously described [13]. We have also found that the feeding response to i.c.v. NPY, galanin and norepinephrine was decreased in CoPP-treated animals. It has been suggested that the reduction in feeding in response to exogenous NPY in CoPP-treated animals is due to a specific decrease in NPY sensitivity [7,28]. This is unlikely, given that the three stimulants we studied act through their own distinct receptors and the feeding response to all three was reduced by CoPP administration.

The interaction between NPY, galanin and norepinephrine is complicated. It is known that α_2 antagonists block galanin- but not NPY-induced feeding, implying that NPY does not act through norepinephrine [18]. In addition, the onset of galanin- and norepinephrine-induced feeding occurs within 7 min of i.c.v. administration [1,24], whereas NPY-induced feeding is delayed by between 12 and 20 min [4,5,23]. It is therefore unlikely that either norepinephrine or galanin have their feeding effect through NPY. The difference in macronutrient selection caused by NPY, which stimulates carbohydrate intake, and galanin, which stimulates fat intake, is further evidence for their independence of action. Our finding of decreased sensitivity to all three compounds following i.c.v. CoPP administration implies that CoPP does not have a specific effect on any individual system. The effect of CoPP may be independent of all three compounds, or it may affect the final common pathway of feeding.

Galbraith et al. reported that NPY mRNA measured at day 2 after i.c.v. CoPP injection was more than doubled and that there was no feeding response to i.c.v. NPY [7]. This increased hypothalamic mRNA at day 2 may reflect a normal compensatory increase, in response to severe hypophagia. Our animals had decreased locomotor activity as well as decreased feeding on the first day following CoPP treatment. It is not clear whether this is because of an early toxic effect, or a compensatory response to decreased energy intake. Hypophagia persisted until day 9 at which time we found no significant change in NPY mRNA levels. Thus the reported rise in hypothalamic NPY mRNA seen at day 2 does not persist despite continued hypophagia.

Since the effect of CoPP has been shown to last for 300 days [13], it has been suggested that a change in body weight set point may occur. The results of experiments utilising pair-fed animals are consistent with this suggestion [10]. When administered systemically in large doses, CoPP has several other metabolic and endocrine effects. These include induction of hepatic heme oxygenase, decreased plasma testosterone without a compensatory rise in luteinising hormone [9,16,17] and suppression of hepatic cytochrome *P*-450 enzyme content and activity [6].

I.c.v. CoPP may exert its effect on food intake and body weight by inducing brain heme-oxygenase or by altering steroid metabolism via inhibition of cytochrome *P*-450 enzyme activity within the hypothalamus. A change in steroid metabolism may alter food intake, as steroids are known to have a permissive effect on the feeding responses elicited by NPY and norepinephrine [27].

In conclusion, i.c.v. CoPP decreases sensitivity to exogenous NPY, galanin and norepinephrine. Its effect on neurotransmitter systems in the brain is therefore more generalised than was previously recognized.

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Adrenomedullin Inhibits Feeding in the Rat by a Mechanism Involving Calcitonin Gene-Related Peptide Receptors

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ABSTRACT

The central effect of adrenomedullin on feeding was investigated in fasted rats. After intracerebroventricular administration, adrenomedullin decreased 2-h food intake in a dose-dependent manner. A dose of 1.7 nmol adrenomedullin decreased 2-h food intake by 57%. Adrenomedullin shares sequence homology with calcitonin gene-related peptide (CGRP), a central anorectic agent, and binding sites for both are present in the hypothalamus. Adrenomedullin competed for [^{125}I]adrenomedullin- and [^{125}I]CGRP-binding sites in hypothalamic membranes. The K_d for the [^{125}I]adrenomedullin-binding site was 0.54 ± 0.07 nM, with a binding capacity of 214 ± 27 fmol/mg membrane protein (n = 3). CGRP and the CGRP receptor antagonist CGRP-(8–37) at concentrations up to 1 μM did not compete at these sites. The K_d for the CGRP-binding site was 0.10 ± 0.02 nM, with a binding capacity of 250 ± 31 fmol/mg, and the K_i values for adrenomedullin and CGRP-(8–37) were 4.6 ± 2.1 and 4.0 ± 1.6 nM, respectively (n = 3). Thus, adrenomedullin showed high affinity binding at both adrenomedullin- and CGRP-binding sites. To establish whether adrenomedullin reduces feeding via CGRP receptors, we coadministered adrenomedullin (1.7 nmol) and CGRP-(8–37) (30 nmol). The reduction in 2-h food intake induced by adrenomedullin was 50% inhibited by CGRP-(8–37). These results show that adrenomedullin decreases food intake in the rat, and this effect is mediated at least in part via CGRP receptors. (*Endocrinology* **137:** 3260–3264, 1996)

ADRENOMEDULLIN is a recently discovered member of the calcitonin family of peptides, isolated from human pheochromocytomas (1). Adrenomedullin shares structural homology and some effects with the calcitonin family of peptides, *i.e.* calcitonin, calcitonin-gene related peptide (CGRP), and islet amyloid polypeptide (IAPP). Immunoreactive adrenomedullin (2, 3) and adrenomedullin messenger RNA have been detected in many human and rat tissues, including heart, lung, and brain (4, 5). Immunoreactive adrenomedullin has been found in both human (6, 7) and rat (2) plasma at concentrations of 2–10 pmol/liter.

Adrenomedullin, like CGRP, is a potent hypotensive peptide when administered peripherally in many species, including the rat (8–10). Early experiments on the vasodilator effects of adrenomedullin in the isolated perfused mesenteric vascular bed (11) and in the isolated rat heart (12) showed marked inhibition by the specific CGRP receptor subtype 1 ($CGRP_1$) antagonist, CGRP-(8–37), indicating that in these vascular beds, the effect of adrenomedullin is via $CGRP_1$ receptors. However, the hypotensive effect of CGRP, but not adrenomedullin, is blocked *in vivo* by CGRP-(8–37) (8, 13). This implies that the effect of adrenomedullin on systemic blood pressure may not occur through $CGRP_1$ receptors.

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Intracerebroventricular (icv) administration of adrenomedullin causes an increase in systemic blood pressure that is inhibited by CGRP-(8–37) (14). Thus, centrally administered adrenomedullin can mediate effects via $CGRP_1$ receptors, which are widely distributed throughout the brain, including the hypothalamus, an area known to be involved in the regulation of food intake (15–17). The calcitonin family of peptides is known to potently decrease food and water intake when administered centrally (18–21). The hypothalamus has been identified as the main target site for calcitonin-induced anorexia, in particular the paraventricular area (22, 23). Calcitonin receptors are found in high concentrations in the hypothalamus (24). Intrahypothalamic injections of IAPP also caused profound anorexia in rats, with the hypothalamus again showing high levels of IAPP binding (19, 25, 26). Other neuropeptides with effects on feeding, including neuropeptide Y and galanin, are most effective when injected into the paraventricular nucleus. Adrenomedullin is present in the brain (3), but its effects on feeding have not been investigated.

Recently, we have shown that specific adrenomedullin receptors are present in rat tissues, especially lung and heart, but also the hypothalamus (27), and that these receptors may be involved in the effect of adrenomedullin on systemic blood pressure (8). Here we established whether adrenomedullin could bind to CGRP receptors in hypothalamic membranes and investigated the effects of icv adrenomedullin on food intake. CGRP-(8–37) was used to determine to what extent any effect on feeding is mediated by $CGRP_1$ receptors.

Materials and Methods

Materials

Human adrenomedullin and human CGRP-(8–37) were synthesized using an Applied Biosystems 431A peptide synthesizer (Foster City, CA). [Tyr^0]αCGRP was supplied by Peninsula Laboratories (Belmont, CA). Rat αCGRP was synthesized by ASG University (Szedgel, Hungary). Peptides were checked for correct mol wt by mass spectrometry. Na^{125}I was supplied by Amersham International (Little Chalfont, UK). All other reagents were supplied by Merck (Poole, UK) or Sigma Chemical Co. (Poole, UK).

In vivo studies

Adult male Wistar rats (200–250 g) were maintained in individual cages under controlled temperature (21–23 C) and light (11 h of light, 13 h of darkness), with *ad libitum* access to food and water. Rats were anesthetized with xylazine (20 mg/kg; Rompun, Bayer, Suffolk, UK) and ketamine (100 mg/kg; Ketalar, Parke-Davis, Pontypool, UK). Permanent 22-gauge stainless steel cannulas (Plastics One, Roanoke, VA) were stereotactically placed 0.8 mm posterior to the bregma in the midline and implanted 6.5 mm below the outer surface of the skull into the third cerebral ventricle. After surgery, a small wire plug was inserted into each cannula to prevent blockage. All animals were allowed at least 7 days to recover before being used in the study. Human angiotensin II (Sigma; 150 ng/rat) was injected icv to confirm the correct position of the cannula, and only animals that showed a sustained drinking response within 2 min were used for the study. Before the study day, animals were sham injected twice to minimize the stress of injection. This was to ensure that any decrease in feeding on the study day was not due to the stress of injection. Cannula placement was verified at the end of the study by a second drinking response to icv angiotensin II, followed by icv injection of dye, removal of the brain, and visual examination of coronal slices. Substances were dissolved in 0.9% saline and injected through a stainless steel injector that protruded 1 mm below the tip of the cannula. After injection, animals were placed back in their home cage with a preweighed amount of food.

Effect of increasing doses of adrenomedullin on fast-induced feeding

Animals (n = 6/group) were fasted for 24 h before injection during the early light phase. Rats received a 10-μl icv injection of adrenomedullin (0, 0.05. 0.17, 1.7, or 5.0 nmol), and food intake was measured at 30 and 120 min. Food intake between groups was compared using ANOVA with *post-hoc* Tukey's test.

Antagonist studies

To establish whether adrenomedullin was producing its effect on food intake via $CGRP_1$ receptors, animals (n = 6–9/group) were icv injected with 30 nmol CGRP-(8–37) immediately before either saline or adrenomedullin (1.7 nmol). Food intake between groups was compared using ANOVA with *post-hoc* Tukey's test.

Receptor binding assays

Membrane preparation. The hypothalamus was removed from male Wistar rats (200–250 g) and frozen in liquid nitrogen. Tissue was homogenized in three 1-min bursts in ice-cold HEPES (50 mM) buffer (pH 7.6) containing sucrose (0.25 M), soybean trypsin inhibitor (10 μg/ml), pepstatin (0.5 μg/ml), leupeptin (0.5 μg/ml), antipain (0.5 μg/ml), benzamidine (0.1 μg/ml), aprotinin (30 μg/ml), and bacitracin (0.1 mg/ml), using an Ultra-Turrax T-25 homogenizer (Merck, Poole, UK). The homogenates were centrifuged at 1,500 × *g* for 10 min at 4 C, and the supernatants were centrifuged at 100,000 × *g* for 1 h at 4 C. The pellets were resuspended in 10 vol homogenization buffer without sucrose by hand homogenization using a glass-Teflon homogenizer and centrifuged at 100,000 × *g* at 4 C. The membranes were resuspended as described above to a final concentration of about 4 mg protein/ml and stored at −80 C. Protein concentration was measured by the biuret method (28).

Peptide iodination. [Tyr^0]αCGRP (12 μg; 2.4 nmol) was iodinated by the Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) method, as previously described (29). Iodinated peptide was separated by reverse phase HPLC (Waters C_{18} Novapak, Millipore, Milford, MA) using a linear gradient of 20–50% aqueous acetonitrile containing 0.05% trifluoroacetic acid over 90 min. Peak fractions were assayed for binding to membranes, and the specific activity of the label, determined by RIA, was 35.9 becquerels (Bq)/fmol.

Rat adrenomedullin was also iodinated by the Iodogen method (27). Briefly, 12.5 μg (2 nmol) peptide in 10 μl 0.2 M phosphate buffer (pH 7.2) were added to an Iodogen-coated (10 μg) polypropylene vial together with 37 MBq Na^{125} I and incubated for 4 min at 22 C. The ^{125}I-labeled peptide was purified by reverse phase HPLC as described for CGRP. Fractions showing binding (SA, 10.4 Bq/fmol) were aliquoted, freeze-dried, and stored at −80 C.

CGRP binding assay. Hypothalamic membranes (100 μg membrane protein) were incubated at 22 C for 45 min with ^{125}I-labeled CGRP (1000 Bq; 56 pM) in binding buffer (20 mM HEPES buffer, pH 7.4, containing 5 mM $MgCl_2$, 5 mM KCl, 1 mM EDTA, 0.25 mg/ml bacitracin, and 1 μM phosphoramidon) containing 0.1% BSA, as previously described (29). The receptor-[^{125}I]CGRP complex was separated from free tracer by centrifugation at 15,600 × *g* for 2 min. Nonspecific binding was determined in the presence of 200 nM unlabeled rat CGRP. Specific binding is defined as total binding minus nonspecific binding. All reaction vessels were silanized before use to reduce the loss of peptide from sticking to plastic.

Adrenomedullin binding assay. Hypothalamic membranes (200 μg) were incubated for 30 min at 4 C in the CGRP binding buffer containing 0.3% BSA and 500 Bq (100 pM) [^{125}I]rat adrenomedullin in silanized microcentrifuge tubes in a final assay volume of 0.5 ml. Nonspecific binding was determined in the presence of 500 nM unlabeled rat adrenomedullin. The receptor-[^{125}I]adrenomedullin complex was separated from free tracer as described above.

In equilibrium competition experiments, the concentration of unlabeled peptide was varied between 0–1 μM. Binding data were analyzed by nonlinear regression to determine the dissociation constant (K_d) or absolute inhibition constant (K_i) and the number of binding sites (B_{max}) using the Receptor-Fit program (Lundon Software, Cleveland, OH). Statistical analysis of one-site *vs.* two-site fits for competition curves were performed within the program by F test. Only $P < 0.05$ was considered significant.

Results

Dose-response study

The food intake in animals after a 24-h fast was inhibited in a dose-dependent manner after icv adrenomedullin (30 min postinjection, by ANOVA, $F_{4,23} = 5.249$ and $P < 0.01$; 120 min, $F_{4,23} = 5.141$ and $P < 0.01$; Fig. 1). Food intake was reduced by 57% with 1.7 nmol and by 66% with 5.0 nmol adrenomedullin at 120 min.

Antagonist study

Adrenomedullin (1.7 nmol) inhibited the 2-h fast-induced food intake by 60% ($P < 0.005$). This inhibition was reduced by 50% when CGRP-(8–37) was injected icv before adrenomedullin at a dose of 30 nmol [Fig. 2; saline, 6.1 ± 0.7 g; adrenomedullin, 2.5 ± 0.5 g; CGRP-(8–37), 5.0 ± 0.7 g; adrenomedullin and CGRP-(8–37), 4.3 ± 0.6 g]. When administered alone, icv CGRP-(8–37) did not have any significant effect on fast-induced food intake. No gross behavioral abnormalities were noted by the investigators, other than a reduction in feeding. This study was repeated twice with similar results (results not shown).

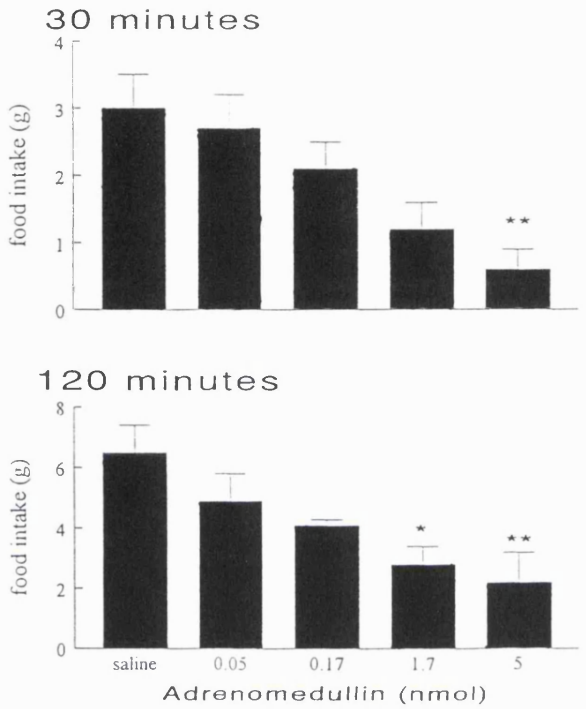

FIG. 1. Effect of icv human adrenomedullin on fast-induced food intake. Male Wistar rats were injected with 10 μl of 0.05, 0.17, 1.7, or 5.0 nmol adrenomedullin, and 30- and 120-min food intakes were measured. Saline was injected as a control. Data are the mean ± SE. *, $P < 0.05$; **, $P < 0.01$.

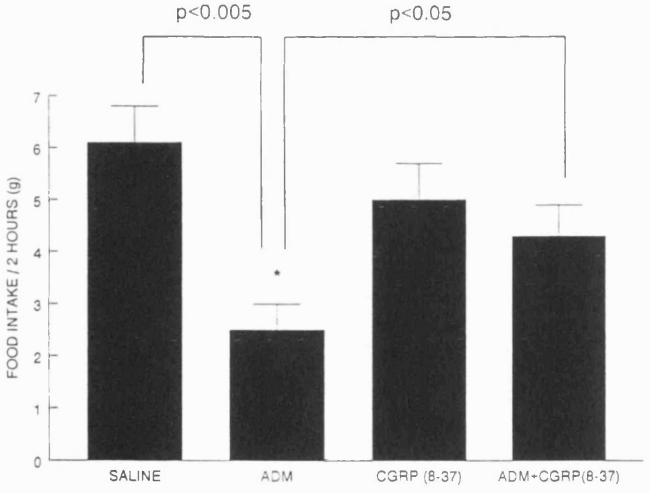

FIG. 2. Effect of hCGRP-(8–37) on icv adrenomedullin-reduced food intake. Male Wistar rats were icv injected with 30 nmol hCGRP-(8–37) in 5 μl before icv injection of 1.7 nmol/5 μl adrenomedullin. Two-hour food intake was measured. Data are the mean ± SE.

Competition of [^{125}I]CGRP binding

For CGRP binding assays, specific binding accounted for 78 ± 2% of the total binding. Unlabeled rat CGRP, hCGRP-(8–37), and human adrenomedullin competed for the [^{125}I]rat αCGRP-binding sites with the following order of potency: rat CGRP > hCGRP-(8–37) > human adrenomedullin (Fig. 3). The K_d of the binding site for rat CGRP was 0.10

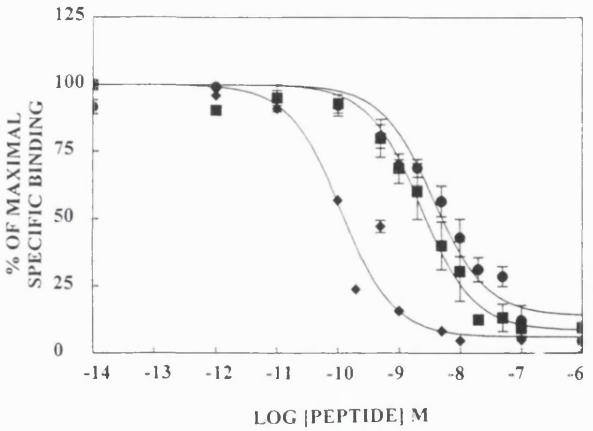

FIG. 3. Effects of rat CGRP (♦), hCGRP-(8–37) (■), and human adrenomedullin (●) on [^{125}I]rat CGRP binding in rat hypothalamic membranes. Values are expressed as a percentage of the maximal specific binding. Each *data point* is the mean of three separate assays performed in triplicate. Where no error bars are shown, they are obscured by the symbol.

± 0.02 nM, with a B_{max} of 250 ± 31 fmol/mg membrane protein (n = 3). The K_i values for human adrenomedullin and hCGRP-(8–37) were 4.6 ± 2.1 and 4.0 ± 1.6 nM, respectively.

Competition of [^{125}I]rat adrenomedullin binding

In the adrenomedullin binding assays, specific binding was 58 ± 2% of the total binding. Adrenomedullin, but not CGRP or CGRP-(8–37), competed for [^{125}I]adrenomedullin binding in hypothalamic membranes (Fig. 4). The K_d of the binding site for human adrenomedullin was 0.54 ± 0.06 nM and the B_{max} was 214 ± 27 fmol/mg membrane protein (n = 3). Nonlinear regression analysis of the competition curves as one- or two-site-fit models revealed both CGRP and adrenomedullin binding to be best explained by single sites.

Discussion

We have shown for the first time that adrenomedullin reduces feeding in a dose-dependent manner when administered centrally. This effect was significantly reversed by the $CGRP_1$ receptor antagonist, CGRP-(8–37), indicating that the adrenomedullin action is mediated at least in part through central $CGRP_1$ receptors. Specific adrenomedullin-binding sites have previously been described, but not characterized in the brain, including the hypothalamus (27), and we have now shown high affinity adrenomedullin binding sites (K_i = 0.54 nM) with a moderate to high concentration of sites (B_{max} = 214 fmol/mg protein). These sites did not bind either CGRP or CGRP-(8–37). Adrenomedullin can also bind to hypothalamic CGRP receptors with high affinity (K_i = 4.6 nM), although with 46 times less affinity than CGRP. It is this binding to CGRP receptors that appears to mediate the effects on feeding, leaving the role of the specific adrenomedullin receptors open until a specific adrenomedullin receptor antagonist is developed. A CGRP receptor subtype, $CGRP_2$, that is not inhibited by CGRP-(8–37) has been reported (30). CGRP binds equipotently to $CGRP_1$ and $CGRP_2$ receptors but the CGRP-induced reduction in feeding is inhibited by CGRP-(8–37) (30). Therefore, it is unlikely that activation of

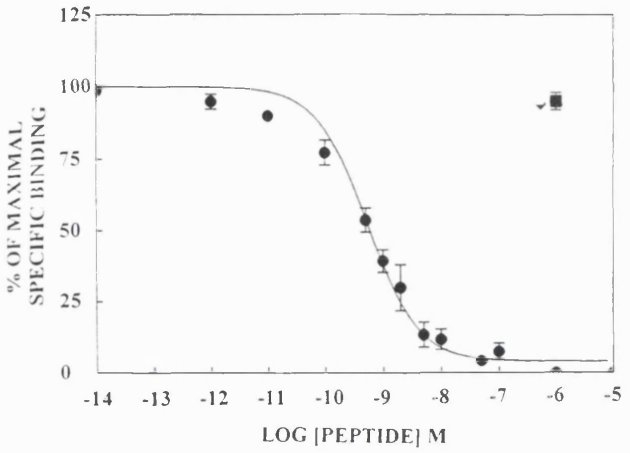

FIG. 4. Effects of rat CGRP (♦), hCGRP-(8–37) (■), and human adrenomedullin (●) on [^{125}I]rat adrenomedullin binding in rat hypothalamic membranes. Values are expressed as a percentage of the maximal specific binding. Each *data point* is the mean of three separate assays, performed in triplicate. Where no error bars are shown, they are obscured by the symbol.

$CGRP_2$ receptors is involved in effects on feeding. It is possible that adrenomedullin has a physiological role in satiety and the control of food intake. Recently, adrenomedullin-immunoreactive neurons were identified in the paraventricular and supraoptic nuclei of the rat (31). These hypothalamic nuclei are known to be important in the regulation of food intake, but further studies (*e.g.* immunoneutralization studies) are required to define a physiological role for adrenomedullin.

Systemic blood pressure was increased by high dose (1–3 nmol/kg) of icv adrenomedullin (14). This hypertensive effect was completely abolished by 3 nmol/kg CGRP-(8–37). The hypertensive effect of icv adrenomedullin is, thus, entirely mediated by $CGRP_1$ receptors. Recently, it has been reported that when adrenomedullin is icv infused into conscious sheep at 100 μg/h (~4 nmol/kg), no significant changes in cardiovascular parameters were observed (32). Murphy and Sampson (33) found that a dose of up to 0.18 nmol adrenomedullin had no effect on blood pressure when given icv. However, they did find that this dose significantly reduced water intake, although they did not investigate the possibility that this effect may be mediated via central $CGRP_1$ receptors. We found that a similar dose (0.17 nmol) reduced feeding by approximately 40%, suggesting that the effects on feeding and drinking may be mediated by similar mechanisms. The fact that a much larger dose of icv adrenomedullin is needed to cause hypertension than to decrease food or water intake in the rat may indicate that the receptors mediating hypertension are further from the ventricular system (14, 33). However, direct brain stem and hypothalamic injections of adrenomedullin are required before the potency of adrenomedullin on these systems can be properly compared.

Recently, new information has emerged concerning the specific adrenomedullin receptor in the rat brain. Kapas *et al.* (34) identified a complementary DNA encoding the rat adrenomedullin receptor and showed that this receptor has binding properties similar to those of specific adrenomedullin receptors described here and by Owji *et al.* (27). This complementary DNA was previously described by Harrison *et al.* in 1992 (35) as an orphan receptor present in a number of brain regions, including the hypothalamus. The molecular cloning of the specific adrenomedullin receptor may be useful in developing antagonists specific to this receptor and eventually unraveling its role in the hypothalamus.

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Conclusions and future work.

The control of feeding and metabolism is an extremely complex area, and this thesis has focussed at a small section of this fascinating area. Many neurotransmitters are likely to be involved, some of which may not have been discovered. Leptin, which seems to be a major player in the control of both feeding and metabolism was not described when the work in this thesis commenced in 1993. In this thesis, I have focussed on the role and effects of three peptides, NPY, GLP-1 (7-36) amide and adrenomedullin.

NPY is a potent stimulus to feeding. It also stimulates the release of ACTH release. These two effects are mediated by distinct receptors. As stated in chapter 3, NPY has many other central actions, including effects on several of the pituitary hormones and effects on blood pressure. I have shown that the NPY receptor that mediates feeding is pharmacologically unlike any of the currently identified NPY receptors. In addition, the NPY receptor that mediates the release of ACTH is unlike the Y1, Y2, Y3, Y4 and Y6 receptors. The fact that [DTrp^{32}]NPY was so potent at stimulating the release of NPY in vivo (figure 3.8) suggests that the NPY receptor that mediates ACTH release is also distinct form the $Y5_{NAT}$ receptor. As newly discovered analogues and non-peptide agonists or antagonists of the various NPY receptors are discovered, further binding curves and in vivo studies looking at the effect of each of these peptides on NPY receptor activation need to be carried out. The eventual aim will be to develop specific agonists and antagonists to each of the receptors. Specific agonists will be required to confirm which of any future cloned receptors mediate NPY induced feeding and NPY induced ACTH release.

I have also postulated that the latency to feeding produced by NPY occurs because CRF, which is released acutely after ICV injection of NPY, acts as a short acting satiety factor (page 35).

I have also shown that GLP-1 is a potent satiety factor, and also that it has effects on peripheral insulin sensitivity when injected into the third cerebral ventricle. Much of this work was made possible because of the existence of the GLP-1 receptor antagonist, exendin (9-39). It is now known that targeted disruption of the GLP-1 receptor (a GLP-1 receptor "knockout mouse") significantly affects glucose metabolism (Scrocchi et al. 1996a) but has no effect on food intake or weight gain. It has been suggested that there may be a novel hypothalamic GLP-1 receptor that is highly related yet distinct from the pancreatic GLP-1 receptor (Scrocchi et al. 1996b).

Others have shown that muscle cells and pancreas express distinct GLP-1 receptors (Yang et al. 1996). Fragments of exendin 4 other than exendin (9-39) have also been shown to be antagonists to GLP-1 receptors stably transfected into CHO cells (Montrose-Rafizadeh et al. 1996). In particular the fragment exendin (5-39) was shown to be 10 times more potent than exendin (9-39) at inhibiting a GLP-1 induced rise in intracellular cAMP (Montrose-Rafizadeh et al. 1996). Unpublished studies carried out since the original draft of this thesis with ICV exendin (5-39) suggest that this peptide does not block the effects of ICV GLP-1 on feeding whereas exendin (9-39) remains completely active. Other fragments and analogues of GLP-1 and the exendins may be useful in determining whether there are indeed further GLP-1 receptors. Indeed Eng (Eng, 1996) has generated chimaeric peptides of GLP-1 and exendin in order to

determine which amino acids are important in agonist and antagonist activity. Such peptides should be studied for their effects on feeding and for their action on pancreatic insulin secretion in order to find selective agonists and antagonists for each receptor, in much the same way that fragments of NPY have been used to distinguish the NPY feeding receptor from the one that mediates ACTH release (chapter 3).

Both NPY and GLP-1 have potent effects on peripheral insulin resistance and glucose metabolism following central injection. These results need to be investigated further. The mechanism of the change in peripheral insulin sensitivity must be investigated using euglycaemic hyperinsulinaemic clamps with central injection of NPY and GLP-1.

Finally I have shown that central injection of adrenomedullin also inhibits feeding, and that this effect occurs at least in part through CGRP-1 receptors. Some authors have suggested that adrenomedullin is a circulating hormone involved in the control of blood pressure. In the final chapter, I have shown that the levels of adrenomedullin in the plasma are too low for the peptide to be a circulating hormone, so that it is likely to be a paracrine agent involved in the control of several processes. It may therefore be locally involved in the hypothalamus in the control of feeding. The next step will be to perform several discrete central injections of adrenomedullin in order to determine its site of action. In order to determine the effects of endogenous adrenomedullin, I would like to develop and use specific adrenomedullin receptor antagonists. There are now several compounds that are known to bind to adrenomedullin receptors, but all of these also bind to other receptors, including IAPP and calcitonin receptors. Adrenomedullin is known to be synthesised in vascular smooth muscle cells and endothelial cells (Sugo et

al. 1994a; Sugo et al. 1994a) and receptors have ben demonstrated in vascular smooth muscle cells(Eguchi et al. 1994). This peptide may therefore be important in the paracrine control of blood pressure as well as in feeding and metabolism.

In summary, neuropeptides have important effects on feeding and metabolism. Neuropeptide Y seems to be a potent hunger factor, GLP-1 a potent satiety factor and adrenomedullin a paracrine agent that may have important effects on feeding.