The Development Of Pancreatic Polypeptide Analogues As A Pharmacotherapy For Obesity.

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Abstract
Pancreatic polypeptide (PP) is a peptide hormone released post prandially from the endocrine F cells of the pancreas. Acute administration of PP to mice reduces both body weight and food intake, and an IV infusion of PP to healthy weight humans reduces food intake over 24 hours. However, the short half life of PP limits its utility as an obesity treatment. This thesis investigates the development of pancreatic polypeptide (PP) analogues, and their possible role as a novel obesity treatment. I hypothesised that specific amino acid substitutions in the PP molecule would increase, enzymatic resistance and affinity for the Y4 receptor, and would thus result in analogues of PP with improved biological function.

I investigated the substitution of amino acids on the breakdown of PP analogues using reverse phase HPLC and MALDI-TOF MS. Receptor binding assays and food intake studies in fasted male C57/BL6 mice were used to investigate the effect of amino acid substitutions on receptor binding affinity and on the anorectic effects in vivo. Finally, long term chronic food intake studies using different models of obesity were used to investigate the long term effects of PP analogue XPP.

The PP analogue XPP was shown to be more enzymatically resistant than PP, with improved binding to the hY4 receptor and greater acute inhibition of food intake when investigated in vivo. When administered chronically in two models of obesity, XPP was shown to reduce body weight in specific models at specific time points. These experiments demonstrated the potential of XPP as an obesity treatment. The development of PP analogues may thus provide a useful and novel treatment for obesity.
Declaration of contributors

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

Chapter 2

The development of breakdown assays were done under the guidance of Mrs Joy Cuenco-Shilto. Matrix-assisted laser absorption/ionization-time of flight analysis was performed by Bachem. All analysis was performed by the author.

Chapter 3

The hY4 and hY2 receptor over-expressing cells were kindly donated by Dr. James Minnion. Rat pharmacokinetic studies were performed in collaboration with Dr. S Ghourab, with both people contributing equally.

Chapter 4

The chronic food intake studies were carried out in collaboration with Dr. James Minnion.

In house radioimmunoassays used in this program of research were performed under the guidance and supervision of Professor Mohammad Ghatei.
Acknowledgements

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I would also like to thank all the lab members in fellows room two for their help and general good nature over the past three years.

Finally I would like to thank my family for their encouragement and support, without which I would never have got this far.
**Abbreviations**

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>AcN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotrophic hormone</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti related protein</td>
</tr>
<tr>
<td>AP</td>
<td>Area postrema</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate nucleus</td>
</tr>
<tr>
<td>BH</td>
<td>Bolton and Hunter</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CART</td>
<td>Cocaine- and amphetamine-regulated transcript</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CLAMS</td>
<td>The Comprehensive Laboratory Animal Monitoring System</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DIO</td>
<td>Diet Induced Obesity</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMH</td>
<td>Dorsomedial hypothalamus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>Dipeptidyl peptidase-IV</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>Fmoc</td>
<td>Fluorenylmethoxycarbonyl</td>
</tr>
<tr>
<td>GEE</td>
<td>Generalised estimating equation</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
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</table>
GIP  Glucose-dependent insulinotropic peptide
GLP-1  Glucagon-like peptide-1
GLP-2  Glucagon-like peptide-2
HPLC  High performance liquid chromatography
ICV  Intracerebroventricular
IP  Intraperitoneal
IV  Intravenous
KO  Knock-out
LHA  Lateral hypothalamic area
MALDI-ToF  Matrix-assisted laser desorption/ionization-time of flight
MCH  Melanin-concentrating hormone
MCR  Melanocortin receptor
MCR2  Melanocortin-2 receptor
MCR3  Melanocortin-3 receptor
MCR4  Melanocortin-4 receptor
MPO  Medial preoptic nucleus
MS  Mass Spectroscopy
NHS  National health service
NEP  Neprilysin
NPY  Neuropeptide Y
NTS  Nucleus of the solitary tract
Ob/Ob  Ob gene knock-out mouse
OXM  Oxyntomodulin
<table>
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<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonylfluoride</td>
</tr>
<tr>
<td>P</td>
<td>Phosphoramidon</td>
</tr>
<tr>
<td>POMC</td>
<td>Proopiomelanocortin</td>
</tr>
<tr>
<td>PP</td>
<td>Pancreatic polypeptide</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
</tr>
<tr>
<td>PYY</td>
<td>Peptide YY</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error Mean</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>VMH</td>
<td>Ventromedial hypothalamus</td>
</tr>
<tr>
<td>VP</td>
<td>Valine pyrolidide</td>
</tr>
<tr>
<td>Y1-Y5</td>
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Body weight remains constant in most human adults despite small daily deviations from an ideal/set point (Jequier and Tappy, 1999). A period of fasting may result in a short term increase in food intake (hyperphagia); this increase in food intake should result in the recovery of lost body weight and ultimately enable body weight to return to baseline levels. This mechanism enables body weight to remain relatively constant over a period of months or years despite occasional mismatches in energy intake and expenditure (Jequier and Tappy, 1999). When energy intake is greater than energy expended, the excess is stored in the form of fat.

1.2 Obesity

Obesity is a medical condition in which excess body fat has accumulated to the extent that it may have an adverse effect on health, leading to reduced life expectancy and/or increased health problems (Walter et al., 2009). An adult with a body mass index (BMI) of greater than 30kg/m² is classified as obese. This is calculated by dividing body weight measured in kilogram (kg) by the square of the person’s height in metres (m). BMI is subdivided into underweight (≤18.5 kg/m²), healthy (18.5-25 kg/m²), overweight (25-29.9 kg/m²), obese (30-34.9 kg/m²), severely obese (35-40.5 kg/m²), morbidly obese (40-44.9 kg/m²) and super obese (≥45kg/m²) (World Health Organisation, 2006).

Currently, the mean BMI for an adult in the UK is 27kg/m², which is above the healthy range (Butland B, 2007). In 2006, 40% of men in the UK in the 25-34 years age group were overweight, and a further 22% classed as obese. In the same age group, 29% of women were overweight and 17% obese (Ali A, 2008). NHS predictions suggest that the number of people who are either
overweight or obese is likely to continue to rise, including 60% of men and 50% of women by 2050 (World Health Organisation, 2006; Butland B, 2007). Obesity has both a social and an economic cost; the Commons Select Health Committee estimates that treatment of obesity alone cost £991 million per year up to 2001 and in 2002 it was £1,124 million; 2.3 and 2.6 percent of the total NHS expenditure for the respective year (Butland B, 2007).

Long term obesity is associated with health problems and premature mortality (Flegal et al., 2005). The co-morbidities associated with obesity include hypertension, Type II diabetes mellitus (T2DM), cardiovascular diseases, osteoarthritis, cancer of the colon, prostate, kidney and gall bladder, stroke, and breathing problems such as sleep apnoea (Butland B, 2007; Fontaine et al., 2003; Krauss et al., 1998; Kopelman, 2000).

The cause of obesity is currently thought to be a combination of genetic and environmental factors. The theory of a genetic predisposition to obesity was first described in 1962 by JV Neel. Neel’s thrifty gene hypothesis suggests that during times of plenty it is advantageous to accumulate fat, as this will aid survival in times of famine. Those people that survive the times of famine were then able to pass on these thrifty genes to the next generation (Neel, 1962). An alternative hypothesis proposes that there has been a genetic drift towards obesity (Ali A, 2008; Stender et al., 2007; Speakman, 2008). Genetic drift is described by Speakman as a process by which a gene which is not under selection but uses a lower and an upper intervention limit to regulate a system loses either the upper or lower intervention limit, and therefore a system drift occurs. There is debate over the importance of genetics in obesity and related conditions. Twin adoption studies demonstrate, genetic factors powerfully influence the development and extent of weight gain (Bouchard et al., 1990). Specific mutations that result in obesity have been identified, including mutations in leptin or the leptin receptor, and mutations in melanocortin 4 receptors (Santoro et al., 2009). However, the increase in the prevalence of obesity is too rapid to be explained purely by genetic change. It is likely that genes influence body weight in relation to the environmental context.
Outside of these putative genetic drives, environmental factors including the decline of manual labour and the rise in abundance of appetising highly calorific foods have certainly contributed to the rise in obesity levels. Understanding the systems regulating energy homeostasis will aid the development of newer, more effective treatments for obesity and obesity related conditions.

Increased energy intake has played an important role in the increase in obesity levels in recent years. An individual’s body weight is usually tightly controlled by a number of systems including the neuroendocrine system and signals from adipose tissue (Berthoud, 2002; Bray, 1980; Farooqi and O’Rahilly, 2009). Day to day there may be small differences between calories ingested and energy expended. Over the space of a year or more, energy expenditure and calories ingested usually remain constant and in balance (Jequier and Tappy, 1999). It is hypothesised that in recent years the change in western diets has unbalanced the body’s regulatory mechanisms (McCrady and Levine, 2009).

A western diet as a whole is very energy dense, with a large proportion of daily calories consumed consisting of carbohydrates and fats (Cordain et al., 2005). Hunter gather societies are thought to have a traditional, low fat, high carbohydrate diet, but are subject to high levels of obesity when their traditional diet is replaced by a more western style of food. A well documented example is the Pima Indians, who have higher levels of obesity and diabetes when exposed to a western diet and lifestyle, but low levels of obesity and diabetes when following a more traditional lifestyle and diet (Ravussin et al., 1994; Schulz et al., 2006; Weyer et al., 2001). In the western world, despite more information on food labelling regarding fat content and health implications, obesity levels have continued to rise. In recent years a greater proportion of food consumed is made outside the home, with increased consumption of convenience and fast food. A typically high fat menu from a fast food restaurant contains 1100KJ/100g (energy in kilojoules per 100 grams consumed), which is considerably higher than average UK diet of 670KJ/100g (Stender et al., 2007). This increase in the number of meals being consumed outside the home, and their high calorie
and fat content is thought to contribute to rising obesity levels (Rosenheck, 2008).

Reduced activity or lack of exercise is also associated with weight gain and obesity. From the mid 1900’s the proportion of the population undertaking a job requiring manual labour declined and the number of sedentary, desk based jobs increased (McCray and Levine, 2009; Gregory et al., 2007). This shift has been suggested to promote weight gain, as energy intake is no longer being balanced by energy output (McCray and Levine, 2009). A recent NHS health survey suggested that men spend 8.0 hours per week taking part in some kind of physical activity and women 5.4 hours per week (Ali A, 2008). These figures are lower than a decade previously where a greater proportion of the population undertook a job which involved physical activity (McCray and Levine, 2009; Gregory et al., 2007).

1.3 Current Strategies For The Treatment of Obesity

 Obesity has been shown to decrease life expectancy by approximately seven years in a number of studies (Allison et al., 1999; Fontaine et al., 2003; Fontaine et al., 2003; Jia and Lubetkin, 2009; Peto et al., 2010; Stewart et al., 2009; Walter et al., 2009). As discussed previously in section 1.2, the social and economic cost of obesity is great. It is therefore advantageous to encourage weight loss in overweight and obese patients. The first strategy is to modify diet and lifestyle. Reducing calories ingested and increasing calories expended promotes weight loss. It has been shown, however, that there is limited compliance with very low calorie or low fat diets (Bray, 2008; Pirozzo et al., 2003), and there is often weight regain approximately 18 months after completion of the diet (Pirozzo et al., 2003). If diet and lifestyle modifications are ineffective, the next step is usually some form of medical treatment to aid weight loss. Medical treatments for the treatment of obesity are discussed in section 1.3.1. The last resort for long term weight loss is a surgical route, usually either gastric banding or gastric bypass. National Institute for Health and Clinical Excellence (Nice) guidelines suggest that obesity surgery should only be carried out if all appropriate non-surgical measures have failed to be effective over 6 months and if the patient has a
BMI \( >40\text{kg/m}^2 \), or \( >35\text{kg/m}^2 \) in conjunction with serious co morbidities such as T2DM (National Institute for Health and Clinical Excellence (NICE), 2006).

### 1.3.1 Medical treatment

At present there is only one drug treatment approved to treat obesity in the UK: Orlistat Xenical (Roche pharmaceuticals, Switzerland) is the prescription only form of Orlistat (120mg three times a day). Xenical is made by Roche pharmaceuticals, Switzerland, and can be prescribed at a dose of 120mg three times a day for patients with a BMI \( \geq 30\text{kg/m}^2 \), or a BMI \( \geq 27\text{kg/m}^2 \) in conjunction with other risk factors. Orlistat is also available as the over the counter treatment Alli (GSK, USA), as a 60mg dose to be taken three times a day. Alli is only sold to patients with a BMI of over 28kg/m².

Orlistat was derived from a bacterial lipase inhibitor. Inhibition of pancreatic lipase impairs triglyceride breakdown (Davidson et al., 1999). Orlistat treatment in conjunction with a calorie controlled diet results in a weight loss of 2.8-3.2kg greater than placebo controls over the period of a year (Davidson et al., 1999; Sjostrom et al., 1998; Hill et al., 1999; Leung et al., 2003). However, a study has shown that those patients who received Orlistat for a year and then received placebo control while maintaining the calorie controlled diet for another year, regained the weight lost in the initial period (Sjostrom et al., 1998). While those patients maintained on orlistat for the second year gained less weight than those receiving placebo, they failed to lose any further weight (Sjostrom et al., 1998). Inhibiting fat digestion and absorption results in excess faecal fat loss. Other side effects include oily spotting, faecal urgency and increased defecation (Guerciolini, 1997; Leung et al., 2003). There is also an associated decrease in absorption of fat soluble vitamins, including vitamins E and \( \beta \)-carotene (Davidson et al., 1999), though levels rarely fall outside of the normal limits (Bray and Tartaglia, 2000). Orlistat has limited efficacy, and therefore limited weight loss occurs (Padwal and Majumdar, 2007; Avenell et al., 2004; Neovius and Narbro, 2008). As mentioned above, once treatment with Orlistat is ceased, weight is often regained, (Svendsen et al., 2008; Sjostrom et al., 1998).
The anti-obesity drug Sibutramine was until recently sold under the trade name Reductil (Abbott laboratories). Sibutramine is a member of the monoamine reuptake inhibitor class of drugs, which inhibits food intake predominately by inhibiting the reuptake of norepinephrine and serotonin, but also by inhibiting the reuptake of dopamine, into nerve terminals. In year-long randomized trials comparing Sibutramine with placebo, subjects in the Sibutramine group lost 9.3% of their initial baseline body weight (30mg dose) whereas those in the placebo groups lost only 1.9% (Bray et al., 1999). In January of 2010, the European Medicines agency recommended its suspension from sale due to concerns over the increased incidence of cardiovascular events in patients taking Sibutramine (Williams, 2010).

Orlistat is the only treatment currently available for the treatment of obesity. However, the amount of weight lost and the tendency of patients to regain weight after treatment is ceased means that it is not a very cost effective option. At present a number of treatments are being developed to treat obesity. Most of these proposed treatments are centrally acting drugs which interact with appetite regulation and hedonic central nervous system pathways. Examples include Lorcaserin (Arena Pharmaceuticals), a 5-HT\(_{2c}\) receptor agonist, and Qnexa (Vivus), a combination of the amphetamine-like drug phentermine and the epilepsy drug topiramate. A phase 3 trial of the drug Qnexa showed a 14kg weight reduction at 1 year (Vivus Pharmaceuticals, 2009). Drugs which act via central pathways often act on systems that do not exclusively regulate appetite, which increases the potential for side effects and adverse reactions. This was demonstrated when rimonabant, a cannabinoid receptor antagonist used as an obesity treatment, was removed from the market due to problems with depression and anxiety (Doggrell, 2008). Currently there is discussion about the use of T2DM treatments such as Exenatide, which also results in weight loss, as possible treatments for obese patients without T2DM (Nayak et al., 2010). Exendatide is an analogue of the gut hormone glucagon-like peptide 1 (GLP-1), and other gut hormones, including oxyntomodulin, peptide YY, and pancreatic polypeptide, have also been proposed as possible treatments for obesity (Field et al., 2008; Neary and Batterham, 2009; Field et al., 2009).
Modifying the physiological satiety system to mimic the post prandial state may help reduce food intake and therefore cause weight loss.

1.3.2 Surgical treatment
The most successful long term treatment for obesity is bariatric surgery (Martins et al., 2010). There are a number of different surgical options depending on the level of obesity, co-morbidities, patient preference and the weight loss required. Laparoscopic adjustable gastric banding (LAGB) is often used for moderate/ less severe cases of obesity, whereas the Roux–en-Y gastric bypass (RYGB) is used for cases in which greater weight loss is required. LAGB is achieved by placing an adjustable band circling the proximal stomach; the gastric restriction is then controlled by inflating the band via a subcutaneous port. The RYGB procedure is more complex and has a greater risk of complications. It is also difficult to reverse, whereas the LAGB can be readily adjusted or removed. The RYGB involves dividing the stomach and creating a small gastric pouch which is attached to the distal limb of the jejunum. This bypasses most of the stomach, the duodenum and some of the jejunum. Both the RYGB and the LAGB result in significant weight loss up to 5 years post surgery.

A meta-analysis comparing LAGB and RYGB showed that at 1,2,3,4 and 5 years post surgery RYGB patients achieved the greatest loss of excess weight at all time points (Christou and Efthimiou, 2009). RYGB results in the loss of up to 70% excess weight at 1 year, whereas LAGB can result in the loss of up to 49% excess weight (Christou and Efthimiou, 2009; Garb et al., 2009). At the 1 year time point, drug treatments on average achieve a 2-3 kg (approximately 8%) weight loss compared to placebo controls (Hill et al., 1999; Rubio et al., 2007). In studies comparing weight loss via drug treatment or surgical treatment, at the sixth month check up point, both groups had lost approximately 13% of their total excess weight. The drug treatment group subsequently maintained their weight loss, whereas those patients in the surgical group continued to lose weight. At the two year check up, patients who had undergone surgery had lost 20kg, compared with
6kg in the drug treatment group (Palazuelos-Genis et al., 2008; Schaeffer et al., 2008).

The RYGB was originally designed to reduce body weight by the malabsorption of nutrients. However, this appears not to be the cause of weight loss in RYGB patients, as following surgery the gut adapts and malabsorption abates (Brolin et al., 2002). The cause of weight loss after bypass surgery remains unclear. Bariatric surgery increases circulating levels of the anorexigenic gut hormones GLP-1, insulin and peptide YY, and decreases levels of the orexigenic gastric hormone ghrelin (Morinigo et al., 2004; Korner et al., 2005). Changes in taste have also been reported following RYGB, which may also contribute to weight loss (Tichansky et al., 2006). Recently a rat model of RYGB has suggested that there may also be an increase in energy expenditure following RYGB surgery, though it is unknown whether RYGB alters energy expenditure in humans (Bueter et al., 2009).

Bariatric surgery is currently the most effective treatment for obesity. However, surgical intervention is not without risk. Bariatric surgery has until recently been performed as an open surgery, which carried with it high risks of infection. With laparoscopic techniques now more widespread, the reduced risks still include anaesthesia complications and post surgical complications such as malnutrition/malabsorption (Gong et al., 2008). Surgery related death can occur in up to 2% of bariatric surgery operations (Adams et al., 2007). In addition, given the size of the obesity problem, it is not economically possible to use bariatric surgery to treat all cases (Avenell et al., 2004; Bessesen, 2008).
1.4 The Hypothalamus

The hypothalamus is a central nervous system (CNS) region important in the regulation of energy homeostasis. It is situated above the pituitary gland and on either side of the third ventricle. The hypothalamus is traditionally sectioned into three zones longitudinally: the periventricular, medial and lateral zones, and into four sections along the rostro-caudal axis: preoptic, anterior, tuberal and mammillary (Berthoud, 2002; Simerly, 1995). Over forty separate nuclei make up the hypothalamus, and these can be further subdivided into subnuclei (Figure 1.1) (Berthoud, 2002). Hypothalamic nuclei exhibit a wide variety of functions, including controlling pituitary hormone secretion, reproduction and body temperature. Nuclei with a role in energy homeostasis will be discussed in sections 1.4.1 to 1.4.4.

The hypothalamus has a large number of afferent and efferent projections which allow it to co-ordinate a wide range of physiological activities, including energy homeostasis. Experiments performed in the 1950’s were the first significant evidence that the hypothalamus regulates energy balance (Anand and Brobeck JR, 1951). Lesioning studies demonstrated that destruction of the ventromedial hypothalamus (VMN) causes hyperphagia in mice, rats, dogs, monkeys and humans (Bernardis et al., 1975; Lindholm et al., 1975; Anand and Brobeck JR, 1951). Conversely, destruction of the lateral hypothalamus (LH) reduces body weight and food intake (Bernardis and Bellinger, 1993; Bernardis et al., 1975; Anand and Brobeck JR, 1951). Electrical stimulation of the LH causes an increase in body weight and body fat in rats (Stellar and Heard, 1976). Early lesioning experiments resulted in the development of the ‘dual centre hypothesis’, which proposed that the VMN acts as a satiety centre and the LH as a feeding centre. The dual centre hypothesis has subsequently been considered over simplified. Recent models of feeding behaviour incorporate the two centre theory, but focus on neural networks spread across multiple hypothalamic nuclei, which send and receive signals of energy balance, often via specific neuropeptides (Elias et al., 1998).
Figure 1.1 A three-dimensional view of the major rat hypothalamic nuclei. (AHA), anterior hypothalamic area; (ARC), arcuate nucleus; (AV3V), anteroventral area of third ventricle; (Cl), capsula interna; (DP), dorsal parvocellular subnucleus of paraventricular nucleus (PVN); (DMN), dorsomedial nucleus; (F), fornix; (LHA), lateral hypothalamic area; (LM), lateral magnocellular subnucleus of paraventricular nucleus; (LPOA), lateral preoptic area; (ME), median eminence; (MP), medial parvocellular (PVN); (MPO), medial preoptic area; (OT), optic tract; (SCh), suprachiasmatic nucleus; (SON), supraoptic nucleus; (SI), substantia inomminata; (ST), subthalamic nucleus; (VMN), ventromedial nucleus; (VP), ventral parvocellular subnucleus of paraventricular nucleus. Adapted from (Berthoud, 2002).
1.4.1 The Arcuate Nucleus (ARC)

The ARC is located on either side of the third ventricle at the base of the hypothalamus. The median eminence, a circumventricular organ with an incomplete blood brain barrier (Broadwell and Brightman, 1976), is adjacent to the ARC and is hypothesised to facilitate circulating factors reaching receptors located on ARC neurons. The position of the ARC next to the third ventricle also enables nutrients and hormones to diffuse from the cerebrospinal fluid into the ARC. In addition, the ARC receives neuronal inputs from other hypothalamic nuclei and the brainstem regarding feeding and satiety (Ricardo and Koh, 1978). The ARC is therefore in a position to integrate signals of nutritional status from the periphery and from other CNS regions to co-ordinate energy homeostasis (Figure 1.2).

Within the ARC, two distinct populations of neurones regulate energy balance (Cone et al., 2001). One population co-expresses neuropeptide Y (NPY) and agouti related peptide (AgRP), and the other co-expresses pro-opiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART). NPY/AgRP neurones are considered to be orexigenic, and project to regions including the paraventricular nucleus (PVN), the lateral hypothalamic area (LHA) and the dorsomedial hypothalamus (DMH), as well as to other neurons within the ARC (Kalra et al., 1999). POMC/CART neurones are anorexigenic and project to similar regions, and also to the perifornical area. These two groups of neurons act antagonistically: activation of NPY/AgRP neurons inhibits POMC/CART neurons, and conversely, POMC/AgRP neurons inhibit NPY/AgRP neurons (Wynne et al., 2005b; Roseberry et al., 2004; Hentges et al., 2004; Cowley et al., 2001). ARC neurones also project to extra hypothalamic brain regions, including the forebrain, brainstem and the ventral raphe nuclei, where they are thought to act to mediate energy homeostasis (Broberger and Hokfelt, 2001).
Figure 1.2 A schematic diagram of the neuronal populations within the ARC of the hypothalamus. One subpopulation of neurons releases NPY and AgRP, whilst the other subpopulation of neurons releases CART peptide and alpha melanocyte stimulating hormone (α-MSH), a product of POMC cleavage. Neurons from the ARC project to other brain regions and hypothalamic nuclei to co-ordinate energy homeostasis. Adapted from (Wynne et al., 2005b).
1.4.2 The Paraventricular Nucleus

The PVN is an essential area of the hypothalamus in the regulation energy homeostasis (Elmquist et al., 1997; Turton et al., 1996), and plays a key role in the integration of signals from the ARC, brainstem and cortex (Sawchenko and Swanson, 1983). A number of neuropeptides associated with the regulation of food intake and energy expenditure are expressed in the PVN. Destruction of the PVN in rodents leads to hyperphagia and weight gain (Aravich and Sclafani, 1983; Leibowitz et al., 1981). Unlike the ARC, the PVN is protected from the general circulation by the intact blood brain barrier. Injection of orexigenic factors, including NPY, AgRP and noradrenalin, into the PVN stimulates food intake (Leibowitz, 1978; Edwards et al., 1999; Kim et al., 2000), whilst injection of anorexigenic factors, including leptin and GLP-1, reduces food intake (McMahon and Wellman, 1998; Shih et al., 2003). Neurons from the PVN project to the median eminence, where they release hypothalamic regulating factors including corticotrophin-releasing hormone (CRH), vasopressin and thyroid-releasing hormone (TRH) into the hypothalamic-pituitary portal system to regulate the secretion of pituitary hormones into the systemic circulation.

1.4.3 The Dorsomedial Nucleus (DMN)

The DMN is situated close to the ARC. The DMN receives projections from NPY neurons from the ARC and neurons from the DMN project to other areas of the hypothalamus involved in the regulation of food intake, including the PVN (Dalton et al., 1981). The destruction of the DMN results in hyperphagia and obesity, and injection of NPY or GABA into the DMN increases food intake (Kelly et al., 1979).

1.4.4 The Ventromedial Hypothalamus (VMN)

The VMN is part of the mid region of the hypothalamus adjacent to the ARC and also thought to play a role in energy homeostasis. Ablation of the VMN results in hyperphagia and obesity in rodents (Bernardis and Border, 1975). The VMN receives projections from ARC NPY/AgRP neurons, and VMN neurons project to the hypothalamic DMN and the brainstem, which are involved in the regulation of food intake (Fu and van den Pol, 2008; Kalra et
al., 1999). The VMN has not been found to produce peptides with well characterised roles in the regulation of food intake, but it does contain receptors for both NPY and leptin.

1.4.5 The Brainstem and Energy Homeostasis
The brainstem is located in the posterior of the brain and is structurally continuous with the spinal cord. The brainstem comprises the medulla oblongata, pons, midbrain and the diencephalon. The brainstem relays information from the cerebellum to the periphery via the cranial/spinal nerves and vice versa. The brainstem regulates a number of essential functions, including breathing, consciousness, temperature regulation and acute nutritional status via the cranial nerves (Broberger and Hokfelt, 2001). Sensory afferent fibres within the vagus nerve activate neurons within the nucleus of the solitary tract (NTS), which is part of the dorsal vagal complex (DVC). These signals are then relayed to various parts of the brain, described in greater detail in section 1.4.5.1.

1.4.5.1 Brainstem and Vagus Nerve
The NTS is located in the caudal brainstem. The NTS receives and integrates inputs related to food intake from a number of peripheral systems via the vagus nerve including leptin and circulating gut hormones such as cholecystokinin-8 (CCK-8) (Yuan and Barber, 1993; Schick et al., 1990). The NTS lies in close proximity to the area postrema, which like the ARC has an incomplete blood brain barrier. Circulating hormones can therefore access receptors in the AP and NTS (Maolood and Meister, 2009). Lesions to the NTS and brainstem can alter feeding behaviour and reduce body weight (Hyde and Miselis, 1983; Miselis et al., 1984). The NTS may therefore be able to detect changes in circulating factors related to food intake. It has been proposed that the brainstem is involved in meal initiation and termination (Grill and Kaplan, 2002).

Gut hormones including peptide YY (PYY$_{3-36}$), PP, CCK, ghrelin and leptin can access the brain. A number of these gut hormones, including PP and PYY$_{3-36}$, are thought to be unable to cross the blood brain barrier, but may act via the vagus nerve or directly on regions of the brainstem, thus bypassing
the blood brain barrier. The vagus nerve, also known as the tenth cranial nerve, extends from the brainstem alongside the internal jugular vein and into the chest and abdomen. Approximately 80-90% of vagus nerve fibres are afferent, conveying sensory information from the periphery to the brain. The vagus nerve plays an important role in the gut brain axis, and vagotomy studies suggest that an intact vagus nerve is essential for the actions of ghrelin, PP and PYY (Le Roux et al., 2005; Gaddipati et al., 2006; Koda et al., 2005; Laskiewicz et al., 2003; Taylor et al., 1978). It has also been suggested that the brainstem can mediate the anorectic effects of gut hormones independent of the hypothalamus, as CCK reduces food intake in decerebrated rats, which lack neuronal communication between the brainstem and hypothalamus (Grill and Smith, 1988; Wynne et al., 2004).

1.4.5.2 Dorsal Vagal Complex

Together, the area postrema (AP), the NTS and the dorsal motor nucleus comprise the DVC. The AP is located posterior to the fourth ventricle. It is thought to play a role in autonomic regulation of the CNS. The AP has an incomplete blood brain barrier which has resulted in the DVC being characterised as a circumventricular organ (McKinley et al., 1990). Due to its lack of blood brain barrier, the AP can detect both peripheral and central chemical mediators, as well as receiving autonomic innervation from the vagus nerve (Contreras et al., 1984; Wang et al., 1998; Schick et al., 1990; Wang et al., 2008). The AP has been shown to play a role in feeding and energy balance. Ablation of the AP attenuates PYY$_{3-36}$ induced inhibition of food intake in rats, and inhibits basal secretion of PP (Deng et al., 2001). Receptors for a number of peripheral satiety factors have been identified in the AP, including the PP Y4 receptor and the GLP-1 receptor (Goke et al., 1995; Trinh et al., 1996; Parker and Herzog, 1999).
1.5 Hypothalamic Neuropeptides

1.5.1 Neuropeptide Y

Neuropeptide Y (NPY) is a 36 amino acid peptide member of the ‘PP’ fold family of peptides, which also contains PYY and PP. It was first isolated from porcine hypothalamic extracts in 1982 (Tatemoto et al., 1982). All members of the ‘PP-fold’ family of peptides bind to Y receptors (Table 1.1). Each of these receptors (numbered 1 to 5) have different substrate affinities and brain expression patterns (Parker and Herzog, 1999). NPY has the highest affinity for the Y1 and Y5 receptors, and it is thought that these receptors mediate the orexigenic effects of NPY (Kanatani et al., 2000).

NPY is the most potent known orexin. Injection of NPY into the third ventricle powerfully increases food intake in rodents (Stanley et al., 1986), and injection of NPY into specific hypothalamic nuclei, including the PVN, also stimulates food intake (Stanley and Leibowitz, 1985). Repeated daily injections of NPY into the PVN results in hyperphagia and weight gain in rodents (Stanley et al, 1986). Despite these orexigenic effects, mice deficient in NPY have a normal body weight and adiposity (Thorsell and Heilig, 2002). However, down-regulation of NPY mRNA in the ARC of adult rats significantly reduces food intake and body weight, suggesting that the lack of phenotype for the NPY knockout mouse may be due to developmental compensation (Marsh et al., 1999; Gardiner et al., 2005). Ablation of NPY/AgRP neurons in adult mice has also been shown to cause anorexia (Luquet et al., 2005; Gropp et al., 2005; Bewick et al., 2005).

The ARC contains the best characterised population of NPY-expressing neurons. These ARC NPY neurons also express AgRP (Hahn et al., 1998). The level of NPY expression in the ARC neurons is dependent on energy status: fasting increases NPY expression (Brady et al., 1990). ARC NPY neurons extend to other areas of the brain associated with energy status, including the PVN and DMN.
1.5.2 Melanocortin System

The melanocortin system is comprised of the peptide products of POMC cleavage, and the endogenous melanocortin receptor antagonists, agouti and AgRP. POMC is a 241 amino acid precursor polypeptide which is cleaved by the prohormone convertase group of enzymes into molecules including adrenocorticotrophin (ACTH) and alpha-MSH. The POMC gene is expressed in the anterior pituitary, the skin, the immune system and the hypothalamus (Castro and Morrison, 1997). In the anterior pituitary, POMC is cleaved into ACTH. In the hypothalamus and the intermediate lobe of the pituitary, ACTH is further processed to generate alpha-MSH. ARC POMC expression is linked to energy status, with expression levels being reduced during periods of fasting (Swart et al., 2002). In humans, POMC mutations can cause early onset obesity, red hair and adrenal insufficiency, whereas in mice, a loss of a copy of the POMC gene makes mice more susceptible to diet induced obesity, as well as causing adrenal insufficiency and a distinctive agouti fur colour (Challis et al., 2004; Krude et al., 1998).

There are five melanocortin receptors, named MC1R-MC5R. It is thought that only the MC3R and the MC4R play a role in energy homeostasis. The main endogenous ligand of the MC3/MC4R is alpha-MSH. Alpha MSH is produced by cells within the ARC of the hypothalamus, and intra cerebroventricular (ICV) administration of alpha MSH inhibits food intake (Watson and Akil, 1979; Murphy et al., 1998). Both MC3R and MC4R are expressed in hypothalamic nuclei including the ARC and the PVN (Mountjoy et al., 1994). Mice lacking the MC4R are obese and hyperphagic (Mencarelli et al., 2008). MC3R null mice are not significantly overweight but do have increased fat mass compared to native litter mates (Butler et al., 2000). Injection of alpha MSH into the third ventricle inhibits food intake and chronically reduces body weight (Semjonous et al., 2009; Benoit et al., 2000). Conversely, AgRP and agouti peptide antagonise the MC3R and MC4R receptors. Agouti peptide is a competitive antagonist of alpha MSH at the MC1R and MC4R, but under normal circumstances is only found in the periphery (Lu et al., 1994). AgRP is expressed in the ARC of the hypothalamus, and is the endogenous antagonist for central MC3R and
MC4R. ARC AgRP expression increases during fasting (Swart et al., 2002). Administration of AgRP into the third ventricles of rats dose dependently increases in food intake (Rossi et al., 1998).

1.5.3 Cocaine- and Amphetamine-Regulated Transcript
CART is the third most abundant mRNA transcript within the hypothalamus (Kristensen et al., 1998; Elias et al., 2001). CART mRNA is expressed in the ARC, the PVN and the LHA (Elias et al., 1998; Hurd and Fagergren, 2000). Within the ARC, CART is co-expressed with POMC (Dhillon et al., 2002; Elias et al., 2001). CART is a putative neuropeptide; it is found in dense core vesicles of neuron dendrites, and its release is calcium dependent (Murphy et al., 2000; Smith et al., 1997). As yet, no receptor for CART has been identified, though data suggest it is linked to an inhibitory G-protein coupled receptor, activation of which leads to an intracellular increase in extracellular signal-regulated kinase (ERK) (Lakatos et al., 2005). Fasted and food restricted animals have reduced CART mRNA expression in the ARC (Sucajty-Szulc et al., 2010; Kristensen et al., 1998). The CART peptide is processed into different fragments, with fragment 55-102 thought to be the active form, and hence being the most widely studied (Dey et al., 2003). Injection of CART (55-102) into the third cerebral ventricle inhibits food intake. ICV administration of a CART antiserum increases night time food intake (Kristensen et al., 1998). However, the injection of CART (55-102) into either the ARC or the VMN increases food intake (Abbott et al., 2001). The differing effects on food intake observed following administration of CART (55-102) into different regions suggests that different populations of CART neurons may play different roles in energy balance (Abbott et al., 2001).
Figure 1.3 Schematic diagram showing the sites of action of hormones that influence food intake. Red arrows and text denotes the pathways by which the anorexigenic gut hormones $\text{PYY}_{3-36}$, OXM, CCK and PP act, black those for the orexigenic gastric hormone ghrelin, and purple those for the adiposity signals leptin and insulin. Adapted from (Wynne et al., 2004).
1.6 Peripheral Signals Of Adiposity

1.6.1 Leptin

Leptin is a product of the ob gene which is predominately expressed in white adipose tissue (Figure 1.3) (Zhang et al., 1994). It is 146 amino acid hormone released into circulation in proportion to adipose tissue levels (Maffei et al., 1995). Leptin has a number of functions, including the regulation of energy homeostasis, immune function and fertility (Chehab et al., 1996). Leptin signals via the leptin receptor (ob-Rb), which is expressed in the ARC, VMH, DMH and LHA of the hypothalamus, and in other areas of the CNS such as the brainstem (Hayes et al., 2010). Leptin acts to maintain long term energy homeostasis and a set level of body adiposity (Elmquist et al., 1998). In the ARC, leptin receptors are located on both orexigenic NPY/AgRP neurons and anorexigenic POMC/CART neurons (Mercer et al., 1996; Elmquist et al., 1997). An increase in white adipose tissue leads to an increase in circulating leptin levels, activating POMC/CART neurons and inhibiting NPY/AgRP neurons. A reduction in adiposity results in a decrease in circulating leptin levels, which disinhibits orexigenic NPY/AgRP neurons. Leptin also acts on leptin receptors in other hypothalamic nuclei. Circulating leptin levels can also reflect short term food intake. Food restriction/fasting can acutely suppress leptin levels. A diet of 35% of average daily calorie requirements for 4 days reduced circulating leptin levels by 40%, but this is rapidly reversed by refeeding (Maffei et al., 1995; Mars et al., 2006).

Absence of leptin signalling results in hyperphagia and obesity in rodents and humans (Montague et al., 1997). However, these mutations are very rare in humans. A condition more commonly associated with abnormal leptin signalling in humans is leptin resistance (Bjorbaek, 2009; Munzberg, 2010; Maffei et al., 1995). Leptin resistance is associated with obesity and may be due to persistently high circulating levels of leptin causing receptor desensitisation (Scarpace and Zhang, 2009). Leptin was once considered a possible target for anti-obesity treatment, as administration of leptin to leptin-deficient mice reduces food intake and body weight (Levin et al., 1996). It was subsequently found that obese subjects have higher circulating levels of
leptin and in many cases are leptin resistant (Segal et al., 1996). Exogenous administration of leptin to the obese is therefore ineffective as a weight loss drug.

There are a number of rodent models of obesity with abnormal leptin signalling. The Ob/Ob mouse and the Db/Db mouse are the best characterised (Coleman, 1978; Ingalls et al., 1950). The Ob/Ob mouse lacks functional leptin, but has a functional leptin receptor. The lack of functioning leptin is due to a recessive mutation, first identified in a group of mice in Jackson laboratories in 1949 (Ingalls et al., 1950). Ob/Ob mice are hyperphagic and obese and develop severe insulin resistance at an early age (Lindstrom, 2007). The Db/Db mouse lacks a functional leptin receptor and is hyperphagic and obese, with poorly regulated blood glucose levels. It is thus also used as a model of obesity and diabetes.

Leptin levels are closely related to fertility; very high and very low levels of leptin have been linked to reduced fertility levels in mouse models (Chehab et al., 1996). The Ob/Ob mouse model is infertile (Mounzih et al., 1997), as the absence of leptin reduces hypothalamo-pituitary-gonadal drive, preventing animals from going through puberty or maintaining normal reproductive function (Goumenou et al., 2003). Interestingly, the infertility of the Ob/Ob mouse is rescued by cross breeding with either the Y2 or Y4 receptor knockout mouse (Sainsbury et al., 2002).

1.6.2 Insulin

Insulin is a 51 amino acid peptide hormone, produced in the beta cells of the pancreatic islets of Langerhans. Insulin regulates glucose homeostasis by stimulating glucose uptake and glycogen storage, and by suppressing gluconeogenesis. Circulating levels of insulin are positively correlated with long-term energy balance, and it has been suggested that insulin acts as a signal of adiposity (Bagdade et al., 1967). Circulating insulin levels inversely correlate with insulin sensitivity in the periphery; long term elevated circulating insulin levels are linked to insulin resistance and T2DM. Insulin is released rapidly after a meal to maintain glucose homeostasis via a tyrosine kinase signal transduction pathway. Insulin crosses the blood brain barrier
via a saturable receptor-mediated process in proportion to circulating levels (Krauss et al., 1998; Baura et al., 1993) and acts as an anorexigenic signal (Won et al., 2009). Administration of insulin into the third cerebral ventricle in rodents reduces food intake (Ikeda et al., 1986). Insulin receptors have been found on POMC neurons in the ARC of the hypothalamus, and central administration of insulin to fasted rats increases POMC mRNA expression (Benoit et al., 2002). The anorexigenic effects of central insulin are blocked by administration of a melanocortin receptor antagonist SHU 9119 (Benoit et al., 2002). This suggests that the central effects of insulin on appetite may be mediated via the melanocortin system.

Due to the increase in numbers of patients with T2DM, and obesity, new treatments are being developed to help maintain better control over blood glucose levels (Bourne J, 2001). Exenatide is an anti-diabetic drug which acts as a GLP-1 receptor agonist to inhibit glucagon release and increase glucose dependent insulin release (Bosi et al., 2008; Davidson et al., 2008). It was developed in collaboration by Amylin pharmaceuticals and Eli Lilly (Nielsen and Baron, 2003). During phase III clinical trials, exendin-4 reduced significantly HBA1c and increased insulin release; it has also been shown to cause weight loss (Buse et al., 2004).
1.7 Peripheral Signals from the Gastrointestinal Tract

1.7.1 Ghrelin
Ghrelin is a 28 amino acid peptide produced in the fundus of the stomach by the chromogranin A immunoreactive X/A-like cells (Kojima et al., 1999). There are two major circulating forms of ghrelin: acetylated ghrelin and des-acyl ghrelin (Hosoda et al., 2000). Acetylated ghrelin is the active form of ghrelin; the addition of the acyl side chain, n-octanoic acid, to the third serine residue of ghrelin is essential for receptor binding and subsequent effects on food intake (Kojima et al., 1999). Ghrelin is an endogenous agonist for the growth hormone secretagogue receptor (GHSR) (Jarkovska et al., 2004), stimulation of which has long been known to cause the release of growth hormone (Kojima et al., 1999). The GHSR is highly expressed in the anterior pituitary, but also in the ARC and the VMN of the hypothalamus (Kojima and Kangawa, 2002). Circulating ghrelin levels rise before a meal and decrease post prandially (Nakazato et al., 2001). Central or peripheral administration of ghrelin dose dependently increases food intake (Lawrence et al., 2002; Kojima and Kangawa, 2002; Wren et al., 2001). Circulating ghrelin levels inversely correlate with long term energy stores. Anorexic individuals have high circulating levels which fall to normal levels on weight gain (Otto et al., 2001). Conversely, obese subjects have low plasma ghrelin levels which normalise after weight loss (Hansen et al., 2002). Unlike lean individuals, obese subjects may not experience the same rapid post prandial fall in circulating ghrelin, which may contribute to their obesity (English et al., 2002). Peripheral administration of ghrelin activates NPY/AgRP neurones in the ARC (Ruter et al., 2003; Nakazato et al., 2001) and has no effect on appetite in NPY/AgRP knockout mice (Chen et al., 2004), suggesting its orexigenic effects are mediated by this neuronal population.

1.7.2 Cholecystokinin
Cholecystokinin (CCK) is produced by mucosal endocrine I cells in the small intestine (Polak et al., 1975). There are a number of active forms of CCK which circulate in the blood stream, including CCK-58, CCK-33 and CCK-8 (Reeve, Jr. et al., 1994; Zhou et al., 1985). Levels of circulating CCK rise
following nutrient intake, and levels remain high for a number of hours (Liddle et al., 1985). CCK is also expressed in the brain, where it can act as a neurotransmitter, and is thought to play a role in a number of processes, including memory, anxiety and reward behaviour, as well as satiety (Crawley and Corwin, 1994).

Peripheral administration of CCK to both humans and rodents inhibits food intake (Smith and Gibbs, 1975; Muurahainen et al., 1988). Peripheral CCK signals via the vagus nerve; peripheral administration of CCK increases c-fos expression in the rat brainstem and does not influence food intake in vagotomised rodents (Zittel et al., 1999). Peripheral CCK has a rapid but relatively short-lived effect on appetite in rodents (Moran, 2000; Thompson et al., 1975). In animals, chronic administration of CCK reduces food intake, but there is a compensatory increase in meal frequency that means there is no effect on body weight (West et al., 1984; West et al., 1987). Chronic administration of a CCK antagonist results in weight gain in rodent models, but interestingly without an increase in food intake (McLaughlin et al., 1985; Meereis-Schwanke et al., 1998).

It has been suggested that any effects on body weight observed following CCK administration may be due to the interaction of CCK with leptin, which enhances the satiating effect of CCK (Matson et al., 2000).

1.7.3 Proglucagon- derived products
The proglucagon gene was originally discovered by Bell et al in 1983. It undergoes tissue specific post translational processing in the intestine, pancreas and central nervous system (Bell et al., 1983). In the L-cells of the small intestine, proglucagon is cleaved by prohormone convertase 1 and 2 into glicentin, oxyntomodulin, GLP-1 and glucagon-like peptide 2 (GLP-2) (Tang-Christensen et al., 2001; Bonic and Mackin, 2003). In the alpha cells of the pancreas, proglucagon is cleaved by prohormone convertase 2 to produce glucagon (Dhanvantari et al., 1996; Patzelt and Schiltz, 1984).
1.7.3.1 Glucagon
Glucagon is a 29 amino acid peptide released from the alpha cells of the pancreatic islets of Langerhans. Glucagon acts to counteract the actions of insulin on blood glucose levels, by increasing glucose synthesis, and the release of glucose from storage as glycogen (glycogenolysis). Levels of circulating glucagon are low post prandially, but rise on fasting. Glucagon binds to the glucagon receptor (GLU-R), which is a G-protein coupled receptor (GPCR) linked to the activation of cyclic AMP, an intracellular secondary messenger (Jelinek et al., 1993). High affinity glucagon binding sites have been identified in the liver, kidneys, brain, and adipose tissue (Moens et al., 1996). GLU-R receptors are also found on the beta cells of the pancreas, where their activation is linked to an increase in insulin release. This allows newly available circulating glucose to be taken up by insulin-dependent tissues, such as liver and muscle. T2DM and the metabolic syndrome are thought to be partly due to the failure of post prandial glucagon suppression, together with insulin deficiency/insulin resistance (Spellman, 2007; Unger and Orci, 1975).

1.7.3.2 Glucagon-like Peptide 1
GLP-1 is a 30 amino acid peptide derived from the proglucagon gene. Physiologically, GLP-1 stimulates glucose-dependent insulin secretion and inhibits glucagon secretion (Drucker, 2001). GLP-1 has also been linked to the inhibition of gastric emptying and food intake (Nauck et al., 1997; Naslund et al., 1999). The L cells of the intestine release two bioactive forms of GLP-1 into the circulation (Herrmann et al., 1995; Orskov et al., 1993). GLP-1 (7-37) and GLP-1 (7-36 amide) are both released post prandially (Orskov et al., 1996). Exogenous GLP-1 (7-36) inhibits food intake when administered peripherally or centrally to rodents (Turton et al., 1996). Daily injections of GLP-1 into the third ventricle reduce body weight in rats (Meeran et al., 1999). Intra venous (IV) infusion of GLP-1 to normal weight volunteers inhibits food intake (Verdich et al., 2001). Circulating GLP-1 levels are inversely correlated with body mass (Ranganath et al., 1996). GLP-1 may play a role in energy expenditure, as a 4 hour infusion of GLP-1 reduced energy expenditure in normal-weight and obese volunteers (Flint et al., 2000).
The GLP-1 receptor is expressed in the hypothalamus and the brainstem (Goke et al., 1995; Uttenthal et al., 1992; Shimizu et al., 1987), where its expression is lower in fed rats than in fasted rats (Zhou et al., 2003). GLP-1 knockout mice show normal feeding behaviour, but are hyperglycaemic when fasted (Scrocchi et al., 1996). GLP-1 is a substrate for the degradative enzyme dipeptidyl peptidase IV (DPPIV), which limits its circulating half life to approximately 2 minutes (Orskov et al., 1993). A number of treatments for diabetes have been developed recently using DPPIV resistant forms of GLP-1 (Burcelin et al., 1999).

1.7.3.3 Exendin-4
Exendin-4 is a 39 amino acid peptide, which acts as a potent agonist for the GLP-1 receptor. It was originally found in the saliva of the Gila monster (Eng, 1992), and has since been developed by Amylin Pharmaceuticals and Eli Lilly and Company as a treatment for T2DM type 2 diabetes under the trade name Exenatide (Nielsen and Baron, 2003). Exendin-4 inhibits glucagon release, and increases insulin secretion in humans. Unlike T2DM treatments such as metformin, treatment with exendin-4 is also associated with weight loss. HbA1c is a measure of glycated haemoglobin, commonly used as a measure of blood glucose levels over an extended period. After 3 years of treatment, patients receiving Exenatide lost an average of 5.3kg compared to placebo, and had a 1% reduction in their glycated haemoglobin (HbA1c) levels (Klonoff et al., 2008; Szayna et al., 2000). However, treatment with GLP-1 and exendin-4 is commonly associated with nausea (Buse et al., 2010; Astrup et al., 2009).

1.7.3.4 Glucagon-like Peptide 2
Glucagon-like peptide 2 (GLP-2) is a 33 amino acid peptide product of the proglucagon gene (Dhanvantari et al., 1996). It is secreted from the L cells of intestines, and like GLP-1 is a substrate for the degradative enzyme DPPIV (Drucker et al., 1997). GLP-2 acts via the GLP-2 receptor, which is expressed in both the small and the large intestine (Munroe et al., 1999). Unlike GLP-1 and other proglucagon products, GLP-2 does not reduce food intake in animals or humans following peripheral administration (Schmidt et al., 2003). However, central administration to rodents has been reported to
reduce food intake (Tang-Christensen et al., 2001). This central anorectic effect may be due to GLP-2 activating central GLP-1 receptors (Lovshin et al., 2001). The main actions of GLP-2 include inhibiting gastric motility and gastric acid secretion, and promoting intestinal growth (Meier et al., 2006).

### 1.7.3.5 Oxyntomodulin

Oxyntomodulin is a 37 amino acid peptide comprised of the 29 amino acids of glucagon with the addition of 8 amino acids at the C terminus (Bataille et al., 1981). Oxyntomodulin is released post prandially from the intestinal L cells (Ghatei et al., 1983; Stanley et al., 2004). Like other members of the proglucagon family, oxyntomodulin inhibits gastric acid secretion and inhibits food intake in rodents and humans (Dakin et al., 2004). A specific oxyntomodulin receptor has not been identified, and it is thought that oxyntomodulin exerts its actions through the GLP-1 receptor. Oxyntomodulin does not reduce food intake in mice which lack the GLP-1 receptor (Baggio et al., 2004). However, there is evidence that there may be a specific oxyntomodulin receptor. Both oxyntomodulin and GLP-1 reduce food intake with a similar magnitude when administered peripherally, but oxyntomodulin has a lower affinity for the GLP-1 receptor than GLP-1 (Dakin et al., 2001). In addition, the anorectic effects of peripherally administered oxyntomodulin are attenuated by administration of the potent GLP-1 receptor antagonist exendin (9-39) into the ARC, but those of peripherally administered GLP-1 are not (Dakin et al., 2004). This provides further evidence that distinct receptors or receptor subtypes may mediate the effects of oxyntomodulin and GLP-1.

In animal studies, oxyntomodulin acutely reduces food intake when administered peripherally and centrally (Dakin et al., 2004; Dakin et al., 2001). Peripheral administration of oxyntomodulin to humans via an IV infusion acutely reduces food intake compared to saline controls up to 12 hours post infusion (Cohen et al., 2003). Chronic daily IP administration of oxyntomodulin over 7 days reduces body weight gain in rats (Dakin et al., 2004). Daily subcutaneous (SC) administration of a long lasting oxyntomodulin analogue has also been shown to reduced body weight gain in rodents (Liu et al., 2010). In humans, administration of 400nmol (SC) of
oxyntomodulin 30 minutes before each meal for a month to healthy over
weight and obese patients significantly reduced body weight compared to
saline control group (Wynne et al., 2005a). Thiakis Ltd has developed an
oxyntomodulin analogue which is more degradation resistant than the native
oxyntomodulin, for the treatment of obesity in humans (Cooke and Bloom,
2006). This molecule is currently undergoing clinical trials. In overweight
patients, acute combined oxyntomodulin and PYY$_{3-36}$ infusion reduces food
intake compared to saline control or infusion with either hormone alone (Field
et al., 2010).

Oxyntomodulin may also regulate energy expenditure. When administered
daily via the third ventricle to rats it reduces body weight compared to ad
libitum and pair fed controls (Dakin et al., 2002). In humans, pre-prandial SC
administration of oxyntomodulin reduced food intake and increased activity
related energy expenditure when examined by indirect calorimetry (Wynne et
al., 2006).

1.7.4 ‘PP-Fold’ Family of Peptides

Peptide YY, NPY and PP are all members of the ‘PP fold’ group of peptides. All
three show high levels of sequence homology (Ekblad and Sundler,
2002). This group are named after the distinct fold in their tertiary structures,
consisting of a “U” shaped extended polyridine helix and an alpha helix
connected by a beta turn. (Figure 1.4 on page 58, shows the basic structure
of PP). These two peptides have NPY as a common ancestor (Blomqvist et
al., 1992). PP is thought to have evolved following a chance duplication of
the PYY gene (Larhammar, 1996a; Cerda-Reverter and Larhammar, 2000).

PP fold peptides exert their effects via a group of G protein coupled receptor
known as the Y receptors. Currently there are 5 known forms of the Y
receptor: Y1, Y2, Y3, Y4 and Y5. Y3 receptors are only found in avian
species. All Y receptors share limited sequence homology but similar
pharmacology. Each receptor has a different affinity for each PP fold ligand,
and different expression patterns within the brain and the periphery. Each of
the different Y receptors can bind at least two of the PP fold peptides
(Table1.1).
The expression of the Y receptors is mainly limited to the brain and the digestive tract, though the Y1 receptor is also found in blood vessels, and Y2 in the heart (Table 1.1) (Dumont et al., 1998; Ferrier et al., 2002). Within the hypothalamus, Y1 receptors are found predominately in the ARC, and Y2 receptors in the PVN and the ARC, (Parker and Herzog, 1999). The Y4 receptor is highly expressed in the area postrema and the dorsal vagal complex of the brain stem (Parker and Herzog, 1999; McTigue et al., 1993) where there is limited expression of other Y receptors.

1.7.4.1 Peptide YY

PYY is a 36 amino acid peptide first isolated in 1982 from porcine intestinal extracts (Tatemoto, 1982). PYY is released from gastrointestinal L-cells post prandially in proportion to calories ingested (Adrian et al., 1985). Levels rise approximately 18 minutes after ingestion of food and remain high for 2 hours (Adrian et al., 1987). PYY is cleaved by the enzyme DPPIV at the N-terminus to generate PYY\textsubscript{3-36}, the major circulating form of PYY (Grandt et al., 1993). PYY\textsubscript{3-36} is a selective agonist for the Y2 receptor, whereas PYY\textsubscript{1-36} is an agonist for the Y1, Y2 and Y5 receptors. Intravenous infusion of PYY\textsubscript{3-36} acutely reduces food intake in humans. Healthy volunteers infused with physiological doses of PYY\textsubscript{3-36} (to mimic post prandial circulating plasma levels), reduced their food intake at a buffet meal by 35% (Batterham et al., 2002). However, supra-physiological doses of PYY\textsubscript{3-36} are associated with nausea (Le Roux et al., 2008).

In rodents, peripheral administration of PYY\textsubscript{3-36} acutely inhibits food intake, and chronically reduces food intake and body weight (Vrang et al., 2006). However, the chronic effects of PYY\textsubscript{3-36} are relatively short lived, with any effect on food intake being abolished after four days of SC or IP administration (Parkinson et al., 2008; Vrang et al., 2006). This may be an administration issue, as intermittent IV infusion (1 hour IV infusion at 30pmol/kg/min every other hour) of PYY\textsubscript{3-36} in obese rats has been shown to reduce body weight by 20% over 10 days (Chelikani et al., 2006).

Nastech and Merck previously developed a nasally administered form of PYY\textsubscript{3-36} to be taken before a meal. This treatment reached phase II clinical
trials, but there was a high dropout rate thought to be due to nausea caused by the super physiological plasma levels of PYY$_{3-36}$ achieved. In the trial, within 26 minutes of administration, plasma PYY$_{3-36}$ concentrations reached approximately 105pmol/L, twice the normal post prandial level (Gantz et al., 2007; MDRNA, 9 A.D.).
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<tr>
<th>Receptor</th>
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<td>Amygdala</td>
<td>Hippocampus</td>
<td>Thalamus, Area postrema</td>
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<td>Blood vessels</td>
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Table 1.1 Y receptor distribution and ligand affinities for the PP-fold family (Ferrier et al., 2002; Jois et al., 2006; Parker and Herzog, 1999; Trinh et al., 1996). Affinities are listed highest to lowest.
### 1.7.4.2 Pancreatic Polypeptide

Pancreatic polypeptide (PP) is a 36 amino acid peptide with an amidated C terminus (Larhammar, 1996a), originally isolated in avian species and first described by Kimmel in 1975 (Kimmel et al., 1975). In humans, PP is found at highest concentrations within the pancreas (123pmol/ml), but is also found at lower levels in other areas of the digestive tract, including the stomach (Adrian et al., 1977; Adrian et al., 1976).

PP is released post prandially from pancreatic endocrine F cells in proportion to the calories ingested. (Adrian et al., 1978; Adrian et al., 1976). PP has also been shown to be released in a diurnal rhythm, with levels reaching their highest at 3pm and returning to their lowest at 9am (Johns et al., 2006). The average fasted plasma circulating level of PP is approximately 30pmol/l, which rises to over 160pmol/l post prandially (Adrian et al., 1976). Meal composition also influences PP release; carbohydrates do not stimulate PP release as effectively as protein or fat (Feinle-Bisset et al., 2005).

The post prandial release of PP from the pancreas is under vasovagal control (Schwartz, 1983). Vagal afferents from the GI tract synapse with neurons in the NTS and the AP of the brainstem, and are relayed to the DMN indicating the presence of food in the stomach and intestines. Vagal efferents from the DMN projecting to the pancreas signal to stimulate PP release (Berthoud et al., 1991). PP and the Y4 receptors are involved in the control of gastric and pancreatic secretion. Infusion of exogenous PP has been shown to stimulate gastric acid secretion, and inhibit pancreatic secretion, as well as to decrease gastric motility (McTigue et al., 1993; Gettys et al., 1992; Okumura et al., 1994; McTigue and Rogers, 1995). Lesions to the AP have been shown to ablate the ability of exogenous PP to inhibit pancreatic secretions (Deng et al., 2001), and vagotomy prevents the post-prandial release of PP and its subsequent effects (Schwartz, 1983).

As previously stated in section 1.7.4, PP has the highest affinity for the Y4 receptor, and its effects on food intake are mediated via its activation of the Y4 receptor (Lin et al., 2009; Sainsbury et al., 2010; Asakawa et al., 1999). The Y4 receptor is linked to cyclic AMP via G protein Gq; increased Y4
receptor activation inhibits cyclic AMP production (Kojima et al., 2007; Hosoda et al., 2000; Dumont et al., 2005; Dumont et al., 1993). In smooth muscle cells, Y4 receptors activate the G protein $G_q$ and stimulates inositol triphosphate ($IP_3$) formation and $IP_3$ dependent calcium release to allow muscle contraction (Misra et al., 2004).

High basal levels of PP, usually caused by F cell endocrine tumours, have been associated with weight loss in a number of patients (Strodel et al., 1984; Adrian, 1986; Martella et al., 1997). Transgenic mice over-expressing the PP gene (resulting in circulating levels of PP twenty times higher than their wild type litter mates) ate significantly less compared to wild type litter mates and had lower body weights (Ueno et al., 1999). Administration of PP anti-serum to these animals returned food intake and body weight to levels similar to wild type animals (Ueno et al., 1999).

Other studies investigating the role of PP in the regulation of appetite have found that patients suffering from the condition anorexia nervosa demonstrated an exaggerated PP response upon eating (Uhe et al., 1992; Fujimoto et al., 1997; Kinzig et al., 2007). Conversely, patients with Prader-Willi Syndrome (PWS), a condition characterised by hyperphagia and obesity, decreased fat free mass, decreased energy expenditure and decreased bone density, have higher than average fasting circulating levels of PP. However, they have a blunted PP response to feeding (Zipf et al., 1981; Zipf et al., 1983). Infusion of PP (50pmol/kg/h) in PWS has been shown to reduce food intake in a free buffet meal by 12%, post infusion (Berntson, 2003). PWS patients have other alterations in gut hormone profiles. Circulating PYY levels are also higher post prandially in PWS patients, and there is a greater rise in plasma PYY levels post prandially (Bizzarri et al., 2010; Gimenez-Palop et al., 2007). Fasting ghrelin levels are higher in PWS patients, and the post prandial fall in ghrelin is more pronounced in PWS patients (Bizzarri et al., 2010; Gimenez-Palop et al., 2007).

Infusion of 10pmol/kg/min of PP to healthy volunteers reduced food intake at a buffet meal by 21.8% compared to saline control, and reduced food intake at 24 hours by 25% (Batterham et al., 2003). A lower infusion rate of
5pmol/kg/min reduced acute food intake by 10.5% and had no effect on 24 hour food intake (Jesudason et al., 2007). Both of these studies resulted in supra-physiological circulating levels of PP with no reported adverse effects, further suggesting indicating that PP may have potential as a treatment for obesity. This thesis describes experiments investigating the utility of PP and PP analogues in the regulation of food intake and the treatment of obesity.
Figure 1.4 A schematic diagram showing the basic structure of PP, with some side chains represented by spheres. Above is a table indicating the amino acids composing the structures which give PP its distinct tertiary shape. Adapted from (Gellman and Woolfson, 2002).
1.8 Hypothesis and Aims of Thesis

**Hypothesis:** Rational modifications to the amino acid sequence of PP will result in a PP analogue more efficacious at inhibiting food intake and promoting weight loss than native PP.

**Aims:** To determine:

- The effect of specific changes to the structure of PP on its susceptibility to breakdown by specific enzymes and tissue preparations, and its binding affinity for the human Y4 receptor.
- The effect of specific changes to the structure of PP on its *in vivo* longevity and efficacy at reducing food intake in rodents following acute peripheral administration.
- The effects of chronic administration of a specific PP analogue (identified in experiments conducted to address aims 1 and 2 above) on food intake and body weight in rodent models of obesity.
Chapter 2

The Breakdown Of PP
And The Design Of
Enzyme-Resistant
Analogues
2.1 Introduction

PP is a member of the ‘PP-Fold’ family of peptides which includes NPY and PYY. These three polypeptides share a strong sequence homology (Larhammar, 1996a), with PP and PYY only differing by the 4 amino acids between amino acids 9 and 12. As previously described in section 1.7.4.2, PP is a 36 amino acid peptide with an amidated C terminus (Larhammar, 1996b) released post prandially from the pancreatic F cells (Adrian et al., 1978; Adrian et al., 1976). When PP was administered to healthy adults via an IV infusion it significantly reduced food intake over 24 hours compared to a saline control (Batterham et al., 2003; Jesudason et al., 2007). This anorectic effect suggests that PP may have potential as an obesity treatment. However, PP is a relatively small peptide which is thus susceptible to a number of degradative enzymes and other mechanisms. PP consequently has a short circulating half life of around 7 minutes (Adrian et al., 1978). This short half life limits the utility of unmodified PP as an obesity treatment.

Previous studies on other gut hormones including GLP-1, oxyntomodulin and PYY3-36 have shown that small regulatory peptides are susceptible to degradation by a number of membrane bound ectopeptidases and other membrane bound enzymes, limiting their circulating half life (Kenny, 1986b; Roques et al., 1993). As the PP family of peptides show strong sequence homology and are thought to be derived from a common ancestor, it is likely that the peptidases that breakdown other members of the PP-fold family will also degrade PP (Ekblad and Sundler, 2002; Blomqvist et al., 1992; Larhammar, 1996a; Larhammar, 1996b). Developing an analogue of PP which can better resist degradation by these enzymes may therefore improve the efficacy of PP at reducing food intake and, ultimately, at reducing body weight.

Analogues of other gut hormones with improved bioactivity have previously been developed. Both the GLP-1 analogue liraglutide, and the oxyntomodulin analogue OXM 6421 have improved efficacy compared to their native hormones, and are now either used or being developed as treatments for T2DM and obesity (Liu et al., 2010; Degn et al., 2004; Astrup et al., 2009;
Green et al., 2003). Inhibitors of enzymes known to inactivate hormones have also been used and developed into treatments for T2DM. DPPIV inhibitors are currently on the market and licensed for the use in T2DM; examples include sitagliptin and vildagliptin (Davidson et al., 2008; Liu et al., 2009). These DPPIV inhibitors increase the circulating levels of GLP-1 to help lower blood glucose levels.

2.1.1 Kidney Brush Border Membrane (KBB)
The exact mechanisms of PP clearance are unknown. PYY is broken-down by a number of enzymes and peptidases located in the kidney (Medeiros and Turner, 1994a; Medeiros and Turner, 1994b; Mentlein et al., 1993a), and it is therefore likely that the kidneys are also a major site of PP clearance. The kidney is thought to play a major role in the degradation of a number of peptide hormones, including atrial natriuretic peptide, bradykinin, angiotensin and substance P (Stephenson and Kenny, 1987b; Stephenson and Kenny, 1987a; Kenny and Maroux, 1982; Vanneste et al., 1988). Enzymes including aminopeptidase N, aminopeptidase A, DPPIV, and nepri lysin are all located within the kidney brush border membranes (Stephenson and Kenny, 1987b; Kenny and Stephenson, 1988; Vanneste et al., 1988; Hupe-Sodmann et al., 1995; Bertenshaw et al., 2001; Medeiros and Turner, 1995). It has been suggested that the levels of certain enzymes within the kidney brush border are dependent upon nutritional status, with, for example, starvation increasing the levels of DPPIV in rats (Ihara et al., 2000). This perhaps suggests a mechanism by which nutritional status regulates circulating gut peptide levels. The kidney brush borders could therefore be a key site for the breakdown of PP, and the reabsorption of amino acids, and kidney brush border preparations may therefore be a useful tool to investigate the breakdown of PP and PP analogues (Kenny and Stephenson, 1988).
2.1.2 Dipeptidyl Peptidase IV (DPPIV)

DPPIV is a member of the serine family of peptidases (s9b) which acts to cleave the first two amino acids at the N-terminus of small to medium sized peptides (Mentlein et al., 1993b; Mentlein, 1999; Drucker et al., 1997). Human DPPIV is 766 amino acid glycoprotein. It has been suggested that 22 of those amino acids, anchor it to the plasma membrane at the N-terminus (VLLG LLGAAALVTI ITVPVVLL), this is flanked by a short intracellular hydrophobic sequence of 6 amino acids (MKTPWK) (Mentlein, 1999). Previously, it has been suggested that for cleavage by DPPIV, a peptide must possess a proline or an alanine at position P1 of the amino acid chain, and a hydrophobic amino acid such as tyrosine at the P2 position (Bongers et al., 1992). DPPIV has a number of substrates, including NPY, PYY and GLP-1 (Mentlein et al., 1993a; Mentlein et al., 1993b). PYY has been shown to be cleaved to by DPPIV to form PYY<sub>3-36</sub>, the predominant circulating form of PYY, and the form with the highest affinity for the Y2 receptor (Grandt et al., 1993).

Recently, DPPIV inhibitors have been developed to increase the half life of GLP-1 to aid in the treatment of T2DM (Davidson et al., 2008). Sitagliptin and Vildagliptin are two DPPIV inhibitors approved for the treatment of T2DM. These two drugs act to inhibit the breakdown of GLP-1 by DPPIV, therefore increasing circulating GLP-1 levels and lowering elevated blood glucose levels. The long lasting GLP-1 analogue exenatide has been modified to prevent breakdown by DPPIV, therefore significantly increasing its plasma half life (Nielsen et al., 2004; Green et al., 2003). As DPPIV has been shown to cleave PYY, and NPY, it is also likely to cleave PP. However, this has not previously been investigated.

2.1.3 Neprilysin (NEP)

NEP is a 742 amino acid metallopeptidase enzyme with a zinc core. It is usually found bound to membranes, and is expressed throughout the body. NEP was originally discovered in purified kidney brush border (Gee et al., 1985; Roques et al., 1993; Hupe-Sodmann et al., 1995), and particularly high concentrations are found in the kidneys, brain, and lungs. NEP has a wide
number of substrates, including GLP-1, Neurokinin A and B, Neurotensin, Oxytocin and Gastrin (Erdos and Skidgel, 1989). NEP cleaves peptide chains on the amino side of hydrophobic residues. This has been demonstrated in GLP-1, in which a number of peptide bonds are cleaved by NEP. Interestingly, the Asp₂₇-Phe₂₈ and the Trp₃₁-Leu₃₂ bonds of GLP-1 were cleaved most readily (Hupe-Sodmann et al., 1995). It has been suggested that exendin-4, a long lasting analogue of GLP-1, is a poor substrate for NEP, which may also partly explain its extended half life (Hupe-Sodmann et al., 1995a; Goke et al., 1993). This suggests that substituting amino acids susceptible to attack by peptidases may increase peptide hormone bioactivity. PYY and NPY have also been shown to be cleaved by NEP, suggesting that PP may also be processed by NEP (Medeiros and Turner, 1994a; Medeiros and Turner, 1994b; Medeiros and Turner, 1996).

2.1.4 Meprin
Meprin is another metalloproteinase highly expressed in the kidney brush border, and also found on intestinal epithelia and mesenteric leukocytes (Bond and Beynon, 1986; Bond et al., 1986). It has been suggested that meprin comprises approximately 5% of total brush border protein (Bond and Beynon, 1986). Meprin peptidase is composed of two sub units which share 50% homology at the amino acid level. These two distinct types of sub unit, are named alpha and beta subunits. Meprin beta is a homooligomer of beta sub units, and meprin alpha is heterooligomer of alpha and beta sub units or a homooligomer of alpha units (Bertenshaw et al., 2002; Becker et al., 2003). Meprin beta is the predominate form in the adult human intestine (Bond et al., 1984). The meprins are similar in size and in substrate requirements to NEP. However, the NEP inhibitor phosphoramidon does not impair meprin beta activity (Bond et al., 1986; Takayama et al., 2008). Meprins are thought to have a wide range of substrates, including gastrin, angiotensin, collagen IV and fibronectin (Bertenshaw et al., 2001). Meprin alpha and meprin beta have different substrate requirements. The best known substrates for meprin beta are gastrin and chemokines (Bertenshaw et al., 2001), whereas the best known substrates for meprin alpha are the cytokines (Kruse et al., 2004). It has been suggested that the meprins have a preference for hydrophobic
amino acids, and for peptide bonds flanked by neutral or hydrophobic amino acids (Butler et al., 1987).

2.1.5 Enzyme Resistant Hormone Analogues
A number of hormone analogues which are more resistant to enzymatic breakdown than the native forms have already been developed. One example is desmopressin acetate (DDAVP) which is a longer lasting, more potent analogue of the hormone vasopressin commonly used in the treatment of diabetes insipidus (Robinson, 1976). The GLP-1 analogue exendin-4 has a significantly increased half life compared to native GLP-1 (Goke et al., 1993), which may be due to increased resistance to peptidases including NEP and DPPIV (Hupe-Sodmann et al., 1995a). The oxyntomodulin analogue OXM 6421 has a longer half life than native OXM following peripheral administration (Druce et al., 2009; Liu et al., 2010), which is probably due to amino acid changes at sites susceptible to cleavage by peptidases including NEP and DPPIV. The kisspeptin-10 analogue, [dY] Kiss-10 has also been shown to be more potent than native kisspeptin-10. Analogue [dY] Kiss-10 significantly stimulated total testosterone at 60 minutes post peripheral injection in mice, when endogenous Kiss-10 did not (Curtis et al., 2010). Currently there are at least two analogues of PYY being developed as treatments for obesity (MDRNA, 9 A.D.; Vivus Pharmaceuticals, 2009). Using analogues of hormones with greater enzyme resistance to treat disease has several advantages, including specificity, which should limit side effects. Using analogues of gut hormones such as PP may allow the mimicking of the normal physiological process of satiation, and may thus be useful in maintaining a stable body weight, or treating obesity.
2.2 Hypothesis and Aims

The breakdown of native human PP will be investigated using KBB membrane preparations and a number of purified recombinant degradative enzymes to establish which amino acid sites are targeted. Once these sites are established, PP analogues with amino acids rational substitutions at these sites will be tested for their ability to withstand enzymatic degradation in vitro.

**Hypothesis:** The substitution of amino acids at key sites targeted by KBB membrane preparations, DPPIV, neprilysin and meprin beta will produce PP analogues resistant to enzymatic degradation.

**Aims:**

1. To determine the effects on native PP of incubation with KBB membrane, DPPIV, neprilysin and meprin beta using high performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectroscopy.

2. To determine the effects of peripheral administration of PP in combination with enzyme specific inhibitors on acute food intake in C57/BL6 mice.

3. To investigate the differences in between the enzymatic breakdown of PP and PP analogues using HPLC and MALDI-TOF mass spectroscopy.
2.3 Materials and Methods

Human PP was made by and purchased from Bachem (St. Helens, UK), divided into aliquots on arrival at Hammersmith and freeze dried. PP analogues were custom synthesised by Bachem bioscience INC. (Philadelphia, USA) or BIOMOL International LP (Exeter, UK). Peptides were synthesised using automated fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis (SPSS), with each amino acid added sequentially from the C to the N terminus.

2.3.1 PP analogue synthesis

Fmoc SPSS uses repeat cycles of coupling and deprotection to create custom made peptides of up to 100 amino acids. The starting block is a single amino acid joined by covalent bonds to a polystyrene resin. The N-terminal amine of the resin embedded amino acid is deprotected by the addition of piperidine in dimethylformamide (DMF); the resin is then washed, removing all the Fmoc from the amine groups, which allows the next amino acid to be coupled to the embedded amino acid when it is added in excess. Each subsequent amino acid is then added in the same fashion. Each amino acid has the Fmoc to protect the reactive amine group, giving greater stability. Once the addition of amino acids is complete the peptide is cleaved from the resin. All peptides were determined to be 90-100% pure by analytical HPLC.
2.3.2 Kidney brush border membrane (KBB)

2.3.2.1 Preparation of KBB membrane

Rat kidneys were placed in cold 0.5M sucrose solution, and the renal cortices dissected and removed. Cortex tissue was then cut into 0.25cm pieces and gently hand homogenised in a solution of 0.5M sucrose (6ml sucrose solution to 1 gram tissue). The tissue was then homogenised using a motorised homogeniser (Jencons) with a tight fitting Teflon coated pestle at 1000rpm (Beckman J2-21, rotor JS-13.1). Following this, 1M MgCl$_2$ was added to the homogenate to produce a final concentration of 10mM. This solution was then stirred in an ice bath for 15 minutes to remove sub cellular structures from the brush border cells. The sub cellular structures were then removed by centrifugation at 1500g (3000rpm, Beckman J2-21, rotor JS-13.1) at 4$^\circ$C for 20 minutes, and centrifugation of the resulting supernatant at 15000g (10000rpm, Beckman J2-21, rotor JS-13.1) for 12 minutes at 4$^\circ$C. The supernatant was then discarded and the pellet resuspended in 100$\mu$l of 300mM mannitol in 12mM Tris-HCL (pH 7.4) with 10mM MgCl$_2$. The solution was then centrifuged for 12 minutes at 2200g (3700rpm, Beckman J2-21, rotor JS-13.1) at 4$^\circ$C and the pellet discarded. A further 500$\mu$l of buffer was added and the solution centrifuged for 12 minutes at 4$^\circ$C at 15000g (10000rpm, Beckman J2-21, rotor JS-13.1). The pellet was then resuspended in 10ml of 300mM mannitol in 12mM N-(2-hydroxyethyl)piperazine-N-(2-ethanesulfonicacid);4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH7.4) and protein concentration measured by biuret assay (Medeiros and Turner, 1994a). Subsequently, 1.5mg aliquots of KBB membrane were aliquoted and stored at -80$^\circ$C, to be defrosted at room temperature when required.

Biuret Test

A biuret test was used to calculate protein content of KBB membrane. A standard solution of BSA at 10mg/ml was made, and increasing volumes of this solution added to tubes (0, 100, 200, 300, 400 and 500$\mu$l), corresponding to 0, 1, 2, 3, 4 and 5mg of protein. The BSA solution in the 5 tubes was then made up to 500$\mu$l with water. Twenty $\mu$l and 200$\mu$l of KBB membrane was
then added to a further 2 tubes and made up to 500µl with water. Three ml of biuret solution was added to each tube, mixed and then placed in a boiling water bath for 2 minutes. Each tube was then allowed to cool and absorbance was measured using a spectrophotometer (UV-160, Shimadzu) at A595nM. The protein content of the samples was interpolated from the BSA standard curve.

2.3.2.2 PP and PP analogues incubation with KBB membrane
A 5nmol vial of freeze dried PP was reconstituted in 250µl of 300mM mannitol in 12mM HEPES buffer (pH7.4). Freeze dried aliquots of peptide were used to ensure peptide concentration and activity was consistent. One hundred µl (2nmol) of peptide solution was then added to 2 microtubes and either ten µl of KBB buffer or ten µl (1.5mg) of KBB membrane added and mixed. The peptide was then incubated at 37ºC in a water bath for 0, 5, 15, 30, 45, 60, 90 and 120 minutes. Five µl of 10% TFA was added to terminate the reactions following incubation. This decreased the pH of the solution to a level which prevented the enzymes from functioning. The solution was then centrifuged at 13,000g for 3 minutes. One hundred and ten µl of supernatant was then removed for analysis by HPLC. The time point where 50% breakdown occurred was identified and used for further experiments.

A 30 minute incubation of 2nmol PP with 1.5mg of KBB membrane resulted in approximately 50% of the PP being broken down. Subsequent experiments were therefore incubated for 30 minutes. PP, GLP-1 and each PP analogue were incubated with KBB as described above in three separate experiments to allow an SEM to be calculated for % breakdown.

2.3.2.3 MALDI-TOF MS analysis
PP was incubated with KBB as described in section 2.3.2.2. Following centrifugation, 110µl of supernatant was dried down using a speed vac savant (Thermo, speed/vac concentrator, SPD 2010) at room temperature for 30 minutes until 5µl of supernatant remained. This was then frozen at -20ºC and sent to Bachem for analysis by MALDI-TOF MS. At Bachem 100µl of 0.1%TFA was added to dilute the sample and mixed. Following sample dilution, 1µl of sample was mixed with 2µl of 10mg/ml matrix (10mg alpa-
cyano-4-hydrcinnanic acid, 495µl ACN, 495µl ethanol and 10µl 0.1% TFA). One µl of sample/matrix was added to an MS plate, and the plate run on MALDI micro MX (Waters, Milford USA) using both linear and reflectron modes. Data was analysed using MassLynx mass spectroscopy software (Waters, Milford USA).

2.3.2.4 Reverse phase HPLC analysis
PP, PP analogues and GLP-1 were incubated with KBB as described in section 2.3.2.2. Following centrifugation 110µl of supernatant placed in a HPLC vial and injected onto the HPLC. A linear gradient of 15 to 55% acetonitrile/water over 30 minutes (both containing 0.5% TFA) was used and absorbance measured at a wavelength of 214nM (Jasco HPLC system, solvent delivery system PU-2080 plus, autosampler AS-2057 plus, degasser DG2080-53, dynamic mixer 2080-32 UV detector uv-2075 using a Phenomenex Gemini C18 (5-µm particles) 250 mm x 4.6 mm column). Percentage degradation was calculated by comparing area under peak in reactions with and without KBB membrane. Percentage degradation values were calculated from a minimum of three experiments and a mean and SEM calculated. Chromatograms shown are representative examples.

2.3.3 DPPIV
2.3.3.1 PP and PP analogues incubation with DPPIV
The effect of DPPIV on PP and PP analogues was investigated as described in section 2.3.2.2, but with porcine DPPIV enzyme purchased from Sigma-Aldrich (Dorset, UK) used instead of KBB membrane. Previously published experiments on OXM determined that 10mU of DPPIV was effective at breaking down OXM (Druce et al., 2009; Liu et al., 2010). I therefore used this amount of DPPIV to examine its effects on PP. DPPIV was diluted with 100mM TRIS-HCL buffer and divided into 10mU aliquots (10mU in 10µl). A 5nmol vial of freeze dried PP was reconstituted in 250µl of 100mM Tris–HCl buffer (pH8). Two nmol of peptide solution in 100µl was then added to 2 microtubes and either 10µl of buffer or 10µl (10mU) of DPPIV added and mixed.
A 120 minute incubation of 2 nmol PP with 10mU of DPPIV resulted in approximately 50% of PP being broken down when analysed by MALDI-TOF MS. The timeline was taken from previous experiments with OXM (Druce et al., 2009; Liu et al., 2010). Subsequent experiments were therefore incubated for 120 minutes. PP and each PP analogue were incubated with DPPIV as described above in three separate experiments to allow an SEM to be calculated for % breakdown.

2.3.3.2 MALDI-TOF MS analysis

PP was incubated with DPPIV as described in section 2.3.3.1. Following centrifugation, 110µl of supernatant was dried down using a speed vac savant (Thermo, speed/vac concentrator, SPD 2010) at room temperature for 30 minutes until 5µl of supernatant remained. This was then frozen at -20°C and sent to Bachem for analysis by MALDI-TOF MS.

2.3.3.3 Reverse phase HPLC analysis

PP and PP analogues were incubated with DPPIV as described in section 2.3.3.1. Following centrifugation 110µl of supernatant was added to a HPLC vial and injected onto the HPLC. HPLC was performed as described in section 2.3.2.4 but using a gradient of 31.5-32.5 acetonitrile/water (both containing 0.05% TFA) 15-40 min. No differences between PP incubated with DPPIV and with DPPIV buffer were detected using HPLC even using this relatively shallow gradient. Chromatograms shown are representative examples. Therefore all further analysis was done by MALDI-TOF MS.

2.3.3.4 The effects of PP administration in combination with a DPPIV inhibitor on the food intake of C57/BL6 mice

All animal procedures were approved by the British Home Office under the UK Animal (Scientific Procedures) Act 1986 (Project Licence 70/6402).

Adult male C57/BL6J mice (Harlan) weighing 22-28g were maintained in individual cages, under controlled conditions with a 12 hours light and 12 hours dark. All animals were allowed ad libitum access to water and RM1 diet (Special Diet Services, Witham, UK) unless otherwise stated. Animals were handled and received sham SC injections of saline on at least two
occasions prior to study days in order to acclimatise them to the experimental procedure.

Feeding studies were carried out during the early light phase (0800-1000) in mice fasted from 1600 the preceding day. Mice following a fast have a greater food intake than their non-fasted counterparts, and are therefore more likely to demonstrate the anorectic effects of an agent. Peptides were administered subcutaneously (SC) in a maximum volume of 0.1 ml of saline, with inhibitors administered intraperitoneally (IP) in a maximal volume of 0.1ml. Animals were weighed and their body weight recorded before IP injection of either the DPPIV inhibitor valine pyrolidide (a gift from Prof. M Ghatei) (75mg/kg) or saline, which was, followed by a SC injection of either saline or PP at a dose of 50nmol/kg. The mice were then returned to their home cage with a known amount of food. Food was reweighed at 1, 2, 4, 8 and 24 hours. Food was removed from the hopper, a visual inspection of the cage made to check for any food spillage/hoarding, and food weighed using balances accurate to 0.01 g. Mouse body weight was measured 24 hours post-injection. All mice feeding studies were completed in conditions where external stressors were minimised, as stress is known to influence feeding behaviour (Marti et al., 1994).

2.3.4 NEP

2.3.4.1 PP and PP analogues incubation with NEP

The effect of NEP on PP and PP analogues was investigated as described in section 2.3.2.2, but with human recombinant NEP 24.11 enzyme purchased from R&D systems (Minneapolis, USA) used instead of KBB membrane. Previously published experiments on OXM determined that 200ng of NEP in 10µl was effective at breaking down OXM (Druce et al., 2009; Liu et al., 2010). I therefore used this amount of NEP to examine its effects on PP. NEP was diluted with 100mM TRIS-HCL buffer and divided into 200ng aliquots (200ng in 10µl). A 5nmol vial of freeze dried PP was reconstituted in 300µl of 100mM Tris–HCl buffer (pH9). Two nmol of peptide solution in 100µl was then added to 2 microtubes and either 10µl of buffer or 10µl (200ng) of NEP added and mixed.
A 30 minute incubation of 2 nmol PP with 200ng NEP resulted in approximately 50% of PP being broken down. Subsequent experiments were therefore incubated for 30 minutes. PP and each PP analogue were incubated with NEP as described above in three separate experiments to allow an SEM to be calculated for % breakdown.

2.3.4.2 MALDI-TOF MS analysis
PP was incubated with NEP as described in section 2.3.4.1. Following centrifugation 110µl of supernatant was dried down using a speed vac savant (Thermo, speed/vac concentrator, SPD 2010) at room temperature for 30 minutes until 5µl of supernatant remained. This was then frozen at -20ºC and sent to Bachem for analysis by MALDI-TOF MS.

2.3.4.3 Reverse phase HPLC analysis
PP, PP analogues and GLP-1 NEP incubations were analysed by reverse phase HPLC as described in section 2.3.2.4.

2.3.4.4 The effects of PP administration in combination with inhibition of NEP on the food intake of C57/BL6 mice
The effects of the inhibition of NEP on the reduction of food intake by PP was investigated as described in section 2.3.3.4, but with the NEP inhibitor phosphoramidon (R&D systems) administered IP at a dose of 1000mg/kg in a maximal volume of 0.1ml, and PP administered SC at a dose of 150nmol/kg in a maximal volume of 0.1ml.

2.3.4.5 The effects of inhibition of NEP on the plasma levels of PP in C57/BL6 mice following SC administration
The effects of the inhibition NEP on the plasma levels of PP following SC administration of PP was investigated as described in section 2.3.3.4, with the following modifications. Mice were housed in groups of up to 5 animals. Animals were weighed and their tails marked, allowing for later identification. Mice were injected IP with either saline or the NEP inhibitor phosphoramidon at a dose of 30mg/kg in a maximal volume of 0.1ml. Immediately following the IP injection, mice were SC injected with either with 25nmol/kg of PP or saline and allowed to return to their cage of origin with free access to water. Animals were then culled at 45 or 90 minutes post injection by inhalation of
carbon dioxide (a schedule 1 method). Once the animals had been culled, cardiac puncture was performed by inserting a 27 gauge needle attached to 1 ml syringe (heparinised using a 100 I.U/ml heparin solution) into the chest cavity and puncturing the heart muscle, and blood removed.

Blood samples were dispensed into 1ml microtubes containing 20µl Trasylol (Nordic Pharma, UK). The tubes were then spun in a microcentrifuge for 10 minutes at 3000g at room temperature. The plasma supernatant was collected and stored in microtubes at -80°C until assay for PP.

2.3.4.6 Radioimmunoassay (RIA) of PP plasma samples

RIA relies on the competition between radioactively labelled ligand and non-radioactively labelled ligand for a fixed number of specific antibody binding sites. The radioactive label used for the RIAs described in this thesis was iodine-125 (I\(^{125}\)). In an RIA, the amount of labelled peptide and antibody are at a constant concentration in each tube. As the amount of unlabelled peptide in a tube is increased (from samples or standards), the amount of labelled peptide which binds to the antibody is reduced. Separation of unbound (free) and bound labelled peptide and counting the proportion of labelled peptide in each fraction in a gamma-counter enables differentiation of the percentage of labelled peptide that has bound to the antibody. A standard curve is established by using known concentrations of unlabelled peptide (standard) to calculate the percentage of labelled peptide bound for each concentration of unlabelled peptide. Concentrations of the peptide in samples can then be determined by interpolation.

The method of separation used in the PP RIA was charcoal absorption. The charcoal was dextran coated to prevent the larger antibody-peptide complexes entering the pellet. Free labelled peptide is bound in the charcoal pellet and is separated from the antibody-bound labelled peptide by centrifugation. Tubes were centrifuged at 2500rpm at 4°C for 20 minutes immediately following addition of dextran-coated charcoal (6mg/tube). The supernatant containing the antibody-peptide complex was aspirated and placed into an empty tube. Both pellet and supernatant were counted in a
gamma-counter (model NE1600, Thermo Electron Corporation) to determine percentage of labelled peptide bound to the antibody.

PP plasma levels were measured using established methods (Adrian et al., 1976). Anti-PP antibody was raised in rabbits against human PP and the radiolabelled PP was prepared by Professor Ghatei by the Bolton and Hunter Method. The RIAs were performed in 0.06M phosphate buffer (54mM Na$_2$HPO$_4$, 6mM KH$_2$PO$_4$, 10mM EDTA, 8mM NaN$_3$, 0.1% (v/v) bovine serum albumin (BSA) pH7.4 ± 0.1) and were incubated for 3 days at 4°C before separating using activated charcoal.

2.3.5 Meprin beta

2.3.5.1 PP and PP analogues incubation with meprin beta

The effect of meprin beta on PP and PP analogues was investigated as described in section 2.3.2.2, but with human recombinant meprin beta enzyme purchased from R&D systems (Minneapolis, USA) used instead of KBB membrane. Previously published experiments on meprin beta determined that 200ng of meprin beta in 10µl may be effective at breaking down 2 nmol of peptide (Butler et al., 1987). I therefore used this amount of meprin beta to examine its effects on PP. Meprin beta was activated as directed by R&D system protocol and then subsequently diluted with 50mM TRIS-HCL buffer (pH 7.5) and divided into 200ng aliquots (200ng in 10µl). A 5nmol vial of freeze dried PP was reconstituted in 300µl of 50mM Tris–HCl buffer (pH7.5). Two nmol of peptide solution in 120µl was then added to 2 microtubes and either 10µl of buffer or 10µl (200ng) of meprin beta added and mixed.

A 30 minute incubation of 2 nmol PP with 200ng meprin beta resulted in approximately 50% of PP being broken down. Subsequent experiments were therefore incubated for 30 minutes. PP and each PP analogue were incubated with meprin beta as described above in three separate experiments to allow an SEM to be calculated for % breakdown.
2.3.5.2 MALDI-TOF MS analysis

PP was incubated with meprin beta as described in section 2.3.5.1. Following centrifugation, 110µl of supernatant was dried down using a speedvac savant (Thermo, speed/vac concentrator, SPD 2010) at room temperature for 30 minutes until 5µl of supernatant remained. This was then frozen at -20°C and sent to Bachem for analysis by MALDI-TOF MS.

2.3.5.3 Reverse phase HPLC analysis

PP, PP analogues and GLP-1 meprin beta incubations were analysed by reverse phase HPLC as described in section 2.3.2.4.

2.3.6 Statistical analysis

The results from acute feeding and plasma breakdown studies were compared using a one-way ANOVA with post hoc Bonferroni test. The breakdown of PP and PP analogues was examined by comparing the area under the curve of the peptide with enzyme/membrane to the same incubation without enzyme/membrane. Enzymatic breakdown experiments were performed a minimum of three times for each peptide and the mean percentage breakdown and S.E.M calculated. An n of three was chosen to give an indication of breakdown, but it is understood that a larger n number would decrease the S.E.M and increase the reliability of the results. The significance compared to native PP was then calculated for each analogue using Student's T test. A p value of less than 0.05 was considered to be significant in all analyses.
2.4 Results

2.4.1 The breakdown of native PP

2.4.1.1 Breakdown of PP by KBB membrane

Following the incubation of PP at 37°C with KBB buffer alone for 30 minutes, a single peak with an identical elution time to that of freshly dissolved PP was detected. This single peak at a retention time of 26 minutes was also observed when PP was incubated with KBB membrane preparation for increasing amounts of time between 15 and 120 minutes (Figure 2.1). PP incubated with KBB for 15 minutes was broken down by 22%. At 45 minutes this had increased to 69% breakdown, and at 60 minutes to 88% (Figure 2.1). By 120 minutes nearly all PP had been degraded (92%) (Figure 2.1). In addition to the reduction in size of the primary PP peak, a number of additional peaks with earlier retention times were observed. In total, twelve major UV-absorbing fractions could be resolved following incubation of PP with 1.5mg of KBB protein for 30 minutes. These fractions were observed at retention times of 9, 12, 13, 14, 15.5, 16, 17, 18, 21, 23, 24 and 25.5 minutes (Figure 2.1). Incubation of PP with KBB for 30 minutes produced 66 ±14% breakdown compared to PP incubated with reaction buffer under the same conditions. This time was subsequently used for PP analogue incubations.

The NEP inhibitor phosphoramidon was added to a number of PP KBB incubations, to investigate what percentage of KBB breakdown of PP could be attributed to NEP. There was no difference in the HPLC elution profile between PP incubated with KBB in the presence of phosphoramidon or without phosphoramidon (Figure 2.2).

Further analysis of PP incubated with KBB using MALDI-TOF MS indicated that there were a number of PP breakdown products detectable following a 10 minute or 30 minute incubation with KBB. A single peak with a mass to charge ratio of 4183M/Z was detected following incubation of PP with buffer for 0 minutes. This peak is consistent with the molecular weight of PP_{1-36}. A number of additional peaks were observed after a 10 minute incubation of PP with KBB, shown in figure 2.3. The number of peaks detected by MALDI-
TOF MS increased when the incubation time was increased to 30 minutes. At 10 minutes, peaks with mass to charge ratios of 2008, 2101, 4015 and 4143M/Z were detected. These masses are consistent with PP and the PP fragments PP$_{9\text{-}26}$, PP$_{3\text{-}21}$, and PP$_{3\text{-}36}$. At 30 minutes, peaks with mass to charge ratios of 1063, 2093, 2871, 4015 and 4143M/Z were detected. These masses are consistent with PP and the PP fragments PP$_{18\text{-}26}$, PP$_{23\text{-}36}$, PP$_{3\text{-}21}$, PP$_{3\text{-}27}$, and PP$_{3\text{-}36}$ (Figure 2.3).
Figure 2.1 Representative HPLC chromatograms of 2nmols PP incubated with 1.5mg of KBB (black line) or with buffer (red line) at 37°C for A) 15 B) 30 C) 60 and D) 120 minutes. Reactions were terminated with 5µl of 10%TFA and the supernatant injected onto HPLC with a Gemini C18 column and a gradient of 15-55% ACN (green line) run over 30 minutes.
Figure 2.2 Representative HPLC chromatograms of 2nmols of PP incubated with 1.5mg of KBB (Black line) and an excess of phosphoramidon or with buffer (red line) at 37°C for A) 15 B) 30 and C)120 minutes. Reactions were terminated with 5µl of 10%TFA and the supernatant injected onto HPLC with a Gemini C18 column and a gradient of 15-55% ACN (green line) run over 30 minutes.
Figure 2.3 Representative MS chromatograms of PP incubated with 1.5mg of KBB for A) 10 minutes and B) 30 minutes at 37°C. Reactions were terminated with 5µl 10% TFA and the supernatant concentrated using a speed vac concentrator and frozen and sent for MALDI-TOF MS analysis. The vertical axis represents percentage (%) and horizontal axis represents mass to charge ratio (M/Z). The arrows indicate the masses of possible PP fragments following breakdown by KKB membrane.
2.4.1.2 Breakdown of PP by DPPIV

Following the incubation of PP at 37°C with DPPIV buffer alone for 120 minutes, a single peak with an HPLC elution time identical to that of freshly dissolved PP was detected (Figure 2.4). This single peak at a retention time of 29 minutes was also observed when PP was incubated with DPPIV enzyme for 120 minutes (Figure 2.4). Using HPLC there was no detectable difference between PP incubated with or without enzyme, and therefore no percentage breakdown could be calculated.

Further analysis of PP incubated with DPPIV using MALDI-TOF MS indicated that the peptide eluting at the same position as native PP was not intact. A single peak was observed at a mass to charge ratio of 4183M/Z following incubation of PP with buffer. This peak is consistent with the molecular mass of PP\textsubscript{1-36}. The single peak detected by HPLC after a 120 minute incubation of PP with DPPIV had a mass to charge ratio of 4014M/Z, as determined by MALDI-TOF (Figure 2.4). This mass is consistent with PP\textsubscript{3-36}. 
Figure 2.4 Representative A) HPLC and B) MALDI-TOF MS chromatograms of PP incubated with 10mU of DPPIV enzyme at 37°C for 120 minutes. Reactions were terminated with 5µl of 10%TFA and the supernatant was either injected onto HPLC with a Gemini C18 column and a gradient of 31.5-33.5% ACN run over 35 minutes, or sent for analysis by MADLI-TOF MS.
2.4.1.3 Breakdown of PP by NEP

Following the incubation of PP at 37°C with NEP buffer alone for 30 minutes, a single peak with an elution time identical to that of freshly dissolved PP was detected. This single peak at a retention time of 25.5 minutes was also observed when PP was incubated with NEP enzyme for increasing amounts of time between 5-120 minutes (Figure 2.5). PP incubated with NEP for 5 minutes was broken down by 27%. At 15 minutes this had increased to 35% breakdown, and at 45 minutes to 81%. By 120 minutes nearly all PP had been degraded (90%) (Figure 2.5). In addition, a number of additional peaks with earlier retention times were observed. In total, seven major UV-absorbing fractions could be resolved following incubation of PP with 200ng of NEP enzyme for 30 minutes (Figure 2.5). These fractions were observed at retention times of 11, 13.5, 15.5, 17, 21, 22 and 24.5 minutes (Figure 2.5) Incubation of PP with NEP for 30 minutes produced 52±5.7% breakdown compared to PP incubated with reaction buffer under the same conditions. This time was subsequently used for PP analogue incubations.

Under the standard conditions (30 minute incubation at 37°C) the addition of the NEP inhibitor phosphoramidon in excess prevented any breakdown of PP by NEP (Figure 2.6). During an extended incubation of PP with NEP (120 minute incubation at 37°C) breakdown was reduced to 20% compared to 90% without inhibitor (Figure 2.6).

Further analysis of PP incubated with NEP using MALDI-TOF MS indicated that there were a number of breakdown products of PP detectable following a 15 or 45 minute incubation with NEP. A single peak with a mass to charge ratio of 4183M/Z was detected following incubation of PP with buffer at both time points examined. This peak is consistent with the molecular weight of PP\textsubscript{1-36}.

A number of additional peaks were observed after a 15 minute incubation of PP with NEP, shown in figure 2.7. The number of peaks detected by MALDI-TOF MS increased when the incubation time was increased to 45 minutes. At 15 minutes, peaks with mass to charge ratios of 803, 1026 and 1324M/Z were detected. These masses are consistent with the PP fragments PP\textsubscript{30-35},
PP_{20-27}, and PP_{9-20}. At 45 minutes, peaks with mass to charge ratios of 803, 1026, 1049 and 1324M/Z were detected. These masses are consistent with PP and the PP fragments PP_{30-35}, PP_{20-27}, PP_{29-36} and PP_{9-20} (Figure 2.7).
Figure 2.5 Representative HPLC chromatograms of 2nmols PP incubated with 200ng NEP (black line) or buffer (red line) at 37°C for A) 15 B) 30 C) 60 and D) 120 minutes. Reactions were terminated with 5µl of 10%TFA and the supernatant injected onto HPLC with a Gemini C18 column and a gradient of 15-55% ACN (green line) run over 30 minutes.
Figure 2.6 Representative HPLC chromatograms of 2nmols PP incubated with 200ng NEP and an excess of phosphoramidon (black line) or with buffer (red line) at 37°C for A) 15 B) 30 and C) 120 minutes. Reactions were terminated with 5µl of 10%TFA and the supernatant injected onto HPLC with a Gemini C18 column and a gradient of 15-55% ACN (green line) run over 30 minutes.
Figure 2.7 Representative MS chromatograms of 2nmols of PP incubated with 200ng NEP for A) 15 minutes and B) 45 minutes at 37°C. Reactions were terminated with 5µl 10%TFA and the supernatant concentrated using a speed vac concentrator and frozen and sent for MALDI-TOF MS analysis. Vertical axis represents percentage (%) and the horizontal axis represents mass to charge ratio (M/Z). The arrows indicate the masses of possible PP fragments following breakdown by NEP.
2.4.1.4 Breakdown of PP by Meprin Beta

Following the incubation of PP at 37°C with meprin beta buffer alone for 30 minutes, a single peak with an elution time identical to that of PP freshly dissolved was detected. This single peak at a retention time of 25.5 minutes was also observed when PP was incubated with meprin beta enzyme for increasing amounts of time between 5-120 minutes (Figure 2.8). PP incubated with meprin beta for 5 minutes caused limited breakdown 0.9%. At 15 minutes this had increased to 39% breakdown, and at 45 minutes this had further increased to 65% (Figure 2.8). By 120 minutes nearly all PP had been degraded (92%) (Figure 2.8). In addition, a number of additional peaks with earlier retention times were observed. In total 4 major UV-absorbing fractions could be resolved following incubation of PP with 200ng of meprin beta enzyme for 30 minutes (figure 2.8). These fractions were observed at retention times of 15, 15.5, 19.5, and 25 minutes. PP, when incubated with meprin beta for 30 minutes, produced 50 ±8.33% breakdown compared to PP incubated with reaction buffer under the same conditions. This time was subsequently used for PP analogue incubations.

Further analysis of PP incubated with meprin beta using MALDI-TOF MS indicated that there were a number of breakdown products of PP detected following a 15 minute and 30 minute incubation with meprin beta. A single peak was observed at a mass to charge ratio of 4183M/Z following incubation of PP with buffer at both time points examined. This peak is consistent with the molecular weight of PP_{1-36}. A number of additional peaks were observed after a 15 minute incubation of PP with meprin beta, shown in figure 2.9. However, unlike PP incubations with NEP and KBB or DPPIV, incubation of meprin beta with buffer alone produced a number of peaks making the chromatograms of PP with meprin beta difficult to interpret (Figure 2.9). At 15 minutes peaks with mass to charge ratios of 1481, 1705, 2093 and 3260M/Z were detected. These masses are consistent with the PP fragments PP_{26-36}, PP_{23-35}, PP_{3-21} and PP_{10-36}. At 30 minutes, peaks with mass to charge ratios of 1704, and 3483M/Z were detected. These masses are consistent with the PP fragments PP_{23-35}, and PP_{4-33} (Figure 2.9).
Figure 2.8 Representative HPLC chromatograms of 2nmols of PP incubated with 200ng meprin beta (black line) or with buffer (red line) at 37°C for A) 15 B) 30 C) 60 and D) 120 minutes. Reactions were terminated with 5µl of 10%TFA and the supernatant injected onto HPLC with a Gemini C18 column and a gradient of 15-55% ACN (green line) run over 30 minutes.
Figure 2.9 Representative MS chromatograms of 2nmols of PP incubated with 200ng meprin beta for A) 15 minutes and B) 45 minutes at 37°C. Reactions were terminated with 5µl 10%TFA and the supernatant concentrated using a speed vac concentrator and frozen and sent for MALDI-TOF MS analysis. Vertical axis represents percentage (%) and the horizontal axis represents mass to charge ratio (M/Z). The arrows indicate the masses of possible PP fragments following breakdown by meprin beta.
2.4.2 The prevention of PP breakdown in vivo

2.4.2.1 The effects of PP administration in combination with inhibition of DPPIV on the food intake of C57/BL6 mice

PP significantly reduced food intake compared to saline at 0-1 hour; the DPPIV inhibitor valine pyrolidide (VP) did not significantly affect food intake when given with saline compared to saline alone (Saline 1.0 ±0.02grams, PP 0.55±0.04grams (p=≤0.001), VP 0.8±0.06grams). However, when PP was administered in combination with VP it significantly inhibited food intake when compared to saline control but significantly increased food intake when compared to PP alone (Saline 1.0 ±0.02grams, PP 0.55±0.04grams (p=≤0.001), PP+VP 0.75±0.03grams (p=≤0.01 and p=≤0.05)). There were no other significant effects on food intake at any other time-points where it was measured (Figure 2.10).
**Figure 2.10** The effect of SC administration of PP (50nmol/kg) with the DPPIV inhibitor valine pyrolidide (VP) (75mg, IP), saline or VP alone on food intake in C57/BL6 mice fasted overnight A) 0-1 hour post injection, B) 1-2 hours post injection, C) 4-8 hours post injection and D) 8-24 hours post injection. PP and PP with inhibitor compared to saline (black *) or compared to PP (red *) using one-way ANOVA with Bonferroni post hoc test *$p \leq 0.05$, **$p \leq 0.01$, or ***$p \leq 0.001$ (n=8-10).
2.4.2.2 The effects of PP administration in combination with inhibition of NEP on the food intake of C57/BL6 mice

PP significantly reduced food intake compared to saline at 0-1 hour; the NEP inhibitor phosphoramidon (P) did not significantly affect food intake when given with saline compared to saline alone (Saline 1.03 ±0.05grams, PP 0.54±0.06grams (p≤0.001), Phosphoramidon 0.92±0.07grams). When PP was administered in combination with P, it significantly inhibited food intake when compared to saline control (Saline 1.03 ±0.06grams and PP+P 0.69±0.07grams (p≤0.01)). There were no other significant effects on food intake at any other time-points where it was measured (Figure 2.11).
Figure 2.11 The effect of SC administration of PP (150nmol/kg) with the NEP inhibitor phosphoramidon (P) (1000mg, IP), saline or P alone on food intake in C57/BL6 mice fasted overnight A) 0-1 hour post injection, B) 1-2 hours post injection, C) 4-8 hours post injection and D) 8-24 hours post injection. PP and PP with inhibitor compared to saline using one-way ANOVA with Bonferroni post hoc test **$p \leq 0.01$, *** $p \leq 0.001$ (n=8/10).
2.4.2.3. The effects of inhibition of NEP on the plasma levels of PP in C57/BL6 mice following SC administration

Following a SC injection of PP in combination with an IP injection of either saline or the NEP inhibitor phosphoramidon plasma levels of PP were measured at 45 and 90 minutes post injection in C57/BL6 mice (Figure 2.12). At 45 minutes PP levels had risen to 11170±2237pmol/L in combination with saline and 30499±2801pmol/L in combination with phosphoramidon. The combination of PP with inhibitor significantly increased plasma PP levels compared to PP alone (p=≤0.01). At 90 minutes post injection, plasma PP levels had decreased from 45 minute levels to 5576±367pmol/L. Ninety minute plasma PP levels had also decreased from 45 minute levels when combined with a NEP inhibitor to 13938±668pmol/L. However plasma PP levels were still significantly higher in mice which received phosphoramidon than those which received saline (p=≤0.001) (Figure 2.12).
**Figure 2.12** The plasma levels of PP following SC administration of PP (25nmol/kg) in combination with either saline or the NEP inhibitor phosphoramidon (30mg, IP) in male C57/BL6 mice. Following SC injection of peptide and IP injection of saline or inhibitor, mice were returned to their cages and blood collected via cardiac puncture at A) 45 and B) 90 minutes. Plasma levels were compared using a one-way ANOVA with Bonferroni post hoc test (**=p≤0.01, ***=p≤0.001) (n=3).
2.4.3 The breakdown of PP analogues

2.4.3.1 The breakdown of PP analogues by KBB

Following the incubation of PP analogues at 37°C with KBB buffer alone for 30 minutes, a single peak with an identical elution time to that of each freshly dissolved PP analogue was detected. This single peak at a retention time of 24 minutes was also observed when the PP analogues were incubated with KBB membrane preparation for 30 minutes (Figure 2.13). GLP-1 was used as a positive control as it is known to be susceptible to a number of degradative enzymes found in the KBB (Stephenson and Kenny, 1987b; Stephenson and Kenny, 1987a; Vanneste et al., 1988). The KBB broke down 73±5.3% of GLP-1 (Figure 2.17).

There was no significant difference between the percentage breakdown of PP and PP analogues Val_{30} and Phe_{30} and Asn_{30} (PP, 66±14%, Val_{30} 68±2%, Phe_{30} 66±2.8%, and Asn_{30} 65±5%) (Figure 2.13, Table 2.1). The PP analogue Lys_{30} was broken down by 85±5% when incubated with KBB (Figure 2.13). The PP analogues Glu_{30} and Cys_{30} had improved resistance to breakdown by KBB than PP. However, this effect did not reach statistical significance (Glu_{30} 30±7% and Cys_{30} 47±3.5%) (Figure 2.13).
Figure 2.13 Representative HPLC chromatograms of PP analogues A) Val$_{30}$ B) Phe$_{30}$ C) Asn$_{30}$ D) Lys$_{30}$ E) Glu$_{30}$ and F) Cys$_{30}$ incubated with 1.5mg of KBB at 37°C for 30 minutes. PP analogues incubated with KBB are represented with a black line, and analogues incubated with buffer with a red line. Reactions were terminated with 5µl of 10%TFA and the supernatant injected onto HPLC with a Gemini C18 column and a gradient of 15-55% ACN (green line) run over 30 minutes.
2.4.3.2 The breakdown of PP analogues by DPPIV

As PP could not be separated from PP_{3-36} using reverse phase HPLC, analysis of PP analogues, breakdown by DPPIV was investigated using MALDI-TOF MS. The two analogues investigated were PP_{Ala 0} and PP_{2-36}

When PP_{Ala 0} and PP_{2-36} were incubated with DPPIV for 120 minutes at 37°C the mass to charge ratios detected by MALDI-TOF MS were consistent with the full length PP analogues (PP_{Ala 0} 4253M/Z and PP_{2-36} 4111KM/Z) (Figure 2.14).
Figure 2.14 Representative MALDI-TOF MS chromatograms of PP analogues A) Ala 0 and B) PP\textsubscript{2-36} incubated with 10mU of DPPIV enzyme at 37\degree C for 120 minutes. Reactions were terminated with 5µl of 10%TFA and the supernatant sent or MALDI-TOF MS analysis. The arrows indicate the masses of uncleaved PP analogues Ala 0 and PP\textsubscript{2-36} following incubation with DPPIV.
2.4.3.3 The breakdown of PP analogues by NEP

Following the 30 minute incubation of PP analogues at 37°C with NEP buffer, a single peak with an elution time identical to that of each PP analogue freshly dissolved was detected. This single peak at a retention time of 24 minutes was also observed when the PP analogues were incubated with NEP enzyme for 30 minutes (Figure 2.15). GLP-1 was used as a positive control as it is known to be susceptible to NEP (Hupe-Sodmann et al., 1995a). NEP broke down 75±9% of GLP-1, higher than the percentage breakdown observed for PP (Figure 2.17).

There was no significant difference between the percentage breakdown of PP and PP analogue Lys 30 (PP, 52±5.7%, and Lys 30 68±2%) (Figure 2.15, Table 2.1). The PP analogues Phe 30 and Cys 30 were less susceptible to breakdown by NEP than PP under the same conditions. However, this did not reach statistical significance (PP, 52±5.7%, Phe 30 36±3 % and Cys 30 27±7.2%) (Figure 2.15). The PP analogues Val 30 Asn 30 and Glu 30 had statistically significant improvements in their resistance to breakdown by NEP when compared to PP (PP, 52±5.7%, Val 30 16±6% (p≤0.05), Asn 30 32±0.6% (p≤0.05 and Glu 30 13±1.9% (p≤0.01))(Figure 2.15).
Figure 2.15 Representative HPLC chromatograms of PP analogues A) Val 30 B) Phe 30 C) Asn 30 D) Lys 30 E) Glu 30 and F) Cys 30 incubated with 200ng of NEP at 37ºC for 30 minutes. PP analogues incubated with NEP are represented with a black line, and analogues incubated with buffer with a red line. Reactions were terminated with 5µl of 10%TFA and the supernatant injected onto HPLC with a Gemini C18 column and a gradient of 15-55-% ACN (green line) run over 30 minutes.
2.4.3.4 The breakdown of PP analogues by Meprin beta

Following the incubation of PP analogues at 37°C with meprin beta buffer alone for 30 minutes, a single peak with an elution time identical to that of each PP analogue freshly dissolved was detected. This single peak at a retention time of 25 minutes was also observed when the PP analogues were incubated with meprin beta for 30 minutes (Figure 2.16). GLP-1 was used as a positive control as it is known to be unstable and susceptible to a number of degradative enzymes found in the kidney brush borders (Stephenson and Kenny, 1987b; Stephenson and Kenny, 1987a; Vanneste et al., 1988). Meprin beta broke down 44±13.2% of GLP-1, slightly lower than the percentage breakdown observed for PP (Figure 2.17).

There was no significant difference between the percentage breakdown of PP and PP analogues Cys$_{30}$ and Asn$_{30}$ (PP, 52±5%, Cys$_{30}$ 61±4.5% and Asn$_{30}$ 42±10.2%) (Figure 2.16, Table 2.1). The PP analogues Lys$_{30}$ and Val$_{30}$ had significantly decreased resistance to breakdown compared to PP when incubated with meprin beta (PP, 52±5%, Lys$_{30}$ 73±5%, and Val$_{30}$ 72±2.8%, (p≤0.05)) (Figure 2.16). The PP analogues Glu$_{30}$ and Phe$_{30}$ had significantly improved resistance to breakdown by meprin beta than PP (PP, 52±5%, Glu$_{30}$ 32±3.7% and Phe$_{30}$ 34±3.6%, (p≤0.01)) (Figure 2.16).
Figure 2.16 Representative HPLC chromatograms of PP analogues A) Val 30 B) Phe 30 C) Asn 30 D) Lys 30 E) Glu 30 and F) Cys 30 incubated with 200ng of meprin beta at 37°C for 30 minutes. PP analogues incubated with meprin beta are represented with a black line, and analogues incubated with buffer with a red line. Reactions were terminated with 5µl of 10%TFA and the supernatant injected onto HPLC with a Gemini C18 column and a gradient of 15-55-% ACN (green line) run over 30 minutes.
Figure 2.17 Representative HPLC chromatograms of GLP-1 with A) KBB membrane B) 200ng NEP and C) 200ng Meprin beta at 37°C for 30 minutes. GLP-1 incubated with enzyme or membrane is represented with a black line, and GLP-1 incubated with buffer with a red line. Reactions were terminated with 5µl of 10%TFA and the supernatant injected onto HPLC with a Gemini C18 column and a gradient of 15-55-% ACN run over 30 minutes.
Table 2.1 The percentage breakdown of PP analogues with amino acid substitutions at position 30 following incubation with KBB, NEP or meprin beta. Breakdown was calculated by comparing the area under the curve of peptide with and without enzyme/membrane (n= 3). Red indicates percentage breakdown higher than PP, blue indicates breakdown lower than PP *p≤0.05, **p≤0.01 compared to PP using Student’s T test.
2.5 Discussion

2.5.1 The breakdown of PP

The studies described in this chapter suggest that PP is a broken down via enzymes present on the KBB membrane. The specific KBB membrane enzymes investigated were DPPIV, NEP and meprin beta. These enzymes were chosen due to their involvement in the breakdown of other regulatory peptides, including other gut hormones (Medeiros and Turner, 1994a; Medeiros and Turner, 1994b; Mentlein et al., 1993a; Ahren et al., 2002; Drucker et al., 1997; Hupe-Sodmann et al., 1995; Kieffer et al., 1995; Mentlein, 1999). In particular, DPPIV, and NEP are known to have a role in the breakdown of PYY. PP and PYY are closely related; they differ by only 4 amino acids and share the PP-fold structural motif (Medeiros and Turner, 1994a; Medeiros and Turner, 1994b; Mentlein et al., 1993a; Cerda-Reverter and Larhammar, 2000; Larhammar, 1996a; Tatemoto et al., 1982; Nygaard et al., 2006). The results from section 2.3 suggest that PP is a substrate for all the enzymes investigated.

The KBB membrane was used as the initial method of investigating PP breakdown due to the high number of degradative enzymes located there (Bond et al., 1986; Carmago et al., 2002; Oneda et al., 2008; Bai, 1993; Vanneste et al., 1988). Previous work indicated that PYY is broken down by NEP and DPPIV from the KBB (Vanneste et al., 1988; Mentlein et al., 1993a; Mentlein et al., 1993b; Medeiros and Turner, 1994a). PP was broken down by enzymes on the KBB: 22% of the PP was broken down following 15 minute incubation with KBB membrane, and by 120 minutes there was almost complete breakdown of PP (98%).

MALDI-TOF MS indicated that PP had been cleaved at a number of positions after 10 or 30 minutes incubation with KBB. PP fragments 3-21 and 3-35 (2101M/Z and 4015M/Z) are the fragments that might be expected if PP was broken down by DPPIV, which cleaves the first two amino acids of small peptides with an Ala or Pro at position P1 and a hydrophobic amino acid at position P2 (Mentlein, 1999; Durinx et al., 2000; Lambeir et al., 2003). PP meets these requirements and is therefore potentially a good substrate for
DPPIV. It is possible that the DPPIV enzyme cleaves at position 2-3 first as this part of the PP amino acid sequence is on the end of the amino acid chain and is therefore putatively more exposed than the rest of the sequence (Blundell et al., 1981; Glover et al., 1983). This initial cleavage will disrupt tertiary structure of PP; the hair pin structure (PP-fold) of PP is created by interactions between the side chains of the proline helix (amino acids 1-8) and the side chains of the amino acids of the alpha helix (amino acids 15-32) (Li et al., 1992; Blundell et al., 1981; Cerda-Reverter and Larhammar, 2000; Glover et al., 1983). Specifically, the residues Pro 2 and Tyr 27, Pro 5 and Leu 24, Pro 8 and Met 17 and Pro 8 and Tyr 20 interact to stabilise the structure of PP (Blundell et al., 1981; Glover et al., 1983). The removal of the Pro at position 2 would therefore likely weaken the tertiary structure and might make the molecule susceptible to breakdown by other enzymes.

Figure 2.18 is a flowchart illustrating a putative sequence of KBB enzymatic breakdown of PP. Once the initial cleavage is made by DPPIV between amino acids 2 and 3, PP$_{3-36}$ may then be cleaved by NEP, meprin beta or other, as yet unknown enzymes, to generate the fragments 3-21, 23-36, 3-27 and 9-26. Fragments 3-21 and 9-26 are detected after only 10 minutes incubation, and therefore are likely to be generated most rapidly after the initial cleavage, and may represent the major breakdown fragments of PP$_{3-36}$. Fragment 18-26 is only observed following 30 minutes incubation with KBB and cannot be produced from cleavage at only 1 site. It is therefore likely to be a secondary or a tertiary breakdown fragment. It may be that PP$_{18-26}$ is produced following cleavage by either NEP or meprin beta at position 17-18. Amino acid 17 is a hydrophobic Met and therefore a substrate for both enzymes.

When PP was incubated with KBB in the presence of excess NEP inhibitor phosphoramidon there was no difference in the percentage breakdown compared to control incubations. This was unexpected, as fragments consistent with PP being cleaved by NEP were detected in the KBB PP incubations. This may be due to the inhibitor itself being broken down by the enzymes present on the KBB membrane, or perhaps due to other enzymes playing a larger role in PP breakdown if NEP is inhibited.
The use of KBB membrane proved useful in investigating PP breakdown. However, following PP KBB incubations it is difficult to assess which enzyme produced which breakdown fragment, and the sequence in which breakdown fragments are generated. It was therefore necessary to investigate specific enzymes, such as DPPIV and NEP, which are present in the KBB and which were hypothesised to generate these breakdown products in isolation.

Figure 2.18 A flowchart for the hypothesised sequence of PP breakdown when incubated with 1.5mg KBB protein at 37°C for 10 or 30 minutes. Purple text indicates primary cleavage, red text indicates a secondary cleavage and orange text indicates tertiary cleavage. Grey text indicates an unknown mechanism and timeframe.

The incubation of PP with KBB produced breakdown fragments consistent with PP being cleaved by the DPPIV enzyme. However, when PP was incubated with DPPIV and analysed using reverse phase HPLC, PP_{1-36} could not be resolved from PP_{3-36}, despite the use of a very shallow gradient (31.5-33.5% ACN over 35 minutes). Further analysis of DPPIV breakdown of PP was thus performed using MALDI-TOF MS. The column used in the HPLC analysis separates by hydrophobicity. This suggests that PP_{1-36} and PP_{3-36} have very similar hydrophobicity. The amino acids shown to be removed using MALDI-TOF MS were Ala and a Pro. These amino acids are not large and do not have large side groups, which explains why there was no
difference in hydrophobicity. The addition or removal of large or hydrophobic residues would potentially change the isoelectric point of the molecule and potentially change the ratio of hydrophobic to non-hydrophobic residues within the molecule. This would change how well the molecule is retained by the HPLC column and thus its retention time. An increase in the number of hydrophobic residues would ultimately increase the retention time of the molecule on the HPLC column, whereas an increase in the number of polar residues would reduce the retention time. PP1-36 has an isoelectric point of 7, and a ratio of hydrophobic to non-hydrophobic residues of 33%. PP3-36 has the same isoelectric point and a slightly higher ratio of hydrophobic to non-hydrophobic residues of 35%. This very small difference makes it difficult to separate the two molecules via reverse phase HPLC.

As previously discussed, the production of PP3-36 following the incubation of PP with KBB was thought to be likely due to the actions of DPPIV (Stewart and Kenny, 1984). DPPIV is also known to breakdown PYY, a closely related hormone from the same family (Grandt et al., 1993; Unniappan et al., 2006). It is possible that DPPIV provides the initial breakdown step, and that preventing breakdown by DPPIV could increase the length of time PP remains intact in circulation. The prevention of PP breakdown by DPPIV on the longevity of the anorectic effects of PP was thus investigated in fasted male C57/BL6 mice. The DPPIV inhibitor VP significantly enhanced the inhibition of food intake by PP at 0-1 hours, and appeared to extend the anorectic effects of PP to 1-2 hours, though the effect at this time point was not statistically significant. These data suggest that the prevention of breakdown of PP by DPPIV may increase the effectiveness of PP at reducing food intake.

NEP is another enzyme that may be responsible for the breakdown of PP. Following incubation with KBB, PP fragments 9-26, 3-21, and 18-26 were generated. These fragments are consistent with cleavage by NEP. These fragments must be secondary or tertiary fragments; they cannot be produced from a single cut in the amino acid sequence, and in accord with this, most were detected in longer incubations. When PP was incubated with NEP enzyme alone, a number of breakdown fragments were identified using
HPLC and MALDI-TOF MS. The fragments generated were different to those produced by incubation with KBB. This may be due to the combination of enzymes present in the KBB working together to produce different breakdown fragments.

Positions 30 and 21 appear susceptible to cleavage by recombinant NEP. The presence of the breakdown fragments 20-27 and 9-20 suggest NEP cuts the amino side of the hydrophobic Ala at position 21. However, positions 20 (Tyr) and 21 (Ala) help to stabilise the tertiary structure of PP by the interaction of their side chains with the Pro at position 8. This makes substitutions difficult, as they may disrupt the tertiary structure. Changes at position 30, which has a limited role within the tertiary structure, may be a more useful position at which to substitute amino acids to make enzymatic resistant PP analogues.

The effect of inhibiting NEP on the anorectic effects of PP was also investigated. It was observed that the addition of the NEP inhibitor phosphoramidon significantly enhanced the inhibition of food intake by PP at 0-1 hours and appeared to extend its anorectic effects to 1-2 hours, though this effect did not achieve statistical significance. Inhibition of NEP significantly increased the levels of PP detected by RIA at both 45 minutes and 90 minutes after PP injection. At 45 minutes, animals which received phosphoramidon had plasma PP levels nearly three times higher than those animals which received a saline control. By 90 minutes, levels of PP had begun to decline in both sets of animals. However, animals which received phosphoramidon still had plasma PP levels double those of controls. This suggests that PP is broken down via NEP in vivo and that the prevention of NEP breakdown can significantly increase the persistence of PP in the plasma. Phosphoramidon has been shown to be a specific NEP inhibitor and has been used previously to investigate NEP both in vivo and in vitro (Oefner et al., 2000). When phosphoramidon was given at a large dose (1000mg) to fasted male C57/BL6 mice, food intake was increased at 1-2 hours. A much lower dose of phosphoramidon was therefore used in the plasma PP experiment (30mg) following a dose finding study (data not shown due to small n number), to avoid the confounding effects of these high doses.
The studies described in this chapter suggest that the enzyme meprin beta breaks down PP, but that it is less important than DPPIV and NEP. When PP was incubated with KBB, the majority of the breakdown fragments detected by MALDI-TOF MS could be attributed to either DPPIV or NEP. PP incubated with meprin beta for 30 minutes produced 50% breakdown and four breakdown peaks, when examined using HPLC. PP incubated with NEP under the same conditions produced 52% breakdown but seven breakdown peaks. This suggests that although meprin beta breaks down PP; PP is a better substrate for NEP than it is for meprin beta.

The incubations with meprin beta produced unexpected peaks with molecular weights suggesting they were not products of PP breakdown. Meprin beta incubated alone in buffer for 15 or 30 minutes resulted in a large amount of background noise on the MS chromatogram. This may be due to enzyme instability. Meprin beta may have been broken down by the temperature of the incubations (37°C) or by the 10% TFA acid used to terminate the reaction. Those masses which corresponded to PP fragments indicated that, as previously suggested (Bond et al., 1986; Butler et al., 1987), meprin beta cleaves at amino acids which are either hydrophobic or which are flanked by hydrophobic amino acids. More PP fragments were detected using the MALDI-TOF MS following 15 minutes incubation than following 30 minutes incubation. This is again unexpected as when PP was incubated with 200ng meprin beta for 15 minutes, only 39% breakdown was detected using HPLC. This increased to 50% when incubated for 30 minutes. A single similar fragment of PP was detected when PP was incubated with KBB that was also detected when PP was incubated with meprin beta. However, these two fragments differ by an amino acid. PP when incubated with KBB generated PP_{26-35} and when incubated with meprin beta it generated PP_{26-36}. This last amino acid may have been cleaved in the KBB incubation by another enzyme.

The experiments detailed in section 2.4 suggest that PP is broken down by enzymes located on the kidney brush border membrane, specifically the enzymes DPPIV, NEP and meprin beta. In experiments where enzyme inhibitors were used in combination with PP, the effects of the inhibition on
food intake in fasted male C57/BL6 mice and the plasma levels of PP were enhanced up to 2 hours. This suggests that the inhibition of PP breakdown will increase the anorectic effects of PP. Analogues of PP which have had susceptible amino acids substituted may therefore have greater enzymatic resistance and thus greater efficacy at reducing food intake \textit{in vivo} compared to unmodified PP.

### 2.5.2 The breakdown of PP analogues

Analogues of PP were designed with amino acid substitutions at the N-terminus to prevent breakdown by the enzyme DPPIV.

The PP analogues PP\textsubscript{2-36} and Ala 0 were shown to be resistant to the enzyme DPPIV. The addition of an amino acid (Ala) or removal of the first amino acid, Ala changes the position of the susceptible Pro at position P1. The addition or removal of an amino acid at the N terminus of peptides has previously been shown to help prevent breakdown by DPPIV (Mentlein, 1999; Druce et al., 2009). The amino acid Pro at position 2 is essential for the correct binding of PP to the hY4 receptor (Gehlert et al., 1996). Therefore, the prevention of cleavage at amino acid 2/3 should aid biological function. This suggests that any PP analogues with either an amino acid addition or substitution at the N-terminus may be more resistant to breakdown by DPPIV and therefore possibly more effective than PP at reducing food intake.

PP analogues with amino acid changes at position 30 were designed to prevent breakdown by NEP. As described in section 2.5.1, amino acids 20, 21 and 30 are sites thought to be susceptible to breakdown via NEP. However, substitutions to amino acids 20 and 21 would be predicted to compromise the correct folding of PP’s tertiary structure (Blundell et al., 1981; Glover et al., 1983), and thus the ability of the analogues to bind to the Y4 receptor.

PP analogues with amino acid substitutions at position 30 were designed. Two replaced the hydrophobic Met with polar uncharged amino acids (Cys or Asn). Another two analogues exchanged the hydrophobic Met for alternative hydrophobic amino acids (Phe or Val). This was done to assess whether
different hydrophobic amino acids were more or less susceptible to NEP. The remaining two PP analogues replaced the hydrophobic Met with either an acidic (Glu) or a basic (Lys) amino acid at position 30.

Substituting Met at position 30 for the basic amino acid Lys increased the percentage breakdown detected in both KBB and meprin beta incubations, and had no effect on breakdown by NEP. Lys is not present in the native PP sequence and only appears once in the sequences of NPY and PYY, at position 4 (Cerda-Reverter and Larhammar, 2000; Larhammar, 1996a). This may be due to the fact that it disrupts the tertiary structure of this group of peptides. In theory the removal of a hydrophobic amino acid and its replacement with a non-hydrophobic amino acid should have increased resistance to breakdown by both NEP and meprin beta (Hupe-Sodmann et al., 1995; Medeiros and Turner, 1994a; Turner et al., 2001; Roques et al., 1993). However, the addition of a Lys may have changed the packing of the amino acids surrounding amino acid 30. Amino acids 15 to 32 form an alpha helix, and the addition of a Lys at position 30 may have disrupted the packing, or increased or decreased the turn within the helix (Blundell et al., 1981; Glover et al., 1983). This may have exposed specific amino acids that under normal conditions would have been protected by the packing of the structure to meprin beta and other enzymes on the KBB, and thus caused the increased breakdown.

Changing the hydrophobic amino acid to a charged Lys had a detrimental effect on breakdown. However, the substitution of the Met at position 30 with the acidic amino acid Glu, decreased breakdown of the Glu_{30} analogue compared to PP in all three preparations. This effect was statistically significant for NEP and meprin beta. Aside from the difference in charge, Lys and Glu are similar in size, though Lys is longer than Glu. This difference in length may mean that Glu does not disrupt the packing of the side chains as much as Lys, therefore conferring resistance to breakdown by degradative enzymes without impairing the tertiary structure.

Substituting a Phe at position 30 conferred some resistance to breakdown by the enzymes NEP and meprin beta. This was surprising, as both NEP and
meprin beta requires hydrophobic residues as substrates (Bond et al., 1986; Butler et al., 1987). The replacement of the hydrophobic Met at position 30 for another hydrophobic amino acid would therefore have been predicted to have little effect on enzyme resistance. The phenol side group of the Phe amino acid may have impaired both NEP and meprin beta cleavage at this point, despite its hydrophobicity. The replacement of Met at position 30 with another hydrophobic residue (Val) had a different effect to replacement with Phe. PP analogue Val$_{30}$ had increased breakdown with meprin beta, and decreased breakdown when incubated NEP, but showed no change in breakdown profile when incubated with KBB membrane. Val is a smaller amino acid than Met, and also lacks the sulphur containing side chain of Met. This may mean that it decreases the turn in the alpha helix around position 30, protecting it from breakdown by NEP (Blundell et al., 1981; Glover et al., 1983). Meprin beta has a larger number of potential substrates in the PP molecule; it can cleave at hydrophobic amino acids and at those amino acids flanked by hydrophobic amino acids (Bond et al., 1986; Butler et al., 1987). This may mean that despite preventing breakdown at position 30 with the substitution of Met for Val, overall breakdown by meprin is increased because other hydrophobic amino acids or amino acids flanking hydrophobic amino acids are exposed to its actions.

The PP analogues Cys$_{30}$ and Asn$_{30}$ had increased resistance to breakdown by NEP compared to native PP. This was expected, as the potential substrate amino acid was replaced with an uncharged polar amino acid. However, Cys or Asn at position 30 had limited effects on breakdown by either meprin beta or by the enzymes on the KBB membrane. This may be because of the greater number of potential targets for meprin beta, and the large number of possible enzymes on the KBB membrane (Butler et al., 1987). Substituting a single amino acid may therefore have little effect on meprin beta or KBB resistance.

In summary, a number of the PP analogues investigated in this section were shown to have increased resistance to breakdown by the KBB membrane preparation and by the enzymes DPPIV, NEP and meprin beta. Further analogues of PP could also be developed by combining amino acid
substitutions. This may confer resistance to multiple enzymes. However, it is not known if these small amino acid substitutions compromise biological function. The substitution of multiple amino acids is more likely to impair binding to the Y4 receptor. Further investigation is therefore required into the ability of these analogues to both bind to the Y4 receptor and inhibit food intake *in vivo*.
Chapter 3

Pancreatic Polypeptide Analogues: hY4 Receptor Binding Affinity And Acute Effects On Food Intake In Mice.
3.1 Introduction

Resistance to degradation by proteolytic enzymes is not the only consideration for the development of a peptide hormone drug. It has previously been reported that amino acid substitutions at specific sites in the oxyntomodulin molecule can prevent degradation by the enzymes DPPIV and neprilysin 24.11 (Druce et al., 2009). For this series of studies on oxyntomodulin analogues, in addition to the amino acid substitutions designed to aid resistance to enzymatic degradation, additional modifications were made to improve the molecule’s affinity for the GLP-1 receptor. Other groups have used primary sequence modifications both to promote degradative enzyme resistance and to improve drug pharmacokinetic profiles (Tomita et al., 2008). Peptide analogues have been used to investigate structure-activity relationships for peptide hormones, including growth hormone releasing hormone (GHRH), CCK and somatostatin (Danho et al., 1992; Rosenthal et al., 1983; Tang et al., 2009). The studies described in the previous chapter demonstrated that individual amino acid changes in the PP primary structure can aid resistance to the breakdown caused by tissue membranes, such as the kidney brush border, and by purified enzymes, such as neprilysin 24.11. Additional modifications to improve affinity for the human hY4 receptor could increase the potential of PP based drugs as anti-obesity treatments.

3.1.1 Analogue development to increase the longevity effect on peptide receptor interaction

Short chain peptides are generally considered to be more susceptible to breakdown by circulating and membrane bound endopeptidases than larger peptide chains, which often posses more stable tertiary and/or quaternary structures (Hupe-Sodmann et al., 1995b). The addition of extra residues to either the C- or N terminus of a peptide may therefore aid its resistance to breakdown by such enzymes. The main disadvantage of this method is that these additional residues may disrupt the overall tertiary structure of the peptide and consequently impair its ability to activate its receptor (Pauling et al., 1949). In the previous chapter, it was described how substituting specific amino acids in the PP sequence could confer relative resistance to
enzymatic breakdown. However, the effects of these substitutions on the binding affinity of these molecules for the hY4 receptor were not investigated.

Modifications to the amino acid sequence may enhance or impair the binding of PP to other Y receptors. Y1 and Y5 receptors are mainly located in the brain (Parker and Herzog, 1999). Given the inability of PP to cross the blood brain barrier (Whitcomb et al., 1990), binding to the Y1 and Y5 receptors is unlikely to result in PP analogues having biological side effects. However, the Y2 receptor is found in similar regions to the Y4 receptor (Dumont et al., 1993) where there is an incomplete blood brain barrier. Any modifications made to the structure of PP which increased affinity for the Y2 receptor might therefore cause side effects, particularly given that Y2 receptor agonist administration can cause nausea (Le Roux et al., 2008). This would make drug development difficult due to compliance issues. It is therefore necessary to determine the effects of modifications to PP structure on both hY4 receptor and hY2 receptor binding.

3.1.2 Analogue development to increase the longevity of the anorectic effect of PP on food intake in male C57/BL6 mice

The amino acid modifications made to improve resistance to degradative enzymes and improve affinity for the hY4 receptor were initially investigated in vitro. It is necessary to determine the effect of these modifications on PP activity in vivo; this can be assessed by determining the effect of the analogues on food intake. Amino acid modifications have been used previously to improve the anorectic effect of a peptide hormone efficacy (Druce et al., 2009) and to investigate structure activity relationships (Caporale et al., 2009; Danho et al., 1992; Rosenthal et al., 1983). PP inhibits food intake in a dose dependent manner for up to 4 hours post IP injection in mice (Asakawa et al., 1999; Kojima et al., 2007; Taylor and Garcia, 1985; Nakajima et al., 1994; Liu et al., 2008), and for up to 24 hours post IV infusion in humans (Batterham et al., 2003; Jesudason et al., 2007). A PP based drug treatment for obesity administered daily or weekly, would be a relatively easy treatment regimen for patients to comply with, and would have a lower cost than more frequently administered peptides. Therefore,
developing a PP analogue which reduced food intake for a longer period than unmodified PP may identify a potentially more effective obesity treatment.

3.1.3 The development of PP analogue XPP and its circulating half life in rodents

PP analogue XPP is more resistant to enzymatic degradation (Appendix IV) than unmodified PP. Analogue XPP has a number of amino acid substitutions which aid its enzymatic resistance. Amino acid changes may also have increased its plasma half life and consequently the longevity of its inhibitory effect on food intake.
3.2 Hypothesis and Aims

PP has a high affinity for the Y4 receptor, and a limited affinity for other Y receptors (Parker et al., 2002). Amino acid substitutions at key sites throughout the PP molecule could help to improve its binding to the hY4 receptor, and ultimately improve the utility of PP as an obesity treatment. The subcutaneous administration of native PP has a well characterised inhibitory effect on food intake in fasted mice (Asakawa et al., 1999; Asakawa et al., 2003; Nakajima et al., 1994). Comparing the effects of PP analogues on food intake to that of native PP will determine whether specific amino acid changes improve or impair bioactivity.

**Hypothesis:** I hypothesise that the substitution of amino acids at key sites in the PP molecule to increase resistance to enzymatic degradation, (section 2.4), and will generate specific PP analogues with a higher affinity for the hY4 receptor and a greater inhibitory effect on food intake in mice than native PP.

**Aims:**

1. To investigate the effect of extending the PP sequence at the N-terminus by a single amino acid and the substitution of amino acids at positions 17, 19, 23 and 30 on the binding affinity of PP analogues for the hY4 and hY2 receptors.
2. To compare the effect of these PP analogues on food intake in mice compared to native PP.
3. To determine the circulating levels of a specific PP analogue, XPP, achieved following subcutaneous administration in mice and rats.
3.3. Materials and Methods

3.3.1 Preparation of membrane from cells over-expressing human Y4 (hY4) receptors

Membrane preparations were made from the HEK 293T cells over expressing the hY4 receptor. Cells were gifted by Dr J Minnion. Cell membrane preparations were made from cells between passage numbers 15 and 35.

HEK 293T cells were cultured in Dulbecco’s modified medium (DMEM, Invitrogen LTD, Paisley UK) supplemented with 10% foetal bovine serum (FBS) (Invitrogen LTD, Paisley UK) and 1% antibiotic (Penicillin (100units/ml) and Streptomycin (100µg/ml), Invitrogen LTD, Paisley UK). Medium was changed every 2-3 days and the cells sub-cultured when 70% confluent using a non-enzymatic cell dissociation buffer (Sigma-Aldrich). The medium was removed from the flask and 2 ml cell dissociation buffer (Invitrogen LTD, Paisley UK) added. Cells were incubated at room temperature until they detached from the flask. Five millilitres of fresh medium was added to each flask and cells recovered by centrifugation for 5 minutes at 100g. The cells were resuspended in fresh medium and transferred to a new flask at a dilution of 1:5.

A minimum of 30 confluent, 175cm² flasks were used for each membrane preparation. The culture medium was removed and the cells detached from the flasks using ice cold 0.02 M phosphate buffered saline (PBS) (Sigma-Aldrich) and scraping. Cells were centrifuged at 2000g for 5 minutes at 4°C, the supernatants discarded and the pellets put onto ice. Pellets were then resuspended in 20 ml of ice-cold 1mM HEPES buffer containing the enzyme inhibitors aprotinin (100KIU/mL), pepstatin (0.5µg/mL), antipain (0.5µg/mL), leupeptin (0.5µg/mL), benzamidine (0.1µg/mL) and SBTI (10µg/mL), and cells homogenised for 1 minute with an Ultra Turrax. Homogenates were further centrifuged at 100000g for 30 minutes at 4°C. Supernatants were discarded and pellets resuspended in homogenisation buffer (0.1M PBS) without sucrose using a hand-held Teflon homogeniser (Potter, Elvehjem). Finally, pellets were resuspended at a final protein concentration of 1-2
mg/ml and stored at -70°C. Protein concentration was measured using Bradford assay. A standard curve was set up using known concentrations of bovine serum albumin protein (Sigma–Aldrich) (0, 0.25, 0.5, 1.0, 1.5 mg/ml) and 0.1 mL was added to 3 mL of Bradford reagent. The solution (0.1ml) of purified hY4 receptors was also added to 3ml of Bradford reagent. All solutions were incubated at room temperature for 45 minutes. The absorbance of each solution at 595nm was measured using a spectrophotometer (WPA Biotech Photometer, UV 1101). A standard curve was then constructed using the correlation of the known absorbance values, and the protein content of the unknown samples interpolated from the standard curve.

3.3.1.1 Receptor binding assay

Analogues of PP were tested for their binding affinity for the hY4 receptor. The amino acid sequences of the PP analogues are shown in table 3.1. Assays were carried out in siliconised eppendorfs in assay buffer containing HEPES (0.02M), CaCl₂ (5mM), and MgCl₂ (1mM). The total volume was 500µl per tube. A standard curve was constructed using known values of unlabelled peptide (200, 10, 2, 0.25, 0.1, 0.05, 0.01, 0.001, 0.0001 pmol). Each tube contained 1000 counts per second of radiolabelled PP (see section 3.3.1.1.1) and 10µl of hY4 membrane (1.67mg/ml). Tubes were incubated for 90 minutes at room temperature. They were then spun in a microcentrifuge at 1500g for 3 minutes, and the supernatant discarded. Pellets were then resuspended in 500µl assay buffer, spun as above, and the supernatant once again discarded. The resulting pellets were counted in a NE 1600 γ counter (NE Technology Ltd, Reading) for 240 seconds. The inhibition coefficient (IC₅₀) was then determined for PP and each PP analogue tested. This was done by reading off the competition binding curve the concentration of unlabelled peptide which displaced 50% of the specific radiolabelled peptide. All curves were performed with points in triplicate. IC₅₀ values were calculated using Prism (Version 4) (GraphPad Software Inc.) and values represented as +/- SEM of the IC₅₀.
3.3.1.1 Preparation of $^{125}$I labeled pancreatic polypeptide

Synthetic human PP (Bachem UK Ltd.) was iodinated using the Bolton and Hunter method (Bolton and Hunter, 1973). Briefly, 5 nmol PP in 50μl 0.1 M borate buffer (pH 8.2) was added to 1 mCi dried Bolton and Hunter reagent (Amersham International, UK). The reaction was incubated at room temperature for 90 mins and the iodinated peptide separated by reversed phase HPLC on a C18 (Waters, Milford, USA.) column using a 10-40% gradient of acetonitrile (AcN)/water/ (vol/vol) acidified with 0.1% trifluoroacetic acid (TFA) over 90 minute. Purified fractions which bound with high affinity to PP antibodies with low non specific binding (NSB) were selected for use in the assays.

3.3.2 Animal studies

All animal procedures were approved by the British Home Office under the UK Animal (Scientific Procedures) Act 1986 (Project Licence 70/6402).

Adult male C57/BL6 mice (Harlan) weighing 22-28g were maintained either in individual cages (feeding studies), or group housed in cages of up to 5 animals (pharmacokinetics studies) under controlled temperature (21-23°C) and lights (12:12 hour light dark cycle lights on at 0700). Adult male Wistar rats (Charles River) weighing 130-180g were maintained in group houses in cages of 5 animals under the same controlled conditions. All animals were allowed ad libitum access to water and RM1 diet (Special Diet Services, Witham, UK) unless otherwise stated. Animals were handled and received sham SC injections of saline on at least two occasions prior to study days in order to acclimatise them to the injection procedure.

3.3.2.1 Acute feeding studies in mice

Feeding studies were carried out during the early light phase (0800-1000) in mice fasted from 1600 the preceding day. Mice following a fast have a greater food intake than their non-fasted counterparts, and are therefore more likely to demonstrate an anorectic effect. Peptides were administered subcutaneously (SC) in a maximum volume of 0.1 ml of saline. Animals were weighed and their body weight recorded before injection. After injection the mice were returned to their home cage with a known amount of food. Food
was reweighed at 1, 2, 4, 8 and 24 hours. Food was removed from the hopper, a visual inspection of the cage made to check for any food spillage/hoarding, and food weighed using balances accurate to 0.01 g. Mouse body weight was measured 24 hours post-injection. All mice feeding studies were completed in conditions where external stressors were minimised, as stress is known to influence feeding behaviour (Marti et al., 1994). A dose of 25nmol/kg was chosen as the starting dose for PP and its analogues as it has been shown to be effective at inhibiting food intake in previous studies (Asakawa et al., 1999; Liu et al., 2008). If 25nmol/kg of PP analogue was ineffective at reducing food intake the dose was increased until food intake was significantly inhibited, up to 500nmol/kg. The amino acid sequences of the PP analogues can be found in table 3.1.

### 3.3.2.2 Pharmacokinetic studies (PK)

Animals were injected with a maximal volume of 0.1ml SC using a 1ml syringe with a 30G needle (Insulin syringe) (Termuo, Elkton, USA). The volume injected was adjusted depending upon the weight of the animal so that each received 50nmol/kg. This dose was used as it has been shown to inhibit food intake in mice (Asakawa et al., 1999; Liu et al., 2008) and should enable plasma levels of PP or its analogues to be detected via radioimmunoassay (RIA) at all time points.

### 3.3.2.2.1 Pharmacokinetics in mice

Mice were housed in groups of up to 5 animals. Animals were weighed and their tails marked, allowing for later identification.

Mice were injected SC with either 50nmol/kg of PP or the PP analogue XPP and allowed to return to their cage of origin with free access to food and water. Animals were then culled at 0, 5, 10, 20, 30 45, 90, 120 and 180 minutes post injection by inhalation of carbon dioxide (a schedule 1 method). Once the animals had been culled, cardiac puncture was performed by inserting a 27 gauge needle attached to 1 ml syringe (heparinised using a 100 I.U/ml heparin solution) into the chest cavity and puncturing the heart muscle, and blood removed. These time points were concentrated before 45 minutes due to PP’s short circulating half life of around 7 minutes (Adrian et
al., 1978) and to establish the peak plasma concentration of PP or its analogue following SC administration. Once a peak plasma level was detected the later time points (90, 120 and 180 minutes) were used to give an indication of the time taken for clearance from the plasma.

Blood samples were dispensed into 1ml eppendorf tubes containing 20µl Trasylol (Nordic Pharma, UK). The tubes were then spun in a centrifuge (3-18K SciQuip, Sigma, USA) for 10 minutes at 3000g at room temperature. The plasma supernatant was collected and stored in an eppendorf at -80°C until assay.

3.3.2.2 Pharmacokinetics in rats

Male Wistar rats were anaesthetised with an intraperitoneal (IP) injection of Hypnorm/Hypnovel at a dose of 2.7ml/kg (0.315mg Fentanyl with 10mg fluanisone/5mg/ml midazolam). Animals were monitored regularly throughout the procedure to ensure the correct level of anaesthesia was maintained.

Cannula tubing was prepared before the start of the cannulation. Portex tubing (internal diameter (ID) 0.86mm, outside diameter (OD) 1.27mm) was cut to 10 cm length. One end had a 1ml syringe with a 27 gauge needle attached to it. A 1ml syringe was filled with heparinised saline (100 I.U/ml) and attached to the needle. The other end of the tube was cut with a scalpel blade to create a bevelled edge.

Once animals were anesthetised they were placed on a heat mat covered with a drape. The animal was orientated with its back on the mat and its head facing the person performing the cannulation. The skin from the neck to the paw on the right side was removed using scissors. Any peripheral fat was removed by blunt dissection. Blunt dissection was then used to clean the right jugular vein of fat and excess tissue until the vein was easily visible. The salivary gland was held out of the area of the vein using hemostats (Fine Science Tools, Heidelberg, Germany) and forceps (Fine Science Tools, Heidelberg, Germany) passed under the vein to remove any remaining connective tissue.
Ideally a 1cm section of vein was visible. A doubled up piece of cotton thread was passed under the vein, and cut to provide 2 threads. One of these threads was moved to the rostral end of the vein and tied tightly to completely occlude the vein. The other thread was moved caudally towards the heart and a loose knot made. The vein was put under slight tension by pulling the thread occluding the vein and a small cut was made in the vein between the 2 pieces of thread. The pre-prepared tubing was inserted into the cut, and the loosely knotted thread tightened to hold the cannula in place. The needle was gentle drawn back to allow approximately 100µl of blood to fill the tube and then 100µl of heparinised saline injected to ensure that the vein had been correctly cannulated.

When blood samples were taken, the saline filled syringe was replaced with an empty 1ml syringe. This was gently drawn back to remove 200ul of blood. Once this was collected the saline filled syringe was reattached, ensuring there were no air bubbles in the tubing, and 50µl of heparinised saline was then injected to help prevent blood clotting within the cannula. Once the time zero blood sample was taken animals were SC injected with 50nmol/kg or PP or analogue XPP, and blood samples taken at 10, 20, 30, 45, 60, 120 and 180 minutes.

Blood samples were dispensed into 1ml eppendorf tubes as described in section 2.3.3.2.1. The plasma supernatant was collected and stored in an eppendorf at -80°C until assay.

Once all the necessary samples had been collected the animals were terminally anesthetised using 1ml (IP) of 30mg/kg Pentobarbitone (Animal Care Ltd).
2.3.4 Statistical Analysis

The results from acute feeding studies were compared using a one-way ANOVA with post hoc Bonferroni test. Plasma pharmacokinetic studies were analysed using the two-way ANOVA with Bonferroni post hoc test comparing the two treatments groups at each time point. A repeated measures one-way ANOVA with post hoc Bonferroni test was also used to compare time points within the same treatment group. Receptor binding studies were completed a minimum of 3 times for each peptide and the mean and S.E.M IC\textsubscript{50} calculated, and significance compared to native PP calculated using Student’s T test. A \( p \) value of less than 0.05 was considered to be significant in all analyses.
<table>
<thead>
<tr>
<th>PP/PP Analogue</th>
<th>Amino acid sequence</th>
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**Table 3.1** The amino acid sequences of PP and its analogues. Amino acid additions and substitutions are highlighted in red. The amino acid structure of XPP is detailed in appendix IV.
3.4 Results

3.4.1 The effect of amino acid substitutions on binding affinity to the hY4 receptor

3.4.1.1 The effect of amino acid substitutions at position 30 on binding affinity to the hY4 receptor

In hY4 competition receptor binding assays, native human pancreatic polypeptide displaced PP$^{125}$ with nanomolar affinity ($IC_{50}$ 0.230 ± 0.03nM) (Figure 3.4).

The native sequence of human PP contains the amino acid methionine (Met/M) at position 30. Substituting a Phenylalanine (Phe/P) or a Glutamic acid (Glu/E), at this position (Figure 3.1) significantly impaired binding to the hY4 receptor compared to PP (Phe 30 $IC_{50}$ 0.66 ± 0.013nM ($p \leq 0.01$)), Glu 30 $IC_{50}$ 0.36 ± 0.02nM ($p \leq 0.01$)). Substitution with Cysteine (Cys/C) or Asparagine (Asn/N) at position 30 (Figure 3.1) had no effect on the analogue's affinity for the hY4 receptor (Cys 30 $IC_{50}$ 0.23 ± 0.01nM, Asn 30 $IC_{50}$ 0.23 ± 0.02nM) (Figure 3.1). Substitution with either a Lysine (Lys/K) or Valine (Val/V) at position 30 (Figure 3.1) improved the analogue’s affinity for the hY4 receptor (Lys 30 $IC_{50}$ 0.12 ± 0.02nM, ($p \leq 0.01$), and Val 30 $IC_{50}$ 0.10 ± 0.04nM, ($p \leq 0.5$)) (Figure 3.1).

3.4.1.2 The effect of amino acid substitutions at positions 0 and 30 on binding affinity to the hY4 receptor

The addition of Alanine (Ala/A) at the 0 position in combination with a Threonine (Thr, T), at position 30 (Figure 3.2) improved binding affinity to the hY4 receptor (Ala 0, Thr 30 $IC_{50}$ 0.15 ± 0.01nM, ($p \leq 0.01$)). The combination of an Alanine (Ala/A) extension at the N terminus with a Leucine (Leu/L), at position 30 (Figure 3.2) had no effect on binding affinity to the hY4 receptor (Ala 0, Leu 30 $IC_{50}$ 0.27 ± 0.05nM). The combination of a Pro at position 0 of PP with a Leu at position 30 had no effect on binding affinity to the hY4 receptor (Pro 0, Leu 30 $IC_{50}$ 0.27 ± 0.03nM) (Figure 3.2). The combination of a Pro at position 0 with a Lysine (Lys, K) at position 30 improved binding
affinity to the hY4 receptor (Pro 0, Lys 30 IC\textsubscript{50} 0.10 ±0.001nM (p≤0.05)) (Figure 3.2).

3.4.1.3 The effect of amino acid substitutions at positions 0, 17 and 30 on binding affinity to the hY4 receptor

The substitution of amino acid Met at position 17 for a Leu, in addition to the addition of Ala at position 0 and the substitution of Met for Leu at position 30 had no affect on the binding of the analogue Ala 0, Leu17, Leu 30 to the hY4 receptor compared to native PP (IC\textsubscript{50} 0.23 ±0.003nM) (Figure 3.3). However, the substitution of Met 17 for an Isoleucine (Ile, I) in addition to Ala at position 0 and Cys at position 30, significantly improved binding to the hY4 receptor (IC\textsubscript{50} 0.13 ±0.001nM, (p≤0.001)) (Figure 3.3).

3.4.1.4 The effect of amino acid substitutions at multiple positions on binding affinity to the hY4 receptor

See appendix IV for XPP hY4 receptor binding information (Figure 3.4).

3.4.2 The effect of amino acid substitution on binding affinity to the hY2 receptor

In displacement human Y2 (hY2) receptor binding assays, native human PP displaced PP \textsuperscript{125} with nanomolar affinity (IC\textsubscript{50} 0.12 ±0.06nM) (Figure 3.5). However, PP only displaced PYY\textsubscript{3-36} \textsuperscript{125} with micromolar affinity (IC\textsubscript{50} 218 ±63nM). Analogue XPP (amino acid changes detailed in appendix IV) has a similar binding affinity for the hY2 receptor (IC\textsubscript{50} 0.036 ±0.1nM).
Figure 3.1 Representative competition binding affinity curves of PP analogues A) Asn 30, B) Cys 30, C) Glu 30, D) Lys 30, E) Val 30 and F) Phe 30 to the hY4 receptor. Radiolabelled PP I\textsuperscript{125} was used to compete with unlabelled peptide for hY4 receptor binding sites. Cell membranes of HEK 293T cells over expressing the cloned hY4 receptor were the source of hY4 receptors. Fifty percent specific binding is represented by the dotted line. IC\textsubscript{50} values were calculated as mean ±SEM of three separate experiments (Table 3.2).
Figure 3.2 Representative competition binding affinity curves of PP analogues A) Ala 0, Leu 30 B) Ala 0, Thr 30 C) Pro 0, Leu 30 D) Pro 0, Lys 30 to the hY4 receptor. Radiolabelled PP$^{125}$ was used to compete with unlabelled peptide for hY4 receptor binding sites. Cell membranes of HEK 293T cells over expressing the cloned hY4 receptor were the source of hY4 receptors. Fifty percent specific binding is represented by the dotted line. IC$^{50}$ values were calculated as mean ±SEM of three separate experiments (Table 3.2).
Figure 3.3 Representative competition binding affinity curves of PP analogue A) Ala 0, Ile 17, Cys 30 and B) Ala 0, Leu 17, Leu 30 to the hY4 receptor. Radiolabelled PP I125 was used to compete with unlabelled peptide for hY4 receptor binding sites. Cell membranes of HEK 293T cells over expressing the cloned hY4 receptor were the source of hY4 receptors. Fifty percent specific binding is represented by the dotted line. IC50 values were calculated as mean ±SEM of three separate experiments (Table 3.2).

Figure 3.4 Representative competition binding affinity curves of A) native PP B) PP analogue XPP to the hY4 receptor. Radiolabelled PP I125 was used to compete with unlabelled peptide for hY4 receptor binding sites. Cell membranes of HEK 293T cells over expressing the cloned hY4 receptor were the source of receptors. Fifty percent specific binding is represented by the dotted line. IC50 values were calculated as mean ±SEM of three separate experiments (Table 3.2).
Figure 3.5 Representative competition binding affinity curves of A) native PP B) PP analogue XPP to the hY2 receptor. Radiolabelled PP $^{125}$I was used to compete with unlabelled peptide for hY2 receptor binding sites. Cell membranes of HEK 293T cells over expressing the cloned hY2 receptor or hY2 receptor were the source of receptors. Fifty percent specific binding is represented by the dotted line. IC$_{50}$ values were calculated as mean ±SEM of three separate experiments.
<table>
<thead>
<tr>
<th>PP/PP Analogue</th>
<th>Average binding to the hY4 Receptor (nM)</th>
<th>SEM</th>
<th>Statistical significance compared to PP binding to the hY4 receptor</th>
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<td>PP</td>
<td>hY4 IC&lt;sub&gt;50&lt;/sub&gt; 0.23 SEM 0.03</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Val 30 0.10 SEM 0.04</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Asn 30 0.23 SEM 0.02</td>
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</tr>
<tr>
<td></td>
<td>Cys 30 0.23 SEM 0.01</td>
<td></td>
<td>Not Significant</td>
</tr>
<tr>
<td></td>
<td>Lys 30 0.12 SEM 0.02</td>
<td></td>
<td>p≤0.05</td>
</tr>
<tr>
<td></td>
<td>Glu 30 0.36 SEM 0.02</td>
<td></td>
<td>p≤0.01</td>
</tr>
<tr>
<td></td>
<td>Pro 0, Leu 30 0.21 SEM 0.03</td>
<td></td>
<td>Not Significant</td>
</tr>
<tr>
<td></td>
<td>Pro 0, Lys 30 0.10 SEM 0.001</td>
<td></td>
<td>p≤0.05</td>
</tr>
<tr>
<td></td>
<td>Ala 0, Leu 30 0.27 SEM 0.05</td>
<td></td>
<td>Not Significant</td>
</tr>
<tr>
<td></td>
<td>Ala 0, Thr 30 0.15 SEM 0.01</td>
<td></td>
<td>p≤0.01</td>
</tr>
<tr>
<td></td>
<td>Ala 0, Leu 17, Leu 30 0.23 SEM 0.03</td>
<td></td>
<td>Not Significant</td>
</tr>
<tr>
<td></td>
<td>Ala 0, Ile 17, Cys 30 0.13 SEM 0.01</td>
<td></td>
<td>p≤0.001</td>
</tr>
<tr>
<td></td>
<td>XPP 0.10 SEM 0.008</td>
<td></td>
<td>p≤0.001</td>
</tr>
</tbody>
</table>

**Table 3.2** The binding affinities of PP and PP analogues for the hY4 receptor *in vitro*. The IC<sub>50</sub> values of the PP analogues were compared to that of native PP IC<sub>50</sub> using Student’s T test, p≤0.05 was considered to be significant.
3.4.3 The effect of acute peripheral administration of PP and PP analogues on food intake in fasted male C57/BL6 mice

3.4.3.1 The effect of PP and PP analogues with substitutions at amino acid position 30 on food intake in fasted male C57/BL6 mice

PP and PP analogues with the substitution of the Met at position 30 in the PP sequence substituted with: Asn, Cys, Lys, Val, or Glu significantly reduced food intake compared with saline control at 0-1 hour (Saline, 1.1g ±0.01grams, Asn 30, 0.55g ±0.01grams (p=≤0.001), Val 30, 0.49g ±0.01grams (p=≤0.01), Lys 30, 0.49g ±0.01grams (p=≤0.001), Glu 30, 0.46g ±0.01grams (p=≤0.001), and Cys 30, 0.78g ±0.01grams (p=≤0.001)). At 0-1 hours PP analogue Asn 30 significantly reduced food intake compared to PP (PP 0.87g ±0.01grams and Asn 30, 0.55g ±0.01grams (p=≤0.001)). PP analogue Phe 30 reduced food intake compared to saline at 0-1 hour, but this effect did not reach statistical significance (0.75g, ±0.01grams) (Figures 3.6 to 3.11).

PP analogue Lys 30 significantly reduced food intake compared to saline control at 1-2 hours (Saline 0.46g ±0.01grams, 0.11g ±0.01grams, (p=≤0.001)). PP analogue Glu 30 increased food intake at 1-2 hours. However, this effect did not reach statistical significance (Saline 0.24g±0.01grams, Glu 30, 0.28g ±0.01grams).

PP analogue Lys 30 significantly reduced food intake compared to PP at 4-8 hours (Saline 0.69g±0.01grams, PP 0.78g ±0.01grams, and Lys 30 0.47g ±0.01grams p=≤0.05)). There were no statistically significant effects of PP analogues at any other time points examined (Table 3.3).
Figure 3.6 The effect of subcutaneous administration of PP (25nmol/kg) or PP analogue Phe 30 (25nmol/kg) on food intake in C57/BL6 mice fasted overnight A) 0-1 hour post injection, B) 1-2 hours post injection, C) 4-8 hours post injection and D) 8-24 hours post injection. PP and PP analogue compared to saline using one-way ANOVA with Bonferroni post hoc test (n=8/10).
Figure 3.7 The effect of subcutaneous administration of PP (25nmol/kg) or PP analogue Val 30 (25nmol/kg) on food intake in C57/BL6 mice fasted overnight A) 0-1 hour post injection, B) 1-2 hours post injection, C) 4-8 hours post injection and D) 8-24 hours post injection. **p≤0.01 compared to saline using one-way ANOVA with Bonferroni post hoc test (n=8/10).
Figure 3.8 The effect of subcutaneous administration of PP (25nmol/kg) or PP analogue Asn 30 (25nmol/kg) on food intake in C57/BL6 mice fasted overnight A) 0-1 hour post injection, B) 1-2 hours post injection, C) 4-8 hours post injection and D) 8-24 hours post injection. ***p≤0.001 compared to saline using one-way ANOVA with Bonferroni post hoc test (n=8/10).
Figure 3.9 The effect of subcutaneous administration of PP (25nmol/kg) or PP analogue Cys 30 (25nmol/kg) on food intake in C57/BL6 mice fasted overnight A) 0-1 hour post injection, B) 1-2 hours post injection, C) 4-8 hours post injection and D) 8-24 hours post injection. *p≤0.05 compared to saline using one-way ANOVA with Bonferroni post hoc test (n=8/10).
Figure 3.10 The effect of subcutaneous administration of PP (25nmol/kg) or PP analogue Lys 30 (25nmol/kg) on food intake in C57/BL6 mice fasted overnight A) 0-1 hour post injection, B) 1-2 hours post injection, C) 4-8 hours post injection and D) 8-24 hours post injection. **p=≤0.01, ***p=≤0.001 compared to saline using one-way ANOVA with Bonferroni post hoc test (n=8/10).
Figure 3.11 The effect of subcutaneous administration of PP (25nmol/kg) or PP analogue Glu 30 (25nmol/kg) on food intake in C57/BL6 mice fasted overnight A) 0-1 hour post injection, B) 1-2 hours post injection, C) 4-8 hours post injection and D) 8-24 hours post injection. *p≤0.05, ***p≤0.001 compared to saline using one-way ANOVA with Bonferroni post hoc test (n=8/10).
3.4.3.2 The effect of PP and PP analogues with an amino acid addition at the N-terminus and position 30 substitutions on food intake in fasted C57/BL6 male mice

3.4.3.2.1 Proline N-terminus extension

PP and PP analogues with a Pro N-terminus extension and the substitution of Met 30 for either Lys or Leu reduced food intake compared to saline control at 0-1 hour (Saline 1.12g ±0.01grams, Pro 0, Lys 30, 0.52g ±0.01grams, \( p \geq 0.001 \) and Saline 0.76g ±0.01grams, Pro 0, Leu 30, 0.71g ±0.01grams) (Figures 3.12 and 3.13). At 1-2 hours both analogues reduced food intake compared to saline (Saline 0.16g ±0.01grams, Pro 0, Leu 30, 0.007g ±0.01grams \( p \geq 0.001 \), and Saline 0.19g ±0.01grams, Pro 0, Lys 30, 0.13g ±0.01grams). PP analogue Pro 0 Lys 30 significantly reduced food intake compared to saline at 8-24 hours (Saline 4.34g ±0.01grams and Pro 0, Lys 30, 3.47g ±0.01grams \( p \geq 0.01 \)). PP analogue Pro 0 Leu 30 significantly reduced food intake compared to PP at 4-8 hours (Pro 0, Leu 30 0.57g ±0.01grams and PP 0.81g ±0.01grams \( p \geq 0.01 \)), (Table 3.3).

3.4.3.2.2 Alanine N-terminus extension

PP and PP analogues with an Ala N-terminus extension and the substitution of Met 30 for Leu at position 30 significantly reduced food intake compared to saline control at 0-1 hour (Saline 0.98g ±0.01grams, Ala 0, Leu 30, 0.71g ±0.01grams \( p \geq 0.05 \), and Saline 0.96g ±0.01grams, Ala 0, Thr 30, 0.736g ±0.01grams) (Figures 3.14 and 3.15). At a dose of 25nmol/kg, PP analogue Ala 0, Thr 30 failed to reduce food intake. The dose was therefore increased to 300nmol/kg, which reduced food intake compared to saline control at 0-1 hour. There was no significant difference between saline control and PP or PP analogues at any further time points (Table 3.3).
Figure 3.12 The effect of subcutaneous administration of PP (25nmol/kg) or PP analogue Pro 0, Leu 30 (25nmol/kg) on food intake in C57/BL6 mice fasted overnight A) 0-1 hour post injection, B) 1-2 hours post injection, C) 4-8 hours post injection and D) 8-24 hours post injection. **p=≤0.01, ***p=≤0.001 compared to saline using one-way ANOVA with Bonferroni post hoc test (n=8/10).
Figure 3.13 The effect of subcutaneous administration of PP (25nmol/kg) or PP analogue Pro 0, Lys 30 (25nmol/kg) on food intake in C57/BL6 mice fasted overnight A) 0-1 hour post injection, B) 1-2 hours post injection, C) 4-8 hours post injection and D) 8-24 hours post injection. **p=≤0.01, ***p=≤0.001 compared to saline using one-way ANOVA with Bonferroni post hoc test (n=8/10).
Figure 3.14 The effect of subcutaneous administration of PP (25nmol/kg) or PP analogue Ala 0, Leu 30 (25nmol/kg) on food intake in C57/BL6 mice fasted overnight A) 0-1 hour post injection, B) 1-2 hours post injection, C) 4-8 hours post injection and D) 8-24 hours post injection. *p≤0.05 compared to saline using one-way ANOVA with Bonferroni post hoc test (n=8/10).
Figure 3.15 The effect of subcutaneous administration of PP (300nmol/kg) or PP analogue Ala 0, Thr 30 (300nmol/kg) on food intake in C57/BL6 mice fasted overnight A) 0-1 hour post injection, B) 1-2 hours post injection, C) 4-8 hours post injection and D) 8-24 hours post injection. ***p=≤0.001 compared to saline using one-way ANOVA with Bonferroni post hoc test (n=8/10).
3.4.3.3 The effect of PP and PP analogues with an Alanine N-terminus extension, and amino acid substitutions at positions 17 and 30 on food intake in fasted male C57/BL6 mice

3.4.3.3.1 PP analogue: Alanine 0, Isoleucine 17, Cysteine 30
PP and PP analogue Ala 0, Ile 17, Cys 30 reduced food intake compared to saline control at 0-1 hour (Saline 0.83g ±0.01grams, Ala 0, Ile 17, Cys 30, 0.59g ±0.01grams) (Figure 3.16). There was no significant difference in food intake of PP analogue Ala 0, Ile 17, Cys 30 and either saline or PP at any time point examined (Table 3.3).

3.4.3.3.2 PP analogue: Alanine 0, Leucine 17, Leucine 30
PP and PP analogue Ala 0, Leu 17, Leu 30 reduced food intake compared to saline control at 0-1 hour (Saline 0.97g ±0.01grams and Ala 0, Leu 17, Leu 30, 0.78g ±0.01grams) (Figure 3.17). At 1-2 hours PP analogue Ala 0, Leu 17, Leu 30 significantly reduced food intake compared to saline (Saline 0.36g ±0.01grams and Ala 0, Leu 17, Leu 30, 0.02g ±0.01grams (**p=≤0.001)). There was no significant difference in food intake between PP analogue Ala 0 Leu 17 Leu 30 and saline at any further time points. There was also no significant differences in food intake between PP analogue Ala 0, Leu 17, Leu 30 and PP at any other time point examined (Table 3.3).
Figure 3.16 The effect of subcutaneous administration of PP (25nmol/kg) or PP analogue Ala 0, Ile 17, Cys 30 (25nmol/kg) on food intake in C57/BL6 mice fasted overnight A) 0-1 hour post injection, B) 1-2 hours post injection, C) 4-8 hours post injection and D) 8-24 hours post injection. Compared to saline using one-way ANOVA with Bonferroni post hoc test (n=8/10).
Figure 3.17 The effect of subcutaneous administration of PP (25nmol/kg) or PP analogue Ala 0, Leu 17, Leu 30 (25nmol/kg) on food intake in C57/BL6 mice fasted overnight A) 0-1 hour post injection, B) 1-2 hours post injection, C) 4-8 hours post injection and D) 8-24 hours post injection. **p=≤0.01, ***p=≤0.001 compared to saline using one-way ANOVA with Bonferroni post hoc test (n=8/10).
3.4.4 The effect of increasing doses of PP and PP analogue XPP on food intake in fasted male C57/BL6 mice

PP and PP analogue XPP significantly reduced food intake at 0-1 hour compared to saline control (Saline 0.75g ±0.01grams and 100nmol/kg PP 0.432g ±0.01grams (*p=≤0.05) and XPP at 20nmol/kg, 0.397g ±0.01grams (**)p=≤0.01) and 50nmol/kg 0.44g ±0.01grams, (*p=≤0.05)) (Figure 3.18). At 1-2 hours 100nmol/kg PP significantly reduced food intake compared to saline control (Saline 0.25g±0.01grams, 100nmol/kg PP 0.03g ±0.01grams (**p=≤0.01)). At 1-2 hours, 50nmol/kg of XPP significantly reduced food intake compared to 5nmo/kg PP (5nmol/kg PP, 0.571g ±0.01grams and 50nmol/kg XPP, 0.44g ±0.01grams (**p=≤0.01)). There was no significant difference in food intake between saline control and PP or analogue XPP at any further time points. There were also no significant differences in food intake between PP and analogue XPP at any further time points examined (Table 3.3).
Figure 3.18 The effect of subcutaneous administration of PP (5, 20 and 100nmol/kg) or PP analogue XPP (5, 20 and 50nmol/kg) on food intake in C57/BL6 mice fasted overnight A) 0-1 hour post injection, B) 1-2 hours post injection, C) 4-8 hours post injection and D) 8-24 hours post injection. *p≤0.05, **p≤0.01, compared to saline using one-way ANOVA with Bonferroni post hoc test (n=8/10)
<table>
<thead>
<tr>
<th>Analogue</th>
<th>Significance of anorectic dose of PP or analogue compared to saline control (*=p&lt;0.05,**=p&lt;0.01, ***=p&lt;0.001)</th>
<th>Significance of anorectic dose of analogue compared to equivalent dose of PP (**=p&lt;0.01 and ***=p&lt;0.001)</th>
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</thead>
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<tr>
<td></td>
<td>Dose (nmol/kg)</td>
<td>0-1 hours</td>
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<td>PP</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>Phe 30</td>
<td>25</td>
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</tr>
<tr>
<td>Glu 30</td>
<td>25</td>
<td>***</td>
</tr>
<tr>
<td>Pro 0, Leu 30</td>
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<tr>
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<tr>
<td>Ala 0, Thr 30</td>
<td>300</td>
<td>*</td>
</tr>
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<td>-</td>
</tr>
<tr>
<td>Ala 0, Ile 17, Cys 30</td>
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<tr>
<td>XPP</td>
<td>5-50</td>
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Table 3.3 PP analogues acute feeding study summary
3.4.5 Plasma levels of PP and PP analogue XPP following acute peripheral administration in rodent models

3.4.5.1 Plasma levels of PP and analogue XPP following SC injection in male C57/BL6 mice

Following a SC injection of either PP or PP analogue XPP (50nmol/kg), plasma levels of PP or analogue XPP in C57/BL6 mice were measured at 0, 5, 10, 20, 30, 45, 90, 120 and 180 minutes post injection using PP and XPP specific RIAs (Figure 3.19). Plasma levels of PP rose to an average maximal plasma concentration at 10 minutes post injection (15397 nmol/ml ±1100 nmol/ml) from undetectable levels at time point zero. Levels of PP were lower 30 minutes after injection (9925 nmol/ml ±1681 nmol/ml at 30 minutes post injection). At 120 minutes post injection levels of PP had continued to decrease (300 nmol/ml ±50 nmol/ml at 120 minutes) and at 180 minutes were at the lower limit of detection by RIA (4nmol/l). The PP RIA used is unable to detect rodent PP. Plasma levels of XPP rose to an average maximal plasma concentration at 20 minutes post injection (23728 nmol/ml ±2183 nmol/ml). The levels of XPP detected by specific XPP RIA fell after 45 minutes (15732 nmol/ml ± 1164 nmol/ml). Plasma levels of XPP were significantly higher than those of PP at 5 (p≤0.01), 10, 20, 30 and 45 minutes (p≤0.001) following administration. There was no significant difference between the maximal plasma levels (10 minutes) of PP and the plasma levels at 20 minutes using a repeated measures one-way ANOVA with Bonferroni post hoc test. However, comparing the remaining time points to the maximal plasma levels, there was a significant difference between 10 minutes and 5 minutes (p≤0.01), 30 minutes (p≤0.01), 45, 90, 120 and 180 minutes (p≤0.001). There was no significant difference between the maximal plasma levels (30 minutes) of XPP and the plasma levels at 5, 10, and 30 minutes using a repeated measures one-way ANOVA with Bonferroni post hoc test. However, comparing the remaining time points to the maximal plasma levels there was a significant difference between 20 minutes and 45 minutes (p≤0.05), 90, 120 and 180 minutes (p≤0.001).
3.4.5.2 Plasma levels of PP and analogue XPP following SC injection in male wistar rats

Following a SC injection of either PP or PP analogue XPP (50nmol/kg), plasma levels of PP or analogue XPP in lean male wistar rats were measured at 0, 10, 20, 30, 45, 60, 120 and 180 minutes post injection (Figure 3.20). Plasma levels of PP rose to an average maximal plasma concentration at 20 minutes post injection (2077 nmol/ml ± 359 nmol/ml), and fell after 45 minutes. At 120 minutes post injection levels of PP had decreased and had reached the lower limit of detection by RIA (4nmol/L). Plasma levels of XPP rose to an average maximal plasma concentration at 60 minutes post injection (9369 nmol/ml ± 547 nmol/ml). The levels of XPP detected by specific XPP RIA fell after 60 minutes. The XPP RIA is unable to detect rodent PP or native human PP and has a lower limit of detection of 0.3nmol/L. However, average plasma levels of XPP were still higher than those of PP at 180 minutes (XPP 95.4 nmol/ml ±12.5 nmol/ml and PP 37.7 ±1.12nmol/ml). Plasma levels of XPP were significantly higher than those of PP at 20 ($p \geq 0.01$), 30, 45, 60 ($p \leq 0.001$) and 120 minutes ($p \leq 0.01$) following administration. There was no significant difference between the maximal plasma levels (10 minutes) of PP and the plasma levels at 20, 30, 45 and 60 minutes using a repeated measures one-way ANOVA with Bonferroni post hoc test. However, comparing the remaining time points to the maximal plasma levels, there was a significant difference between 10 minutes and 120, and 180 minutes ($p \leq 0.001$). There was no significant difference between the maximal plasma levels (60 minutes) of XPP and the plasma levels at 20, 30, and 45 minutes using a repeated measures one-way ANOVA with Bonferroni post hoc test. However, comparing the remaining time points to the maximal plasma levels there was a significant difference between 60 minutes and 10 minutes ($p \leq 0.05$), 120 and 180 minutes ($p \leq 0.001$).
Figure 3.19 The plasma levels of PP or XPP following subcutaneous administration in male C57/BL6 mice (50nmol/kg). Following SC injection of peptide, mice were returned to their cages and blood collected via cardiac puncture at 0, 5, 10, 20, 30, 45, 90, 120, and 180 minutes. **=p≤0.01, ***=p≤0.001 plasma levels were compared at each time point using a two-way ANOVA with Bonferroni post hoc test (n=4-7).
Figure 3.20 The plasma levels of PP or XPP following subcutaneous administration in male wistar rats (50nmol/kg). Following anaesthesia, rats were given SC injection of PP or XPP, blood collected via jugular vein cannula at 0, 10, 20, 30, 45, 60, 120 and 180 minutes. **=p≤0.01, ***=p≤0.001 plasma levels were compared between groups at each time point using a two-way ANOVA with Bonferroni post hoc test (n=3).
3.5 Discussion

The experiments described in section 2.3 demonstrated that specific amino acid substitutions can increase the resistance of PP to enzymatic breakdown \textit{in vitro}. PP, unlike other small peptides, has a tertiary structure (Blundell et al., 1981; Gellman and Woolfson, 2002), which results in the “PP-fold” characteristic of PP, PYY and NPY. The tertiary structure of PP is reliant upon the primary amino acid sequence to form correctly \textit{in vivo}. Small changes to the amino acid sequence of a peptide such as PP can therefore affect receptor interaction and bioefficacy. I hypothesised that the amino acid changes made to the structure of PP would increase the ability of the analogues to bind to the hY4 receptor and inhibit food intake. This would be likely due to increased bioavailability, as less peptide would be broken down by enzymatic cleavage, increasing the amount of peptide available to interact with the receptor, and might also reflect the altered structure of the molecule binding more efficaciously to the receptor. These alterations would hopefully ultimately result in a PP analogue which circulates for longer in plasma than native PP, in a form which interacts more effectively with the hY4 receptor, therefore inhibiting food intake for longer.

I investigated the effect of single and multiple amino acid substitutions to the PP sequence on hY4 receptor binding, using competition binding assays, and on \textit{in vivo} bioefficacy, using acute food intake studies in lean male mice.

3.5.1 The effect of changes to the PP molecule on binding affinity for the hY4 receptor.

The enzymes NEP and DPPIV were found to be important in the breakdown of PP. Amino acids were subsequently substituted to prevent degradation by both these enzymes and to stabilise the tertiary structure of PP. These analogues were then examined to ensure that bioefficacy was maintained or improved.

A number of amino acids have previously been found to be essential for receptor interaction and activation. The amidated tyrosine at position 36 of PP is essential for hY4 receptor binding (Schwartz et al., 1990; Walker et al., 1997; Gingerich et al., 1991; Gehlert et al., 1996). The whole PP molecule is
necessary for high affinity binding to the hY4 receptor; small sections of PP have been shown to have impaired hY4 receptor binding compared to full length PP (Gingerich et al., 1991; Gehlert et al., 1996; Walker et al., 1997). The studies described in section 3.4.1 demonstrated that amino acid changes at position 30 affected the ability of PP to displace radiolabelled PP in binding assays. It has been shown previously that oxidation of the native Met improved the ability of the analogue to bind to the hY4 receptor (Gingerich et al., 1991). It was suggested that this was due to increased Van de Waals forces between the oxidised Met and the amino acid side chains of other amino acids in the alpha helix. Amino acid 30 (Met), as well as being a key site for enzymatic cleavage by NEP 24.11, is also likely to play a role in the stability of the tertiary structure. Therefore Met 30 needs to be substituted carefully to allow better enzymatic resistance whilst maintaining structural stability.

Replacing Met at position 30 with Asn, Cys, Lys, or Val improved binding to the hY4 receptor compared to native PP. The substitution of Met for these amino acids improved resistance to breakdown by NEP, with limited effect on KBB or meprin beta breakdown (Section 2.4). In avian PP, position 30 is occupied by a Val and has been shown to cause Van de Waal interactions between Val 30 and Pro 2, providing extra cross links between the alpha helix and the proline helix side chains, and thus helping to maintain the hair pin bend shape (Blundell et al., 1981). Amino acids Asn and Cys are both similar in size to Met in relative molecular mass (Asn 132, Cys 121 and Met 149) and, despite being polar molecules, are unlikely to disrupt the tertiary structure. Conversely, the addition of a Glu at position 30 impaired binding to the hY4 receptor, despite significantly improving resistance to breakdown by both NEP and meprin beta and the KBB membrane (Section 2.4). This may be due to the change in charge from a neutral Met to a negative Glu. A change in charge may have disrupted the packing of the proline helix hydrophobic side chains, altering overall tertiary structure. Alternatively, it may have impaired the degree of turn in the amphipathic alpha helix. If the degree of the alpha helix turn is changed this could cause the uncoiling of the entire alpha helix, as hydrophobic residues may no longer be facing into
the hair pin bend. The tertiary structure of the molecule would then have been destroyed. A further theory is that a charge at this position may somehow effect the interaction of the substrate for the receptor, therefore impairing binding. Substituting a Phe at position 30 also increased the IC\textsubscript{50} compared to native PP. This may be due to the phenol group side chain disrupting the side chain interactions which play a major role in the secondary structure of PP (Schwartz et al., 1990). A Phe at position 30, however, improved resistance to the degradative enzymes examined (Section 2.4). PP analogues Glu\textsubscript{30} and Phe\textsubscript{30} are examples of analogues which despite improved enzymatic resistance have impaired biological function.

The Pro at position 2 has also been described as critical for the binding of PP to the hY4 receptor (Gehlert et al., 1996). This Pro is cleaved by the enzyme DPPIV, as shown in section 2.4. DPPIV cleaves between the Pro at position 2 and the Leu at position 3 to generate PP\textsubscript{3-36}. It was shown in section 2.4 that the addition of another amino acid at position 0 inhibited cleavage of PP by DPPIV. It was also shown in section 2.3 that the addition of Ala at the N-terminus of PP slightly inhibited binding to the hY4 receptor compared to native PP. In section 3.4.1.1, competition binding assays demonstrated that the addition of Ala at position 0 of the PP in combination with either Leu or Thr at position 30 did not inhibit binding to the hY4 receptor, as these analogues have an IC\textsubscript{50} lower than or equal to that of unmodified PP.

The hair-pin bend in the ‘PP-fold’ family of peptides is highly conserved in all species and throughout all the ‘PP-fold’ peptides (Blomqvist et al., 1992; Cerda-Reverter and Larhammar, 2000; Larhammar, 1996a). This indicates that the hair pin bend is necessary for the PP family of peptides to correctly bind to the Y family of receptors. The hair pin bend is created by the beta turn (amino acids 9-14). However, without the interactions of the poly-proline helix side chains (amino acids 1-8) with the amphipathic alpha helix (amino acids 15-31), this bend would not be maintained (Blundell et al., 1981). This suggests that both the C and the N terminus of the peptide are required for receptor interaction. This is further supported by the limited affinity of PP fragments for the hY4 receptor (Gingerich et al., 1991; Gehlert et al., 1996;
Walker et al., 1997). The stabilisation of the hair pin structure may therefore improve receptor binding affinity.

Alanine N-terminus extensions in combination with position 30 changes were investigated with the addition of either a Leu or an Ile at position 17. These two additional amino acids changes were chosen to putatively stabilise the alpha helix which runs from amino acids 14 to 31 (Blundell et al., 1981; Glover et al., 1983). Isoleucine and leucine are both hydrophobic, and thus may stabilise the alpha helix by positioning themselves inside the helix. The stabilisation of the alpha helix may also mean that the analogue is less likely to be degraded by circulating and membrane bound enzymes. Circulating plasma levels might therefore remain higher for longer after administration, possibly leading to a greater anorectic effect. Having a more stable structure may also enable a greater number of analogue molecules to be in the correct form to bind to the hY4 receptor at any specific moment, creating more receptor substrate interactions in a shorter period of time.

Most amino acid changes at position 30 significantly improved hY4 receptor binding affinity. However, substituting Met at position 30 with Phe or Glu significantly impaired hY4 receptor binding. The combination of an N-terminus extension and changes at position 30 had a range of affects on affinity for the hY4 receptor. Lys at position 30 and a Pro N-terminus extension significantly decreased the IC₅₀ compared to native PP. Using the amino acid Ala to extend the N-terminus had a different effect, in combination with a Leu 30 or a Thr 30 on hY4 receptor binding. Ala 0, Thr 30 significantly increased affinity for the hY4 receptor compared to PP, whereas Ala 0, Leu 30 had only slightly improved binding to the hY4 receptor. The combination of an Ala extension with an Ile at position 17 and a Cys at position 30 significantly improved hY4 receptor binding, whereas an analogue with the substitutions Ala 0, Leu 17, Leu 30 had slightly, and non-significantly, impaired hY4 receptor binding. Overall, displacement assays suggest that an N terminus extension can aid hY4 receptor binding and that specific amino acid substitutions of the Met at positions 17 and 30 can either impair or enhance binding to the hY4 receptor compared to native unmodified PP.
3.5.2 The effect of changes to the PP molecule on food intake in overnight fasted male C57/BL6 mice.

Displacement assays indicate how well a peptide binds to the receptor. However, they do not indicate the bioefficacy of a peptide. PP binding to the hY4 receptor has been shown previously to inhibit intracellular cyclic AMP levels (Kojima et al., 2007; Hosoda et al., 2000; Dumont et al., 2005; Dumont et al., 1993). This secondary messenger system could therefore be used to measure the level of receptor activation by each analogue compared to PP. However, the need to stimulate cyclic AMP levels in order to observe the suppressant effects of PP and PP analogues can lead to high variation between studies. Analogues which do not bind well to the hY4 receptor or act as antagonists will not inhibit food intake in mice as effectively as native PP. Measuring the effect of PP and PP analogues on food intake in fasted C57/BL6 mice is a simple and effective measure of bioefficacy. Fasted mice were used as PP analogues were predicted to have an anorectic effect. Mice following a fast would be expected to have a greater food intake than their non-fasted counterparts, and therefore to more clearly demonstrate the anorectic effect of a test agent.

PP has a well characterised inhibitory effect on food intake when administered peripherally to mice (Asakawa et al., 1999; Katsuura et al., 2002; Kojima et al., 2007; Liu et al., 2008; Ueno et al., 1999). In a number of the experiments detailed in section 3.4.3 native PP did not significantly inhibit food intake as had been demonstrated in previous studies. In previous studies a dose of between 25nmol/kg and 30nmol/kg had been shown to be effective at inhibiting food intake in fasted male mice (Asakawa et al., 1999; Liu et al., 2008; Ueno et al., 1999). There was no apparent reason for the differences in PP’s ability to inhibit food intake. The experiments were carried out under the same conditions in the same mice. The PP used in all the studies was from a single batch freeze dried prior to the studies and reconstituted before each experiment. The body weights within each of the groups were matched, and randomised for each experiment therefore the mice in each group had all received different peptides/saline in the previous experiments. All mice had a minimum of a 24 hour wash out period between
once experiment and the next. However, it is possible that mice which had in the previous experiment received an anorectic peptide had too little body weight to lose in the subsequent experiment. Thus, making food intake inhibition by PP harder to achieve, possibly causing PP to be less effective in some experiments.

To ensure that analogues maintained or improved upon the bioefficacy of PP, the ability of PP analogue to inhibit food intake in fasted lean C57/BL6 male mice was investigated. The results of these studies suggest that some of the changes made to the PP structure had little or no effect on the ability of PP analogues to inhibit food intake. Examples of analogues which had improved receptor binding affinity but were no better than native PP at inhibiting food intake in fasted mice included PP analogue Ala 0, Leu 30 and PP analogue Ala 0, Thr 30.

The inhibitory effect of PP analogues on food intake did not always reflect their hY4 receptor affinity. PP analogue Glu 30 would be expected to have a limited effect in vivo due to the significant decrease in binding affinity for the hY4 receptor. However, at 0-1 hours Glu 30 significantly inhibited food intake compared to saline control. This was unexpected and may be due its improved enzymatic resistance enabling more Glu 30 to remain intact in the circulation, allowing more analogue molecules to interact with the hY4 receptor, and thus compensating for its relatively low affinity (Section 2.4). Alternatively it may be that Glu 30 has improved binding to the mouse Y4 receptor, but not the hY4 receptor. This could be checked by developing a binding assay which uses a membrane which over-expresses mY4 receptor, rather than hY4 receptors. This may indicate why PP analogues which do not bind well to the hY4 receptor in vitro and would not be expected to inhibit food intake greatly in vivo, achieve a significant inhibition of food intake in fasted male mice. However, the similarities between the human and mouse Y4 receptor and human and mouse PP suggest this is unlikely. Conversely PP analogue Ala 0, Thr 30 had significantly improved affinity for the hY4 receptor and yet when it was administered SC, a dose of 300nmol/kg had to be given before a significant food intake inhibition was observed at 0-1 hours. This analogue may for example, be binding tightly to plasma proteins such
as albumin which would then prevent it from interacting with the receptor as it is trapped in the circulation (Gokara et al., 2010; Ghuman et al., 2005).

The majority of analogues investigated did not inhibit food intake in fasted male mice as effectively as would be expected from their binding to the hY4 receptor in vitro. The food intake results suggest that despite improved affinity for the hY4 receptor, some PP analogues do not have improved abilities to inhibit food intake in fasted male mice. This may be due to the differences between the human and mouse Y4 receptor; human PP analogues may reduce food intake more effectively in human studies, but it is unfortunately impractical to test a large number of such analogues in humans. However, other unknown factors may also be impairing the actions of PP analogues in vivo, including their ability to enter the circulation from the SC depot. This could be impaired by analogues binding to plasma proteins such as albumin (Ghuman et al., 2005). All of these factors would impair biological functioning, despite improved affinity for the hY4 receptor in vitro.

3.5.3 PP analogue XPP: hY4 and hY2 receptor affinity, food intake inhibition and circulating plasma levels.
XPP amino acid sequence details, Appendix IV.

Analogue XPP required a 50% lower dose than native PP to inhibit food intake in fasted mice. This may reflect its greater affinity for the Y4 receptor, or a combination of improved affinity and improved resistance to enzymatic breakdown. Food intake inhibition by XPP was significantly greater than PP (5nmol/kg) at 1-2 hours (p≥0.01); this may be due to the higher circulating plasma levels of XPP at this time. At 0-1 hours PP significantly inhibited food intake at a dose of 100nmol/kg (p≤0.5). However, XPP significantly inhibited food intake at the same time point at both 20 and 50nmol/kg (p≤0.01 and p≤0.5). At 4-8 hours, animals receiving XPP at doses of 20 and 50nmol/kg had lower food intakes than animals receiving saline or PP (5 and 20nmol/kg), but this effect did not achieve statistical significance. These details suggest that XPP has a longer effect on food intake than unmodified PP. This may be due to the combination of increased enzymatic resistance, improved affinity for the hY4 receptor and maintenance of a low affinity for
the hY2 receptor. In addition to the increased time in circulation, it would appear that XPP levels take longer to reach the maximum plasma concentration than native PP following SC administration. Both a longer circulation time and a delay in reaching maximal plasma concentrations could help to explain why XPP inhibits food intake for longer, and at a lower dose than unmodified PP.

Interestingly, XPP levels peaked at 60 minutes in rats and 30 minutes in mice. These differences may be explained by the differences in experimental procedure, with rats being anaesthetised and mice being returned to home cages following SC administration. During anaesthesia, basal metabolic rate slows, which may explain why both XPP and PP levels detected were higher in mice which remained conscious following SC injection (Choi et al., 2002). In addition, the body temperature of the anaesthetised rats may have dropped (despite being on a heat mat) which may have reduced circulation to the skin, reducing the uptake of both PP and XPP from the SC depot. In addition, rats and mice have different metabolic rates, which is likely to influence drug metabolism. However, the time when maximal levels of PP were detected in the circulation was the same in both rats and mice (10 minutes).

It is possible that analogue XPP exerts its effects on food intake via other Y receptors, for example, the Y2 receptor. This was shown to be unlikely as XPP has a lower affinity for the Y2 receptor than native PP. However, XPP might potentially exert its effects via an Y1 or Y5 receptor mediated mechanism. The Y1 receptor has the highest binding affinity for NPY (Kanatani et al., 2000; Dumont et al., 1993; Parker and Herzog, 1999), which is orexigenic. If XPP acts as an antagonist for the Y1 receptor, this could decrease food intake. However, as the Y1 receptor is found only in the brain and PP is not thought at present to be able to cross the blood brain barrier this is also an unlikely mechanism (Whitcomb et al., 1990; Allen et al., 1983; Flynn et al., 1999; Trinh et al., 1996; Parker and Herzog, 1999). Further studies are required to determine whether XPP can access the brain from the circulation, and whether it binds to and inhibits the Y1 and/or Y5 receptor.
3.5.4 Conclusions

PP analogue XPP was chosen as the lead analogue for further studies. XPP has improved affinity for the hY4 receptor and unaltered affinity for the Y2 receptor compared to PP. It remained at higher levels in circulating plasma following peripheral administration than unmodified PP in both rats and mice. XPP reached its peak plasma concentration later than native PP following SC administration. XPP also inhibited food intake in fasted male mice at half the dose required of PP. The experiments described in this chapter only investigated the acute effects of XPP on food intake. I therefore investigated the effects of XPP on energy homeostasis following chronic administration in different models of obesity, as described in the following chapter.
Chapter 4

The Effect Of Chronic Administration Of A Pancreatic Polypeptide Analogue On Energy Homeostasis In Mice.
4.1 Introduction

The studies described in chapters 2 and 3 demonstrated that small changes to the amino acid sequence of PP alter the resistance of PP to degradative enzymes and the affinity of PP analogues to the hY4 receptor. These studies resulted in the development of PP analogue XPP. PP analogue XPP has improved enzyme resistance and improved affinity for the hY4 receptor binding, without increased affinity for the hY2 receptor. These changes are thought to be responsible for the more potent and longer acting anorectic effects of XPP compared to PP, following acute peripheral administration.

It is necessary to demonstrate that potential obesity treatments are effective at chronically reducing food intake and body weight. The effectiveness of chronic daily administration of the candidate treatment at reducing food intake and body weight in animal models of obesity gives a valuable indication of how likely a treatment is to be effective in obese humans. Two well established models of obesity in rodents are the leptin knockout mouse (Ob/Ob mouse) (Moriya et al., 2009; Vrang et al., 2006; Ravinet et al., 2003; Pocai et al., 2009), and the diet induced obese mouse (DIO mouse) (Vrang et al., 2006).

The leptin deficient Ob/Ob mouse is hyperphagic and obese (Zhang et al., 1994; Drel et al., 2006; Ingalls et al., 1950), and was first observed in the 1950’s following a chance mutation in the leptin gene (Ingalls et al., 1950). The Ob mutation is also associated with a number of other hormonal and metabolic alterations, including poor temperature regulation, poor fertility, decreased expression of $\beta_3$ adrenergic receptors and lower oxygen consumption in comparison with heterozygous litter mates (Breslow et al., 1999; Zhang et al., 1994; Kaplan and Leveille, 1974). In humans, leptin deficiency has been observed in a small number of obese adults and children (Montague et al., 1997; Strobel et al., 1998). Ob/Ob mice gain weight quickly and are often used as a model of obesity. The Ob/Ob mouse has previously been used to elucidate the role of NPY in obesity (Erickson et al., 1996; Beck, 2006; Hollopeter et al., 1998). The Ob/Ob mouse has also been used to investigate the effects of chronic administration of gut
hormones, including PYY, DPPIV resistant GLP-1 analogues, and PP (Neary et al., 2005; Irwin et al., 2007; Asakawa et al., 2003), on food intake and body weight.

However, animals lacking a particular gene may undergo adaptive changes to overcome the loss of this gene. For example, embryonic knockout of NPY or AgRP has little effect, but adult animals with knockdown of NPY or knockout of the NPY/AgRP neurons are hypophagic, demonstrating the ability of the hypothalamus to compensate for the loss of specific signals within specific temporal windows (Lin et al., 2004; Challis et al., 2004; Thorsell and Heilig, 2002; Yang et al., 2009; Bi et al., 2007; Bewick et al., 2005b). The Ob/Ob mouse lacks an operational form of the gene which controls the production of leptin, and although it is obviously unable to completely compensate for the lack of leptin, it may have developmental or adaptive changes secondary to the loss of leptin. Therefore, although the Ob/Ob mouse is a useful model, it does not mimic the pathophysiological mechanisms responsible for the majority of human obesity, and may have altered energy homeostasis-regulating systems which do not reflect normal physiology.

The diet induced obese (DIO) mouse model is usually used on a C57/BL6 background. DIO mice are fed high fat diet, typically with 40-60% of their calories derived from fat from an early age, for between 8-16 weeks (Harlan Laboratories, 2010). The obese phenotype is typically observed after a month of feeding with a high fat diet. After 16 weeks of feeding on a high fat diet, mouse body weight gain begins to slow and they are considered to be relatively weight stable. DIO mice are often used as a non leptin deficient model of obesity, as DIO mice have a 93% increase in fat mass compared to control diet fed mice at 16 weeks (Surwit et al., 1995; Reuter, 2007). DIO mice have a similar obesity phenotype to humans, including visceral adiposity, insulin resistance, hyperinsulinemia, hyperleptinemia, and leptin resistance (Petro et al., 2004; Rossmeisl et al., 2003; Van et al., 1997; Surwit et al., 1995). Numerous studies have used the DIO model of obesity to examine novel therapeutic interventions for the treatment of obesity,
including the CB1 receptor antagonist Rimonabant (Reuter, 2007; Ahren et al., 2000; Hildebrandt et al., 2003).

Currently available obesity treatments have limited efficacy, as detailed in section 1.3.1. Initially, leptin was investigated as a potential drug target (Zhang et al., 1994; Campfield et al., 1995). However, subsequent studies suggested that obesity leads to leptin resistance, and thus that any exogenous administration of leptin would have little effect on weight loss (Bjorbaek, 2009; Scarpase and Zhang, 2009; Heymsfield et al., 1999). In recent years, a number of gut hormones have been proposed as potential treatments for obesity, including Oxyntomodulin, GLP-1, CCK, PYY$_{3-36}$ and PP (Druce and Bloom, 2006; Field et al., 2008; Neary and Batterham, 2009; Small and Bloom, 2005). All of these gut hormones reduce food intake following peripheral administration in animal models and in healthy human volunteers (Cohen et al., 2003; Dakin et al., 2002; McMahon and Wellman, 1998; Scott and Moran, 2007; Vrang et al., 2006; Batterham et al., 2003).

Gut hormones have potential as obesity treatments as they often act via discrete receptors or neuronal systems accessible from the periphery, reducing the potential for adverse effects. They also mimic the physiological appetite-regulating system, again limiting the possibility of harmful side effects. It has recently been suggested that the administration of gut hormones may also mimic the bariatric post operative state, (currently the most effective treatment for obesity) (Le Roux et al., 2007; Karra et al., 2009; Morinigo et al., 2006; Morinigo et al., 2008; Holdstock et al., 2008). Bariatric surgery achieves on average a 13% loss of excess weight over a 6 month period (Adams et al., 2007; Christou and Efthimiou, 2009; Kolotkin et al., 2009). It was originally assumed that malabsorption of nutrients drives the weight loss following bariatric surgery. However, it has now been shown that this malabsorption lasts only a few weeks (Holdstock et al., 2008). The longer lasting effects of bariatric surgery may result from changes in circulating gut hormones, with surgery associated with increased levels of the anorexigenic gut hormones PYY$_{3-36}$ and GLP-1 and reduced levels of the orexigenic gastric hormone ghrelin (Karamanakos et al., 2008; Kolotkin et al., 2009; Neary and Batterham, 2009). Mimicking the post-surgery gut hormone
profile may therefore have potential as an obesity treatment. Such drug treatment poses less risk to the patient than invasive bariatric surgery, and removes the time and costs associated with surgical recovery (Pories, 2008). There are, however, a number of limiting factors, including half life and route of administration, which make gut hormones difficult to develop as obesity treatments (Bays and Dujovne, 2002). If the short half life of gut hormones could be overcome allowing a once daily or weekly preparation to be used, gut hormones may have clinical utility as obesity treatments.

PP has potential to improve on currently available treatments for obesity as it has previously been shown to be well tolerated in acute infusion studies (Jesudason et al., 2007; Batterham et al., 2003). It has a specific receptor target and limited affinity for any other receptors or systems (Dumont et al., 1998; Berglund et al., 2001), therefore limiting potential side effects. High circulating levels of PP in patients with PPomas are reasonably well tolerated (Adrian, 1986). Currently, two analogues of PP are being developed by 7TM Pharma for the treatment of obesity, each targeting different receptor systems. These are obinepitide (targeting Y2 and Y4 receptors) and TM30339 (targeting Y4 receptors alone). A recent phase I/II double blind placebo controlled study evaluating obinepitide has found that there is effective inhibition of food intake in obese subjects, and a phase II study is being carried out at present (7TM Pharma, 2007). If PP analogues were to be developed as a treatment for obesity, an IV infusion (as used by acute studies to date (Batterham et al., 2003; Jesudason et al., 2007)) would not be suitable and an alternative route of administration would need to be established.

PP, however, has a short half life of around 7.5 minutes (Adrian et al., 1978) which limits its utility as an obesity treatment in its endogenous form. In breakdown studies (Appendix IV), PP analogue XPP had greatly improved resistance to a number of enzymes thought to play a role in the breakdown and deactivation of PP. In studies investigating circulating plasma pharmacokinetics, plasma levels of XPP were maintained at higher concentrations for longer than the same dose of unmodified PP in both mouse and rat models (Section 3.4.5). In acute studies, XPP inhibited food
intake by around 50% at 2 hours (Section 3.4). If this effect was maintained over an extended period it would result in significant weight loss, possibly greater than that achieved using Orlistat (Hogan et al., 1987; Ackroff and Sclafani, 1996). The studies undertaken in this section investigate the effect of chronic SC administration of XPP on food intake and body weight in animal models.
4.2 Hypothesis and aims

PP acutely inhibits food intake and causes weight loss when administered peripherally (IP). An acute IP injection of PP at 30 nmol/kg has been shown to decrease food intake for up to 6 hours, with increasing doses decreasing food intake for up to two days in mice (Liu et al., 2008; Asakawa et al., 2003; Katsuura et al., 2002; Kojima et al., 2007; Lin et al., 2009).

If this treatment could be adapted to cause greater food intake inhibition following a single daily subcutaneous injection it could provide a putative new treatment for obesity with greater efficacy, and fewer side effects than current therapies.

**Hypothesis:** I hypothesise that chronic daily administration of PP analogue XPP will reduce food intake and body weight in DIO and Ob/Ob mice.

**Aims:**

1. To investigate the effect of chronic daily SC injection of XPP on food intake and body weight in male Ob/Ob mice.
2. To investigate the effect of chronic daily SC injection of XPP on food intake and body weight in two different sets of male DIO mice.
4.3 Materials and Methods

4.3.1 Custom designed analogues and peptide
PP analogue XPP was custom synthesised, purified and purchased and as described in section 2.3.1.

4.3.2 Animals
All animal procedures were approved by the British Home Office under the UK Animal (Scientific Procedures) Act 1986 (Project Licence 70/6402).

Three sets of mice were used for these experiments: male Ob/Ob mice, male DIO C57/BL6 mice fed on a medium fat diet (study 2) and male DIO C57/BL6 mice fed on a high fat diet (study 3). Animals were maintained as described in 3.3.2. All animals were allowed ad libitum access to water and ad libitum access to RM1 diet (Special Diet Services, Witham, UK) unless otherwise stated. Animals were handled and received sham SC injections of saline on at least two occasions prior to study days in order to acclimatise the animals to the injection procedure. All mice feeding studies were completed in conditions where external stressors were minimised as stress is known to influence feeding behaviour in rodents (Marti et al., 1994).

4.3.3 Chronic administration of XPP in ob/ob mice.

4.3.3.1 Study/Injection procedure
Male Ob/Ob (Harlan, USA) mice were randomised into weight controlled groups (average weight 43.2 grams, (n=4/5)). Mice were SC injected during the early dark phase (1800-1900), in a maximum volume of 0.1 ml of saline. Before an animal was injected it was weighed and body weight recorded. After each injection, mice were returned to their home cages with a known amount of food. Food was reweighed at the same time each day at the point of injection. Food was removed from the hopper, a visual inspection of the cage made to check for any food spillage/hoarding and food weighed using balances accurate to 0.01 g. Acute food intake was measured on day one at 4 hours post injection. This was done to observe any initial acute effect on food intake.
4.3.3.2 Dosage
Animals received daily SC injections of either saline or XPP at 50nmol/kg or 150nmol/kg for 7 days. In a dose finding study 40nmol/kg of XPP was the most effective and highest dose used. Therefore 50 nmol/kg of XPP a dose slightly higher than the dose which was the most effective was used to try and ensure that a reduction in food intake was observed. 150nmol/kg was also used as 3 times the predicted lowest effective dose to establish the maximal effective dose.

4.3.4 Chronic administration of XPP in diet induced obese mice
DIO models of two different levels of obesity were used. Mice of an average weight of around 46grams were considered morbidly obese and leptin insensitive (Study 2). Mice used in the subsequent study had an average weight of around 36grams and were characterised as mildly obese (Study 3). These two groups of mice were used to help represent the broad spectrum of obesity in the human population.

4.3.4.1 High fat diet induced obesity- Study 2

4.3.4.2 Diet
C57/BL6 mice were group housed in cages of 10 and fed on a high fat diet containing 45% energy as fat from seven weeks of age and were maintained on this diet throughout the study (% energy content: 30% Carbohydrate, 25% Protein and 45% Fat) (Research Diets, USA). Mice were maintained as described in section 3.3.2. At week 11, mice were transferred from group housing to individually ventilated cages, and then given 2 weeks to acclimatise to the new cages before the start of the study.

4.3.4.3 Study/Injection procedure
Male DIO (Harlan, USA) mice were randomised into weight controlled groups (average weight 46.45 grams, n=7/8). Mice were SC injected during the early dark phase (1800-1900) in a maximum volume of 0.1 ml of saline. Before an animal was injected it was weighed and body weight recorded. After each injection, mice were returned to their home cages with a known amount of food. Food was reweighed at the same time each day at the point
of injection. Food was removed from the hopper, a visual inspection of the cage made to check for any food spillage/hoarding and food weighed using balances accurate to 0.01 g. Acute food intake was measure on day 1, and day 27 at 4, hours post injection. This was done to observe any initial acute food intake effect and to establish if an acute food intake effect was still observed following around 4 weeks of daily administration.

4.3.4.4 Dosage
Animals received daily SC injections of either saline or XPP at 66nmol/kg or 200nmol/kg of peptide for 62 days. These doses were used as they are higher than those used in the previous study which proved ineffective at reducing food intake.

4.3.4.5 Glucose tolerance testing (GTT)
Glucose tolerance tests were performed on day 63 of the study to assess if treatment with XPP influenced glucose tolerance. Mice were fasted overnight from 1600 on day 62. At 0900 on day 63, mice were removed from their cages and 10µl of blood was collected via venopuncture of the tail vein, and a baseline glucose measurement taken via a glucose metre and glucose strips (Acc-check, Roche, Switzerland). After a baseline glucose level was established, mice received a 10ml/kg IP dose of 20% dextrose solution (glucose challenge) and were returned to their home cages. Blood samples were then taken via the initial venous puncture at 15, 30, 45, 60, 90 and 120 minutes post dextrose injection. Blood glucose levels were plotted and area under the curve, between 0 to 120 minutes was calculated for each mouse.

4.3.5 High fat diet induced obesity- Study 3

4.3.5.1 Diet
C57/BL6 mice were group housed in cages of 10 and fed on a high fat diet containing 60% of its energy as fat for 13 weeks prior to the study (% energy content: 20% Carbohydrate, 20% Protein and 60% Fat) (Research Diets, USA). Mice were maintained as described in section 3.3.2. At week 13, mice were transferred from group housing to individually ventilated cages, and
then given 2 weeks to acclimatise to the new cages before the start of the study.

4.3.5.2 Study/Injection procedure
Male DIO (Harlan, USA) mice were randomised into weight controlled groups (average weight 37.15 grams, n=8/10). Mice were SC injected during the early dark phase (1800-1900), in a maximum volume of 0.1 ml of saline. Before an animal was injected it was weighed and body weight recorded. After each injection mice were returned to their home cages with a known amount of food. Food was reweighed at the same time each day at the point of XPP injection. Food was removed from the hopper, a visual inspection of the cage made to check for any food spillage/hoarding and food weighed using balances accurate to 0.01 g. Acute food intake was measured on days 1 and 30 at 4 hours post injection. This was done to observe any initial acute food intake effect and to establish if an acute food intake effect was still observed following around 4 weeks of daily administration.

4.3.5.3 Dosage
Animals received daily SC injections of either saline or XPP for 32 days. Due to the large dose being administered the doses were ramped; on day 1-9 animals received either 200nmol/kg or 1000nmol/kg, from day 10 onwards doses were trebled, with animals receiving 600nmol/kg or 3000nmol/kg for the remaining 22 days. These higher doses of XPP were used as previous doses of 200nmol/kg were not effective at either reducing body weight or food intake. Dose ramping or escalating doses have been used previously within the laboratory. In previous work on exendin-4, dose escalation helped to prevent dose related side effects. This hopefully gave the mice time to acclimatise to each dose step before the final largest dose was given, therefore providing protection against the problems previously observed with administration of large doses of anorexigenic agents (Fineman et al., 2004; Gentilella et al., 2009).
4.3.6 Statistical analysis
The results from acute feeding studies were compared using a one-way ANOVA with post hoc Bonferroni test. Chronic body weight and food intake data was compared using the general estimating equation (GEE). If the GEE indicated a significant difference between a treatment group and saline, a Mann Whitney U test was used to assess the time point at which significance was reached. The glucose tolerance tests results were analysed using a one-way ANOVA with repeated measures. A $p$ value of less than 0.05 was considered to be significant in all analyses.
4.4 Results

4.4.1 Study 1: The effect of daily SC injections of analogue XPP in male Ob/Ob mice.

4.4.1.1 Body weight and food intake
Daily administration of 50nmol/kg and 150nmol/kg of PP analogue XPP to Ob/Ob mice did not significantly reduce body weight compared to saline controls (Figure 4.1). However, daily doses of XPP at 150nmol/kg significantly reduced cumulative food intake over 7 days compared to saline control (Day 1 to day 5 $p=0.02$, day 5 to day 7 $p=0.0087$). XPP at a lower dose of 50nmol/kg reduced food intake compared to saline control over 7 days. However, this effect did not reach statistical significance.

4.4.1.2 Acute food intake
XPP at both 50nmol/kg and 150nmol/kg did not significantly reduce acute food intake compared to saline control at 4 hours on day 1 (Saline 0.46+/-0.073grams, 50nmolkg XPP 0.371 +/- 0.06grams, 150nmol/kg XPP 0.353 +/- 0.04grams) (Figure 4.2).
Figure 4.1 The effect of daily SC administration of PP analogue XPP at 50nmol/kg, and 150nmol/kg on A) body weight and B) cumulative food intake in Ob/Ob mice over 7 days. (n=4/5, *p ≤0.05 and **p ≤0.01 (treatment group Vs saline control)).
Figure 4.2: The acute effect of SC administration of XPP at 50nmol/kg and 150nmol/kg on 4 hour food intake on day 1 in Ob/Ob mice (n=7/8).
4.4.2 Study 2: The effect of daily SC injections of XPP in male diet induced obese C57/BL6 mice.

4.4.2.1 Body weight and food intake
Daily administration of 66nmol/kg and 200nmol/kg of PP analogue XPP to male DIO mice did not significantly reduce body weight or food intake over 62 days (Figure 4.3).

4.4.2.2 Acute food intake
At 4 hours post injection, on days 1 and 27, 66nmol/kg of XPP and 200nmol/kg did not significantly reduce acute food intake compared to saline control (Day 1, Saline 0.68 +/-0.09grams, XPP 66nmol/kg 0.59 +/-0.08grams, and 200nmol/kg 0.59 +/-0.06grams. Day 27, Saline 1.34 +/-0.1grams, XPP 66nmol/kg 1.68 +/-0.1grams, XPP 200nmol/kg 1.12 +/-0.1grams). (Figure 4.4)

4.4.2.3 Glucose tolerance tests
There was no significant difference in glucose tolerance between saline control group and the two treatments (66nmol/kg group, 908.6mmol/L and 200nmol/kg, 970.07mmol/L) (Figure 4.5).
Figure 4.3: The effect of daily SC administration of XPP at 66nmol/kg and 200nmol/kg on A) body weight and B) cumulative food intake in DIO C57/BL6 mice over 62 days. (n= 7/8).
Figure 4.4: The acute effect of SC administration of XPP at 66nmol/kg and 200nmol/kg on 4 hour food intake on A) day 1, and B) day 27 in DIO C57/BL6 mice (n= 7/8).
Figure 4.5: Glucose tolerance tests in DIO C57/BL6 mice following 62 days of daily administration of 66nmol/kg XPP or 200nmol/kg XPP on A) blood glucose levels at 0 to 120 minutes B) incremental area under curve for blood glucose values (n= 7/8).
4.4.3 Study 3: The effect of daily subcutaneous injections of analogue XPP in male diet induced obese C57/BL6 mice.

4.4.3.1 Body weight and food intake
Daily administration of 600nmol/kg and 3000nmol/kg of PP analogue XPP to DIO mice reduced body weight over 32 days (600nmol/kg, average body weight decreased by 2.77grams, and 3000nmol/kg average body weight decreased by 1.88grams). However, this effect did not reach statistical significance. There was no statistically significant reduction in food intake in either of the two treatment groups compare to saline control (Figure 4.6).

4.4.3.2 Acute food intake
On day 1 there was no significant acute food intake reduction in either treatment group compared to saline control at 4 hours (Saline 0.48 +- 0.05grams, XPP 600nmol/kg 0.36 +-0.06grams, and 3000nmol/kg 0.48 +- 0.04grams). On day 30 there was a significant increase in acute food intake at 4 hours in both treatment groups compared to saline control (Saline 0.33 +-0.04grams, 600nmol/kg, 0.65+-0.05grams, and 3000nmol/kg 0.88 +- 0.05grams, ***p=<0.001) (Figure 4.7).
Figure 4.6: The effect of daily SC administration of XPP at 200/1000nmol/kg day 0 to 8 and 600/3000nmol/kg day 9 to 32 on A) body weight and B) cumulative food intake in DIO C57/BL6 mice over 32 days. (n= 8/10 per group).
Figure 4.7: The acute effect of SC administration of XPP at 200/1000nmol/kg day 0 to 8 and 600/3000nmol/kg day 9 to 32 on 4 hour food intake on A) day 1, and B) day 30 in DIO C57/BL6 mice (n= 8/10, ***p<0.001).
4.5 Discussion

The studies described in this chapter were carried out to assess the effect of chronic peripheral administration of PP analogue XPP on body weight and food intake in two different models of obesity. In chapter 3 the acute effects of increasing doses of XPP were examined compared to PP in lean C57/BL6 mice. It was shown that XPP significantly reduced food intake at 2 hours post-injection at 50nmol/kg whereas 100nmol/kg of PP significantly reduced food intake at the same time point. This suggested that XPP may be useful as an obesity treatment if given over a longer period of time and at a higher dose. Daily administration of XPP was used to investigate the effectiveness of XPP at reducing food intake and body weight over periods of 7 to 62 days.

The daily administration of PP or the PP analogue XPP has not been investigated previously. However, PP’s acute effects on food intake have been demonstrated in a number of studies (Nakajima et al., 1994; Ueno et al., 1999; Asakawa et al., 1999; Asakawa et al., 2006), with PP reported to reduce food intake for over 24 hours. A number of mechanisms have been suggested to mediate the effects of PP on food intake. It has been proposed that PP acts via Y4 receptors to activate POMC neurons and GABAergic neurons in the ARC of the hypothalamus, stimulating an increase in alpha MSH release by removal of GABAergic inhibition, and by a direct increase in POMC neuronal activity. An increase in POMC activity in the ARC leads to an increase in activation of MC4 receptors by the agonist alpha MSH (Lin et al., 2009). It has also been suggested that PP acts on the Y4 receptor to modulate an orexin pathway in the LHA and a BDNF pathway in the VMH to reduce food intake (Sainsbury et al., 2010). As yet it is unclear how PP exerts these effects as it is unable to cross the blood brain barrier (Pieribone et al., 1992). Repeated peripheral injection of PP over 24 hours reduces hypothalamic NPY and orexin mRNA expression (Asakawa et al., 2003), again suggesting a central mechanism of action. The minimal amino acid changes and improved Y4 receptor affinity of XPP suggest its mechanism of action is likely similar to that of unmodified PP.
Daily SC administration of XPP at various doses reduced body weight in 2 different models of obesity, though these effects did not reach statistical significance in all studies. However, in all 3 chronic studies there was an initial trend of reduced body weight followed by a small body weight regain, and a period of weight stability. Interestingly, in the 2 DIO studies the level of body weight lost does not appear to correlate with a decrease in food intake. In study 2 and 3, acute food intake post day 10 is higher in the animals receiving treatment compared to saline treated animals. This may not be an accurate assessment as it was only the food intake up to 4 hours post injection, and the animal's receiving XPP treatment may be eating less than saline treated controls later on in the dark period. However, cumulatively there is little difference in food intake between the saline animals and those animals in the treatment groups. It is possible that desensitisation to the effects of XPP at anorectic receptors reveals milder agonistic effects of XPP at orexigenic Y receptors. Whatever the mechanism, the lack of obvious anorectic effects suggests that XPP may be increasing energy expenditure, most likely by increasing locomotor activity as PP is reported to (Liu et al., 2008). It has been suggested previously that lean individuals have a higher rate of spontaneous physical activity (SPA) compared to overweight individuals (Teske et al., 2008). XPP or PP may promote an increase in locomotor activity leading to an increase in SPA, allowing the animals to lose weight without a reduction in food intake.

In the Ob/Ob study, the animals in the treatment groups significantly reduced their food intake compared to the saline treated animals. This suggests that perhaps these animals have different mechanisms with regard to weight control and food intake than animals used in studies 2 and 3 with a functioning leptin system. This could be due to the genetic differences between the two groups of animals. Ob/Ob mice lack the Ob gene which codes for leptin therefore they are leptin deficient from birth (Ingalls et al., 1950). Conversely the DIO mice have a functioning leptin system, but are obese due to overfeeding. The DIO model may be considered to be a more physiological model, as few humans have a leptin deficiency, whereas many humans over eat and become obese.
In both DIO studies, XPP reduced body weight up to 10 days. The body weights of the animals in the treatment groups then plateaued for around 10 days, and subsequently increased from day 35 in study 3. In study 3, body weight again plateaued from day 10 to day 20. The acute food intake measured on day 30 (study 3) and day 27 (study 2) showed that XPP does not have a profound effect on the acute food intake following SC administration. This was unexpected as, in acute studies using smaller doses, XPP significantly reduced food intake over 24 hours. The overall body weight regain observed after days 10 in studies 2 and 3 in the XPP treatment groups may be due to tachyphylaxis, that is, the mice in these treatment groups become desensitised to XPP. Receptor internalisation may be one of the mechanisms by which the mice are adapting to high levels of XPP. However, previous studies using chinese hamster ovary (CHO) cells over-expressing human Y4 receptors have shown the receptor internalisation does not occur when cells are exposed to high concentrations of PP in vitro (Parker et al., 2007).

Conversely another hormonal mechanism may be compensating for the high levels of XPP, preventing further weight loss. The plateau observed in these studies may be the point where the counter acting mechanism is beginning to work, and the subsequent weight gain is the body attempting to return to its set point. It has been shown previously that daily administration of PYY₃₋₃₆, has a delayed orexigenic action (Parkinson et al., 2008), that study suggested that an increase in hypothalamic NPY and AgRP mRNA may be the cause. A similar mechanism may act to balance the overall actions of both PP and PYY due to their close evolutionary background (Larhammar, 1996a; Blomqvist et al., 1992; Cerda-Reverter and Larhammar, 2000). As detailed previously, α-MSH containing neurons within the POMC are activated following acute peripheral PP, as they are by PYY (Lin et al., 2009). However, as yet there is no mechanism showing neuronal activation following chronic administration of PP. It may be that chronic administration somehow changes the neurons activated by PP with the POMC or other areas of the brain, therefore preventing long term weight loss.
At present the most effective treatment for obesity is bariatric surgery. For a new drug treatment to be classed as an effective alternative to bariatric surgery or to Orlistat (the only available pharmacological obesity treatment licensed in Europe) it would need to be as effective with reduced side effects or risks, or significantly more effective. Treatment of DIO mice with XPP at either 200nmol/kg or 3000nmol/kg resulted in a 30% reduction in excess weight for a period of time during each study (based on average weight of a mouse being 25 grams, the mice in study 2 were 22 grams overweight and the mice in study 3 were 12 grams overweight). However, this weight loss was not sustained, and the average body weight of the mice gradually rose, perhaps reflecting the mice becoming less sensitive to XPP.

The initial weight loss achieved by XPP in each of the studies which used DIO mice indicates that XPP may have potential as an obesity treatment but that either the treatment regime or the formula needs to be modified to allow XPP to cause maximal weight loss. Dose ramping is a mechanism used previously to help prevent desensitisation and overcome any initial nausea problems (Fineman et al., 2004; Gentilella et al., 2009). This strategy appears to have prevented the mice from feeling unwell. The animals were observed daily and particularly after each dose ramp, and showed no signs of ill health. However, as weight loss plateaued in all 3 studies it would suggest that dose ramping does not prevent desensitisation to XPP. A slow release preparation of XPP, which allows the minimum activation of the Y4 receptors to cause food intake inhibition or an increase in locomotor activity without causing desensitisation of the system, might prove more effective than XPP dissolved in saline.

The use of slow release preparations of insulin have allowed once daily administration to be used in the control of blood glucose in both type I and type II diabetes (Kato et al., 2010; Meneghini et al., 2010). The methods used to develop long lasting slow release preparations could be adapted to develop XPP into a once daily preparation which maintains low circulating levels without causing desensitisation of the Y4 receptors. Insulin glargine uses amino acid substitutions to allow insulin to precipitate once injected SC, allowing it to be released slowly throughout the day (True, 2010; Kato et al.,
Further amino acids could be substituted into XPP to allow precipitation once in the SC depot. However, there is potential for further amino acid substitutions to have a detrimental effect on binding to the Y4 receptor and the biological effects of any such structural changes would therefore need to be examined carefully. Another option would be the addition of a fatty acid to the XPP sequence. The insulin analogue Levemir has a myristic fatty acid attached to the Lysine at the B29 position which allows it to bind to serum albumin (Russell-Jones et al., 2009; Russell-Jones et al., 2009). This Levemir-albumin complex then disassociates slowly over a long period of time, elevating Levemir levels over 24 hour profile. Addition of a similar fatty acid to the XPP molecule might allow the long term maintenance of a low circulating concentration of XPP, possible preventing desensitisation of the Y4 receptor.

In summary, daily XPP administration at doses of 200nmol/kg to 3000nmol/kg (SC) reduces body weight in DIO mice and a dose of 150nmol/kg significantly reduces food intake in Ob/Ob mice. Unfortunately, this reduction in body weight is not sustained when treatment is continued up to 62 days. Due to its ability to inhibit food intake acutely, and the initial body weight reduction observed in the DIO mice, XPP may have some utility as an obesity treatment if a slow release preparation could be developed.
Chapter 5

Final Discussion
The number of people in the UK categorised by the world health organisation (WHO) as obese (BMI 25-30) is rising. The NHS predicts that the number of people classed as either overweight or obese is likely to increase to 60% of men and 50% of women by 2050 (World Health Organisation, 2006). This will ultimately put the health service under pressure due to the cost of obesity treatment, and the treatment of obesity associated conditions such as T2DM (Avenell et al., 2004). As mentioned in section 1.3.1, the medicinal treatments currently available to treat obesity are limited (Neovius and Narbro, 2008). The surgical strategy for treating obesity is contentious due to the cost and risk benefit profiles, and is impractical to use it to treat the increasing numbers of obese patients (Christou and Efthimiou, 2009; Garb et al., 2009; Martins et al., 2010). This suggests that new, more effective treatments for obesity are necessary (Field et al., 2009; Pournaras and Le Roux, 2009; Neovius and Narbro, 2008; Clapham et al., 2001; Butland B, 2007; Bourne J, 2001; Bays and Dujovne, 2002). Gut hormones such as PP, PYY, and OXM have potential as obesity pharmacotherapies, due to their physiological actions on food intake, and a number are currently in development (Bays and Dujovne, 2002; Clapham et al., 2001; Cooke and Bloom, 2006; Druce et al., 2004; Druce and Bloom, 2006; Field et al., 2008; Vivus Pharmaceuticals, 2009; Wynne et al., 2005a).

PP has been shown in mouse models and in humans with a normal BMI to reduce food intake after acute administration over a period of up to 24 hours (Asakawa et al., 1999; Asakawa et al., 2006; Batterham et al., 2003; Jesudason et al., 2007; Katsuura et al., 2002; Kojima et al., 2007; Liu et al., 2008). PP acts via the Y4 GPCR to inhibit cyclic AMP production (Dumont et al., 2007; Dumont et al., 2005; Kojima et al., 2007). The Y4 receptor is located in a number of locations within the brain (area postrema and DVC) and the peripheral nervous system (vagus nerve) (McTigue et al., 1993; Larsen and Kristensen, 1997; Parker and Herzog, 1999). Studies using transgenic PP over-expressing mice have indicated that PP levels 20 times higher than those levels detected in their wild type litter mates can be well tolerated, with the transgenic mice eating significantly less than wild type mice, but otherwise behaving healthily (Ueno et al., 1999). In human infusion
studies, healthy volunteers have been shown to eat up to 22% less than saline control over 24 hours (PP 10pmol/kg/min) (Batterham et al., 2003). These studies suggest that PP may have potential as an obesity treatment. Unmodified PP, however, has little clinical utility due to its short circulating half life of around 6 minutes (Adrian et al., 1978).

I have demonstrated that the substitution of enzyme sensitive amino acids within the PP sequence can increase resistance to certain enzymes and tissue preparations. These modifications in combination with modifications thought to increase affinity for the hY4 receptor produced an analogue of PP with increased resistance to enzyme and tissue degradation and increased affinity for the hY4 receptor \textit{in vitro}. The ability of PP analogue XPP to inhibit food intake was investigated in two different models of obesity and indicated potential to decrease food intake and ultimately reduce body weight over the long term.

5.1 PP analogue XPP as a potential treatment for obesity.

PP analogue XPP is resistant to all the enzymes and tissue preparations examined in this thesis (<5% breakdown, appendix IV). It has been shown to have significantly improved affinity for the hY4 receptor (section 3.4). XPP also inhibited food intake compared to PP in acute feeding studies (section 3.4). It has been shown previously that peripheral administration of PP reduces food intake and body weight over 48 hours (Liu et al., 2008). Chronic studies investigating food intake and body weight in Ob/Ob mice receiving daily SC injections of XPP showed that XPP significantly inhibited food intake over 8 days but that this food intake inhibition did not correspond with a body weight reduction. It was observed that despite the food intake reduction detected in Ob/Ob mice, the average body weight within all the groups increased. It was decided that Ob/Ob mice with their genetic lack of leptin, may have disturbed weight reduction mechanisms, and that these mice were therefore not an ideal model of obesity for studies in this thesis (Pelleymounter et al., 1995).

The DIO mouse is considered to be a more reflective model of human obesity, and therefore was used for the longer term chronic studies detailed
in this thesis. These studies showed that body weight was reduced in all animals which received daily SC injections of XPP. The food intake and body weight of mice receiving XPP did not differ significantly from those animals receiving saline treatment. Despite this, mice receiving 66nmol/kg of XPP over 45 days lost approximately 6 grams of body weight (starting weight of 47g to end weight of 40.8g). The average weight of a C57/BL6 mouse is 25 grams therefore a 6 gram weight loss is approximately 28% excess weight lost. In the same study, mice receiving saline also lost weight and on day 45 had lost 5 grams (approximately 23% of excess weight). Therefore overall on day 45 XPP has enabled an extra 5% of excess weight to be lost compared to saline. Further studies are required to examine whether this is a real biological effect that could be demonstrated statistically using, for example, larger group sizes.

5.2 Proposed mechanism of action of XPP.

XPP in both DIO chronic studies reduced food intake for a short period of time. XPP also decreased body weight up to day 30. However, after this point body weight was observed to be slowly returning to baseline levels. This may be explained by a change in NPY levels within the hypothalamus, which previous studies suggested should occur following PP administration (Sainsbury et al., 2010; Asakawa et al., 2003). A decrease in NPY mRNA levels in the ARC of the hypothalamus would allow for an increase in activation of the MC4R (Cone et al., 2001). Previous studies have linked the MC4R system with the BDNF system within the hypothalamus (Sainsbury et al., 2010). Animals which lack BDNF are obese and hyperphagic, and mimic the phenotype observed when the MC4R is knocked out (Rios et al., 2001; Bariohay et al., 2005). Increased MC4R activation could potentially increase BDNF mRNA, which may subsequently decrease food intake and increase energy expenditure (Xu et al., 2003; Wang et al., 2010; Xu et al., 2003; Bariohay et al., 2005; Wang et al., 2010).

Further investigation is required to decipher the mechanism by which XPP causes a reduction in body weight. The use of the comprehensive laboratory animal monitoring system (CLAMS) system would enable the energy
expenditure part of the proposed mechanism to be investigated. The use of a conditional knock-out animal model would also be a useful tool to investigate, for example, whether animals which lack BDNF in the hypothalamus lose weight following chronic administration of XPP.

5.3 Future formulation modifications to XPP

In the two long term chronic studies using DIO mice it was observed that the mice body weights plateaued and then rose again. As mentioned in section 4.5, this may be due to receptor down regulation. Receptor down regulation is thought to occur when receptors are over exposed to stimuli and the number of receptors is reduced as part of a negative feedback mechanism. SC administration has been shown to cause a rapid increase in circulating levels of substance administered. For XPP, this rapid increase in combination with the reduced breakdown (shown in section 2.4) may mean that there is a large sustained spike in plasma XPP levels following injection which over stimulates the Y4 receptor system and leads to receptor down regulation. To overcome this, a constant low level of receptor activation could be maintained using, for example, a SC pump or a slow release form of XPP. These two different methods would enable more consistent plasma levels to be established compared to the sharply peaking levels commonly observed following SC injection. Long lasting slow release formulations of insulin are currently available and have been shown to more effective at controlling blood glucose levels and HbA1C levels in the long term than shorter acting counterparts (Heinemann et al., 2000; Heise et al., 2011; Swinnen et al., 2010; Hollander et al., 2008). The process by which insulin was manipulated to create a longer lasting form is similar to the process by which I have created a longer lasting analogue of PP, i.e. modifications to the primary amino acid sequence. Amino acids were modified in insulin to change the isoelectric point from 5.4 to 6.7. This made insulin more soluble at a slightly acidic pH, and less soluble at a physiological pH. These changes to insulin, in combination with a zinc based formulation, enabled insulin glargine to be soluble at pH 4, therefore enabling it to be injected. After entering the SC depot the surrounding pH changes from 4 to nearer pH 7, causing the precipitation of the insulin. This allows the insulin to be stored at the depot
and released slowly, and thus for basal blood insulin levels to be maintained for a period of up to 24 hours (Raslova, 2010; Heinemann et al., 2000). If this formulation could be utilised for XPP, constant levels of XPP at doses low enough to activate the receptor and cause weight loss, could potentially be achieved, whilst avoiding the over stimulation and subsequent down regulation of the system putatively caused by a bolus SC injection.

Insulin can also be administered via SC dwelling pumps which allow for longer term control of blood glucose without the need for daily injections. However, it is unknown whether PP or XPP would be stable enough to be administered via this method.

An alternative method to the two currently used for insulin is PEGylation (Veronese and Mero, 2008). This is a process by which a polymer of polyethylene glycol is attached to a molecule. This method prevents elimination from the body by the kidneys and liver and thus often increases the biological half life of the resulting molecule (Kang et al., 2009). A poly ethylene glycol polymer can be added to the molecule by targeting an amino acid within the peptide, or by the addition of a His tag which already has the PEG attached at either end of the peptide. In the case of XPP, the His tag could be added instead of the Gly at position -1, perhaps also inhibiting cleavage by DPPIV. At present there are a number of products utilising PEG technology including hepatitis, and haemophilia treatments (Mei et al., 2010; Rasenack et al., 2003). For example, PEGASY is a version of interferon alpha used in the treatment of chronic hepatitis A and B. Studies have shown that PEGASY is longer lasting, and therefore more effective, than unmodified interferon alpha (Rasenack et al., 2003).

Modifications which allowed XPP to be administered less frequently would improve treatment compliance, and might help prevent maladministration or over use. It may also reduce the cost of treatment.

The effect of XPP on the food intake and body weight of human volunteers needs to be investigated in the future. The in vitro work carried out in this thesis investigated the affinity for the hY4 receptor, whilst the animal models used examined the effects in vivo. The mouse version of the Y4 receptor
differs by only a few amino acids to the human form (Berglund et al., 2001). However, these small differences could mean that XPP given chronically is more effective in humans than in mouse models.

### 5.4 The use of analogues as investigative tools

Analogues of hormones and peptides have been used previously to investigate structure activity relationships. An example is oxyntomodulin, where analogues were used to investigate the parts of the molecule necessary to bind to either the GLP-1 receptor or the glucagon receptor (Druce et al., 2009; Estall and Drucker, 2006; Pocai et al., 2009), and for its biological activity (Pocai et al., 2009; Druce et al., 2009; Wynne et al., 2006). Analogues have been developed previously to overcome biological constraints which make the native hormone or peptide ineffective as a potential treatment. Useful analogues mimic the biological function of the native hormone but lack its limitations, such as rapid breakdown or short half life. Analogues can also provide information regarding both receptor binding requirements and the substrate requirements for degradative enzymes (Hupe-Sodmann et al., 1995a; Matsas et al., 1984).

In this thesis I have used analogues to improve the resistance of PP to certain degradative enzymes and tissue preparations whilst improving affinity for the hY4 receptor. These experiments have highlighted the complicated structure activity relationship between PP and the hY4 receptor and overall biological function. For example, PP analogue Lys 30 has improved hY4 receptor affinity compared to PP, but is more susceptible to breakdown by KBB membrane and meprin beta, which would suggest that it might not be as effective as PP at inhibiting food intake in vivo. However, in fasted male C57/BL6 mice Lys 30 significantly inhibited food intake at both 0-1 and 1-2 hours. This suggests that although enzyme resistance is an important factor in overall biological function, other factors such as receptor affinity may be more important. Conversely, analogue Ala 0, Thr 30 has improved affinity for the hY4 receptor compared to native PP, which suggested that it would have greater anorectic effects in vivo. On investigation this was found not to be the case; analogue Ala 0, Thr 30 required a dose of 300nmol/kg to inhibit food
intake whereas previously PP has been shown to inhibit food intake at 25 nmol/kg.

Amino acid substitutions have also suggested enzyme substrate preferences. For example, the enzyme NEP is known to require hydrophobic residues as its substrates (Erdos and Skidgel, 1989; Kenny, 1986a; Medeiros and Turner, 1994b). However, when I replaced the hydrophobic Met 30 residue of PP with Val another hydrophobic residue it resulted in a statistically significant improvement in breakdown by NEP. This suggests that there may be a hierarchy of substrate preferences for enzymes such as NEP. However, when the hydrophobic Met 30 was replaced by the basic amino acid Lys, which should have provided enzyme resistance at this site, it increased enzymatic breakdown. This highlights the complexity of the tertiary structure of PP, and that the use of analogues can identify which amino acids are necessary for biological function and which are needed to maintain tertiary structure.

In conclusion, this thesis has developed an analogue of PP with improved enzyme resistance, and improved affinity for the hY4 receptor. In vivo experiments have indicated that XPP inhibits food intake acutely. Chronic administration experiments suggest that modifications may be needed to the formulation of XPP to develop it into a useful obesity treatment. During the development of XPP, a number of PP analogues were generated which were useful in elucidating the complex structure activity relationship between PP and the hY4 receptor. Finally, further development of XPP may provide a new obesity treatment with greater utility than those currently on the market.
Appendices
Confidentiality:
THE FOLLOWING INFORMATION IS RESTRICTED, CONFIDENTIAL AND LEGALLY PROTECTED. The information detailed in the following appendices will not be in the publically available document for 5 years.
Appendix I – Development of XPP RIA

XPP was conjugated to BSA by the gluteraldehyde method and lyophilized by freeze drying overnight. Sheep were immunised with the XPP conjugate which had been made up in water and complete Freund’s adjuvant (Sigma-Aldrich, USA). Sheep were given a primary injection SC, followed by a booster every 2 months after injection. One week after each injection the sheep were bled to test the antibody titre. Blood was centrifuged at 1600g at 4°C for 15 minutes and the plasma separated and stored at -20°C. Serial dilutions were used to determine a suitable antibody titre for RIA. These antibodies were used to develop the RIA used in section 3.4.

XPP label was iodinated using the Bolton and Hunter method, as previously detailed in section 3.3.

The standard procedure for all XPP assays:

Tubes (3mls) were labelled in specific RIA racks holding up to 128 tubes. The first 34 tubes in all assays were NSB, 1/2 x label, 2x label, Zeros x 2, standard curve x 10, zeros x2 all in duplicate. All samples were assayed in duplicate, separated by 2 zeros in duplicate at least every 50 samples. Additional standard curves, flanked by 2 zeros in duplicate were inserted every 400 tubes and at the end of the assay. Assays were carried out in 0.06M phosphate buffer (pH 7.2) (appendix II), containing 0.3% BSA. Each tube was buffered with 500µl of this assay buffer, and the volume was adjusted for further additions.

The standard concentration for all XPP assays was 0.25pmol/ml, achieved by adding 4mls to a 1pmol standard vial. The volume of buffer in the standard curve tubes was adjusted to allow the addition of 1µl, 2µl, 3µl, 5µl, 10µl, 15µl, 20µl, 30µl, 50µl, and 100µl of standard solution in duplicate. The antibody was diluted in buffer to the correct titres for 100µl additions to every tube apart from the NSB. The excess tube received 600µl. The radioactive label was diluted in assay buffer to give 250 counts/ 10 seconds/ 100µl after subtraction the background radiation for 100µl addition to every tube except the ½ x and the 2x which received 50 and 100 respectively. The
assays were then incubated for 3 days at -4°C. After the 3 days the free bound antibody was separated from the label bound antibody by charcoal separation as detailed in section 3.3. After separation, the supernatant tubes were wax sealed and counted in 16 well gamma counters as detailed in section 3.3.

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</table>

Table A1: Contents of XPP RIA assay tubes
Appendix II – Solutions used in this thesis

Dextran coated charcoal:
Add 2.4g charcoal and 0.24g dextran to 100ml phosphate buffer with gelatine and mix for 20 minutes at 20°C.

DPPIV assay buffer:
1.2g of Tris-HcL in 50mls GDW, confirm pH to be 8±0.1 and store at 4°C.

1M HEPES:
Add 35.75g HEPES to 150mls of GDW, confirm pH 7.4±0.1 and store at 4°C.

HPLC buffers:
Add 1.25ml of trifluroacteic acid to 1.25L GDW.
Add 1.25ml of trifluroacteic acid to 1.25L acetonitrile

KBB assay buffer:
0.546g mannitol, and 0.038g HEPES in 10mls GDW, confirm pH to be 7.4±0.1 and store at 4°C.

Meprin beta assay buffer:
0.3g Tris-HcL in 50mls GDW, confirm pH to be 8±0.1 and store at 4°C.

Neprilysin assay buffer:
0.3g Tris-HcL in 50mls GDW, confirm pH to be 7.5±0.1 and store at 4°C.

Phosphate buffer (RIA buffer):
Dissolve 48g of Na2HPO4.2H2O, 4.13g KH2PO4, 18.61g C10H14H2O8Na2.2H2O, 2.5g NaN3 in 5L GDW, which had been boiled and allowed to cool, confirm pH to be 7.6±0.1 and store at 4°C.

Phosphate buffer with gelatine:
Dissolve 12.5g gelatine in boiling GDW, allow to cool before addition of the other ingredients.
10% Trifluoroacetic acid solution:

Add 1ml of Trifluoroacetic acid to 9mls of GDW, store at room temperature.

Receptor binding assay buffer:

Add 0.736g CaCl2, 0.204g MgCl2, 20mls of 1M HEPES to 980mls of GDW, store at 4°C.
# Appendix III – Amino Acid codes

<table>
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<tr>
<th>Amino Acid</th>
<th>Three Letter</th>
<th>Single Letter</th>
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<tr>
<td>Proline</td>
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<tr>
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<td>Threonine</td>
<td>Thr</td>
<td>T</td>
</tr>
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</table>
Appendix IV – XPP structure and supporting information

Figure A1: Representative HPLC chromatograms of 2nmols XPP incubated with A) 1.5mg of KBB, B) 200ng NEP and C) 200ng meprin beta at 37°C for 30 minutes. The black line represents XPP with enzyme/tissue preparation and the red line represents XPP with buffer. Reactions were terminated with 5µl of 10% TFA and the supernatant injected onto HPLC with a Gemini C18 column and a gradient of 15-55% ACN (green line) run over 30 minutes. All incubations showed less than 5% breakdown compared to buffer control.
The amino acid sequence of PP analogue XPP:

For the amino acid sequence of XPP or details of its structure affinity relationship, please contact Prof. Steve Bloom at:

Section of Investigative Medicine,

Division of Diabetes, Endocrinology and Metabolism,

Department of Medicine,

Imperial College London.
Appendix V – List of Suppliers
Bachem Ltd, Merseyside, UK
Beckman Coulter, High Wycombe, Buckinghamshire, UK
BIOMOL International L.P., Exeter, UK
Charles River laboratories INC, Wilmington, USA
Du Pont Ltd., Cambridgeshire, UK
Fine Science Tools, Heidelberg, Germany
GraphPad Software Inc., San Diego, USA
Harlan, Oxon, UK
Invitrogen, Paisely, Scotland
Jencons, East Grinstead, West Sussex, UK
Jasco, Essex, UK
Millipore, Milford, MA, USA
NE Technology Ltd., Reading, Berkshire, UK
Nordic Pharma, UK
Phenomenex, Macclesfield, Cheshire, UK
R&D Systems Europe Ltd., Abingdon, UK
Research Diets Inc. New Brunswick, USA
Sigma Aldrich, Poole, Dorset, UK
Special Diet Services Ltd, Witham, Essex, UK.
Thermo Scientific INC, Waltham, USA
Termuo, Elkton, USA
A kispeptin-10 analog with greater in vivo bioactivity than kispeptin-10
doi:10.1152/ajpendo.00426.2009

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International Union of Basic and Clinical Pharmacology. LXXVII. Kisspeptin Receptor Nomenclature, Distribution, and Function
Helen R. Kirby, Janet J. Maguire, William H. Colledge and Anthony P. Davenport
[Abstract] [Full Text] [PDF]

Updated information and services including high resolution figures, can be found at:
http://ajpendo.physiology.org/content/298/2/E296.full.html

Additional material and information about AJP - Endocrinology and Metabolism can be found at:
http://www.the-aps.org/publications/ajpendo

This information is current as of May 9, 2011.
A kisspeptin-10 analog with greater in vivo bioactivity than kisspeptin-10


Department of Investigative Medicine, Imperial College London, Hammersmith Hospital, London, United Kingdom.

Submitted 7 July 2009; accepted in final form 18 November 2009

Curitue AE, Cooke JH, Baxter JE, Parkinson JR, Bataveljic A, Ghatel MA, Bloom SR, Murphy KG. A kisspeptin-10 analog with greater in vivo bioactivity than kisspeptin-10. Am J Physiol Endocrinol Metab 298: E290–E303, 2010. First published November 24, 2009; doi:10.1152/ajpendo.00426.2009.—The kisspeptins are neuropeptides that stimulate the hypothalamic-pituitary-gonadal (HPG) axis. The smallest endogenous kisspeptin, kisspeptin-10 (KP-10), binds to the receptor KISS1R with a similar affinity to the full-length peptide, kisspeptin-54 (KP-54), but is less effective in vivo, possibly because of increased enzymatic breakdown or clearance. The kisspeptin system may have therapeutic potential in the treatment of reproductive disorders and endocrine cancers. We have rationally modified the structure of KP-10 and tested the binding affinity of three analogs for the KISS1R. Those analogs that bound with relatively high affinity to KISS1R were tested for ability to stimulate ERK1/2 phosphorylation in vitro and for their ability to stimulate the HPG axis in vivo. One analog, [Dty]KP-50, bound to KISS1R with lower affinity to KP-10 and exhibited similar bioactivity in vitro. However, in vivo peripheral administration of [Dty]KP-10 increased plasma LH and testosterone more potently than KP-10 itself at 20 min postinjection in mice. In addition, 60 min postinjection, 0.15 nmol [Dty]KP-10 significantly increased total testosterone levels in mice whereas the same dose of KP-10 had no significant effect. Should multiplication of the kisspeptin/KISS1R signaling system prove therapeutically useful, long-lasting analogs such as [Dty]KP-50 may have greater therapeutic potential than endogenous forms of kisspeptin.

KISS-1: KISS1R; agonist; luteinizing hormone; testosterone

The kisspeptins are potent neuropeptide stimulators of the hypothalamic-pituitary-gonadal (HPG) axis, acting via the G protein-coupled receptor KISS1R (also known as GPR54) (11, 17, 23). Absence of kisspeptin signaling in rodents and humans results in hypogonadotropic hypogonadism (HH) and lack of sexual maturation (4, 29). Conversely, administration of exogenous KP-10 to immature rats stimulates sexual maturation and induces precocious puberty (21). Furthermore, kisspeptin stimulates the release of LH, FSH, and testosterone when administered centrally or peripherally to male rats or primates (7, 14, 18–20, 30, 33), and peripheral administration of kisspeptin stimulates gonadotropin release in male and female humans (5, 8). Studies have shown that kisspeptin is likely to have its main action at the level of the hypothalamus (7, 10, 15, 20, 30, 33), although direct actions on the pituitary have been suggested (20).

The KISS-1 gene that encodes kisspeptins was first discovered as an antimestatasis gene (12), and kisspeptins have been suggested as a possible treatment for endocrine-related cancers (22). Manipulation of the kisspeptin signaling system has therapeutic potential. Drugs based on the kisspeptin molecule are potential treatments for delayed puberty and HH, as well as metastatic cancer (3, 13, 26). However, the kisspeptin them-
Table 1. Sequences and binding affinities of KP-10 and analogs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>pIC50, nM</th>
</tr>
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<tbody>
<tr>
<td>KP-10</td>
<td>YNWNSFOLF-KRF-9</td>
<td>1.0 ± 0.3</td>
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<tr>
<td>Native screened analogs</td>
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<tr>
<td>ANA1</td>
<td>ANWNSFOLF-KRF-9</td>
<td>2.3 ± 1.3</td>
</tr>
<tr>
<td>ANA2</td>
<td>YAWNSPFLR-KRF-9</td>
<td>4.0 ± 0.9</td>
</tr>
<tr>
<td>ANA3</td>
<td>YANASQGKL-KRF-9</td>
<td>109.5 ± 89.8</td>
</tr>
<tr>
<td>ANA4</td>
<td>YNWASFOLF-KRF-9</td>
<td>6.0 ± 0.4</td>
</tr>
<tr>
<td>ANA5</td>
<td>YNWNSFOLF-KRF-9</td>
<td>8.7 ± 0.8</td>
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<tr>
<td>ANA16</td>
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<tr>
<td>ANA17</td>
<td>YNWNSFOLF-KRF-9</td>
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<tr>
<td>ANA18</td>
<td>YNWNSFOLF-KRF-9</td>
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<td>Exchanger exchange analogs</td>
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<td>3.6 ± 0.3</td>
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</tr>
<tr>
<td>ANA21</td>
<td>YNWNSFOLR-KRF-9</td>
<td>8.7 ± 0.8</td>
</tr>
</tbody>
</table>

#Preparation of CHO-K1S1R Membranes

Chinese hamster ovary (CHO) cells, which had been stably transfected with the human KISS1R (CHO-K1S1R) receptors kindly donated by Prof. M. Parmentier, IRIBN, Brussels, Belgium, were cultured in Gibco Ham's F12 medium (Invitrogen, Paisley, UK) containing 10% fetal bovine serum and 100 IU/ml penicillin and 100 μg/ml streptomycin. Membranes were prepared by homogenizing and differential centrifugation, as previously described (25). Briefly, cells were scraped into PBS, added to 100 ml of 1 M NaH2PO4 buffer, and centrifuged for 15 min at 4°C at 1,450 g. The resulting pellet was resuspended in 50 ml HEPS buffer, by use of a motorized homogenizer (IKA). The mixture was then centrifuged as previously described, and the resulting supernatant was ultracentrifuged (Sorvall, Slawance, UK) at 110,000 g at 4°C for 1 h. The resulting membrane was used for receptor binding assays as described below.

#Receptor Binding Assay Using CHO-KISS1R Membranes

Assays were carried out in silanized Eppendorf tubes in 500 μl of buffer (2 mM MgCl2, 6.5 mM CaCl2, 20 mM HEPS pH 7.4, and 1% BSA). Unlabeled peptide (at a final concentration of 200, 20, 5, 1.02, 0.02, and 0.002 nM) was incubated with 50 pl radiolabeled [125I]KP-54 iodinated by the iodogen method (25), and 100 μg CHO-K1S1R membrane protein at 30°C for 30 min. Following incubation, tubes were centrifuged at 16,000 g for 3 min and the supernatant was discarded. The pellets were washed in 500 μl of assay buffer and centrifuged at 16,000 g for 4 min. The supernatant was discarded. Pellets were counted in a gamma counter (model NE1800, Thermo Electron) for 240 s.

Effect of KP-10 and Analogs on ERK1/2 Phosphorylation In Vitro

In the CHO-K1S1R cells used, KISS1R receptor activation results in increased ERK1/2 phosphorylation (23). The test kit (techo-test, Northampton, UK) uses a cell-based ELISA technique to determine relative levels of phosphorylated ERK1/2 within plated cells. CHO-K1S1R cells were plated into 96-well plates (Nunc) and allowed to grow to confluence overnight. Cells were then washed with 100 μl of serum-free Ham's F12 media containing 100 IU/ml penicillin and 100 μg/ml streptomycin (GIBCO). In initial experiments, cells were treated with 100-1,000 nM KP-10 and 1,000 nM analogs in 100 μl for 5 min. Subsequently, the dose-response effects of KP-10 and [A(Y)]KP-10 (ANA18) were compared by incubating cells in 0.1, 1, 10, 100, 1,000, or 10,000 nM of either peptide for 5 min. Following treatment, the medium was then removed and the cells were fixed with 4% formaldehyde buffer and stored at 4°C until measurement of ERK1/2 phosphorylation.

The ELISA was carried out according to the manufacturer's instructions. Briefly, fixed cells were incubated with 50 μl of supplied anti-phosphoERK or anti-phosphoERK primary antibody solution at room temperature for 1 h. They were then treated with secondary antibody and finally treated with a color-changing substrate. The absorbance of each well was measured at 450 nm via a spectrophotometer (Lab-system Multiskan MS, Cambridge, UK). Finally, cells were stained with the supplied cell-staining solution to determine relative cell number in each well, and absorbance was read at 595 nm. The 450-nm reading was then normalized by dividing it by the 595-nm reading of the same well to reflect the cell number.

Animals

Studies were carried out on adult male C57Bl/6 mice. Animals were housed in groups of 8–11. All animals were maintained under controlled temperature (21–23°C) and a 12:12-h light-dark cycle (lights on at 0700) with food (Rodent diet, SDS) and water available ad libitum. Animal procedures carried out in the department were conducted under the British Home Office Animals Scientific Procedures Act 1986 (Project License 70/0402) and were authorized by the Home Office after successful completion of the local ethical review process.

LH and Total Testosterone Response Following Application of KP-10 in Mice

Mice were injected intraperitoneally (ip) at time 0 on the study day with either saline or KP-10 at 0.5, 1, 10, or 30 nmol. At 20 min, mice were euthanized by CO2 inhalation, and blood was collected immediately by cardiac puncture. Blood was stored on ice until centrifugation at 15,000 g for 7 min, and plasma was stored at −80°C until assay. Plasma was assayed for LH, FSH, and total testosterone.

Initial Study To Determine In Vivo Effect of Selected Kisspeptin Analogs

Under the protocol described above, male C57Bl/6 mice were ip injected at t = 0 either with 0.5 nmol KP-10 or with kisspeptin analogs with IC50 < 10 nM in our initial receptor binding studies. Analogs were administered at doses chosen to reflect their binding affinity to KISS1R relative to KP-10 itself, as determined in initial receptor binding studies (data not shown). Analogs were selected to reflect a variety of receptor affinities, and LH and testosterone were measured by RIA.

During the course of these studies, a study investigating the effect of a number of KP-10 analogs on a spontaneous-response Lactate reporter gene system in yeast was published (22). The study did not test the analogs for receptor binding affinity or in vivo efficacy at stimulating gonadotrophin or testosterone release. However, their data did suggest that [A(Y)]KP-10 (ANA18 in our studies) might act as a...
KISS1R superagonist and that [dY]KP-10 (ANA18 in our studies) did not activate KISS1R in their system. Given that ANA18 did bind to KISS1R in our experiments, we hypothesized that it might act as a KISS1R antagonist. Therefore, as previously described, C57Bl/6 mice were ip injected at t = 0 with 0.5 nmol KP-10, or with kispeptin ANAS at 0.5 nmol or ANA18 at 0.5 or 10 nmol. At t = 20 min, mice were euthanized, blood was collected and separated, and LH and testosterone were measured by RIA.

**Comparing the Dose-Response Effect of ip [dY]KP-10 and KP-10 on Plasma LH and Testosterone**

As previously described, male C57Bl/6 mice were ip injected at t = 0 with either KP-10, or with kispeptin analog 19 ([dY]KP-10) at 0.015, 0.05, or 0.15 nmol. At t = 20, mice were euthanized, blood was collected and separated, and LH and testosterone were measured by RIA.

**Comparing the Time Course of the Effect of ip [dY]KP-10 and KP-10 on Plasma LH and Testosterone**

As previously described, male C57Bl/6 mice were ip injected at t = 0 with either KP-10, or with [dY]KP-10 at 0.5 nmol. At t = 20, t = 60, mice were euthanized, blood was collected and separated, and LH and testosterone were measured by RIA. A subsequent study was performed using doses of 0.5 nmol of KP-10 and [dY]KP-10 and including an additional t = 120 time point.

**RIA**

LH and FSH plasma levels were measured by use of reagents and methods obtained from the National Hormone and Pituitary program (Dr. A. Parl, University of California, Harbor Medical Center, Los Angeles, CA), and the radiolabeled peptides were prepared by the chloramine-T method (28). The RIAs were prepared in 0.06 M phosphate EDTA buffer (pH 7.4) and left to incubate for 3 days at 4°C before separation by immunoprecipitation. Results were calculated in terms of a National Institute of Diabetes and Digestive and Kidney Diseases standard preparation. The intra- and interassay coefficients of variation were 8.2 and 13.8%, respectively, for LH and 8.3 and 15.4%, respectively, for FSH. Plasma total testosterone was measured by using a commercially available kit (Siemens Biomedical). Assays were carried out according to the manufacturers’ instructions. Briefly, standards ranging from 0 to 55 ng/ml, as provided, and plasma samples were added to antibody coated tubes (as provided) in 50 μl volumes. Radiolabeled [%3H]-testosterone was added to tubes in 1-ml volumes (~1,000 cpm tube). The tubes were then vortexed and incubated at 37°C for 3 h before counting and data reduction. The intra- and interassay coefficients of variation were <10%.

**Statistical Analysis**

Curves and I.C50 values were calculated by use of the Prism 4 program (GraphPad Software, San Diego, CA). I.C50s were determined as means ± SE of three or more independent assays carried out in triplicate. All ELISA and in vivo studies were analyzed by one-way ANOVA with Tukey’s post hoc test. In all cases, P < 0.05 was considered significant.

**RESULTS**

**Initial Receptor Binding Screen**

Initial receptor binding studies were carried out on analogs using membranes prepared from CHO-KISS1R cells. Binding studies proceeded for 1 h, using 1,000 ± 344 nmol for KP-10 (Fig. 1). Initial analog binding studies showed that only one analog, ANAS, had an IC50 lower than that of KP-10 itself. Other analogs had IC50 ranging from 3.2 to 447 nM, and ANA10 did not bind at concentrations up to 200 nM. Subsequent studies suggested that ANA19 ([dY]KP-10) was a KISS1R superagonist, and the receptor binding curve for [dY]KP-10 is thus shown in Fig. 1.

**Effect of KP-10 and Analogs on ERK1/2 Phosphorylation In Vitro**

The effect of KP-10 on ERK1/2 activation was established. Treatment of plated cells with KP-10 at 100 or 1,000 nM induced phosphorylation of ERK1/2 in a dose-dependent manner at 5 min (0.52 ± 0.014 arbitrary units (AU) (control), 0.732 ± 0.037 AU (KP-10 100 nM) and 0.931 ± 0.0789 AU* (KP-10 1,000 nM); n = 3; *P < 0.001).

All analogs were tested for their ability to induce ERK1/2 phosphorylation at 1,000 nM. The experiments were carried out on 3 separate study days, and the data are therefore represented as % basal response. [dY]KP-10 and KP-10 significantly increased ERK phosphorylation compared with control. The effect of KP-10 compared with [dY]KP-10 was not significantly different. Although several other analogs tested increased ERK phosphorylation, these changes did not reach statistical significance (Fig. 2A).

Following this study, a dose-response study was carried out to compare the effects of [dY]KP-10 and KP-10 on ERK phosphorylation. Treatment with KP-10 or [dY]KP-10 at 1,000 or 10,000 nM doses significantly increased ERK phosphorylation compared with control (Fig. 2B). There was no significant difference between the effects of KP-10 and [dY]KP-10 at equal doses.

**Studies to Determine Efficacy of Analogos In Vivo**

**To determine a dose-response for ip KP-10 in mice.** Administration of 10 or 30 nmol KP-10 significantly increased plasma LH and administration of 1, 10, or 30 nmol KP-10 significantly increased plasma testosterone at t = 20 (Table 2). Because a dose of 1 nmol KP-10 appeared to result in a maximal stimulation of testosterone, a lower dose of 0.5 nmol was used for future analog studies.

Plasma FSH was not significantly changed by KP-10 administration at any dose at this time point (data not shown).
In vivo screen of analogs with IC<sub>50</sub> < 10 nM. Administration of all analogs and KP-10 increased total testosterone at t = 20 min, but the relatively low number meant that only the effects of ANA14, ANA17, and ANA19 ([d]-KP-10) were statistically significant (Fig. 3).

ANA5 and ANA8 had previously been tested in vitro for their ability to activate KISSIR in a LtrZ reporter gene assay in yeast (22). ANA5 had been reported to have superagonistic activity in vitro, and, in contrast, ANA8 had been reported to have no agonistic activity at a concentration of 10 μM. Since our studies suggested that ANA8 did bind to the KISSIR, and that ANA5 was thus a potential KISSIR antagonist, we investigated the effect of both ANA5 and ANA8 in vivo.

ANA5 stimulated the release of testosterone at 20 min. However, this effect was not as great as that of KP-10. At a dose of 0.5 nmol, ANA5 did not influence testosterone release. However, at a higher dose of 10 nmol, ANA8 significantly stimulated testosterone release (Fig. 4), suggesting that ANA8 is a weak agonist of the KISSIR, rather than an antagonist.

Table 2. Effect of intraperitoneal administration of KP-10 on plasma levels of LH and testosterone in male mice 20 min postinjection in vivo

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LH (ng/ml)</th>
<th>Testosterone (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.45 ± 0.046</td>
<td>0.55 ± 0.246</td>
</tr>
<tr>
<td>KP-10 0.3 nmol</td>
<td>0.705 ± 0.778</td>
<td>4.430 ± 4.445</td>
</tr>
<tr>
<td>KP-10 1 nmol</td>
<td>2.27 ± 0.016</td>
<td>36.953 ± 1.270*</td>
</tr>
<tr>
<td>KP-10 10 nmol</td>
<td>3.124 ± 1.049*</td>
<td>36.728 ± 0.936*</td>
</tr>
<tr>
<td>KP-10 30 nmol</td>
<td>4.494 ± 0.134*</td>
<td>37.067 ± 4.028*</td>
</tr>
</tbody>
</table>

Results are means ± SE. *P < 0.01 vs. saline (ANOVA with post hoc Tukey's adjustment), n = 6.
Comparing the time course of the effect of ip [dY]2KP-10 and KP-10 on plasma LH and testosterone. [dY]2KP-10 has a lower binding affinity for the KISS1R than KP-10, suggesting that its increased potency in vivo could be due to increased longevity in the circulation.

Thus we compared the time course of the effects of 0.5 nmol KP-10 and [dY]2KP-10 at 20 and 60 min postinjection on LH and total testosterone levels in mice. At 20 min postinjection, [dY]2KP-10 significantly increased LH. LH was elevated following KP-10 at 20 min postinjection, but this did not reach statistical significance. However, both KP-10 and [dY]2KP-10 significantly increased testosterone at 20 min [t = 20, plasma LH: 0.649 ± 0.203 ng/ml (saline), 2.535 ± 0.773 ng/ml (KP-10), 3.115 ± 0.758 ng/ml* [dY]2KP-10]; testosterone: 3.373 ± 1.101 nmol/l (saline), 74.499 ± 17.903 nmol/l* (KP-10), 85.185 ± 30.860 nmol/l* [dY]2KP-10; n = 4–6; *P < 0.05 vs. saline]. At 60 min postinjection, neither KP-10 or [dY]2KP-10 significantly raised LH, but [dY]2KP-10 did significantly raise testosterone [t = 60, plasma LH: 0.720 ± 0.110 ng/ml (saline), 1.049 ± 0.316 ng/ml (KP-10), 1.401 ± 0.226 ng/ml [dY]2KP-10]; testosterone: 3.815 ± 0.789 nmol/l (saline), 50.116 ± 22.474 nmol/l (KP-10), 94.572 ± 29.471 nmol/l* [dY]2KP-10; n = 4–6; *P < 0.05 vs. saline].

To better examine potential differences between the effects of KP-10 and [dY]2KP-10, we compared the time course of the effects of a lower dose of 0.15 nmol KP-10 and [dY]2KP-10 at 20, 60, and 120 min postinjection on LH and total testosterone levels in mice.

At 20 min postinjection [dY]2KP-10 significantly increased LH, but KP-10 did not. There was a trend toward an increase in LH following [dY]2KP-10 at 60 min postinjection, which did not reach statistical significance (Fig. 6A). At 120 min, plasma LH had returned to baseline in all groups (data not shown). [dY]2KP-10 significantly increased plasma testosterone levels at 20 and 60 min postinjection. Testosterone was raised in response to KP-10, but this change did not achieve statistical significance at any time point investigated (Fig. 6B).
residues at position 6 and position 10 appear critical for KISS1R binding. Exchanging these phenylalanines for alanine, a similar amino acid, or o-phenylalanine greatly reduced the molecule’s affinity for KISS1R.

During the course of these studies, several papers were published investigating the effect of sequential manipulation of KP-10 on in vitro and in vivo activity (8, 22, 24, 27). Nida et al. (22) investigated the effects of a number of the human KP-10 analogs that we were also studying, specifically ANA1–10 (which they also designated ANA1–10) and 19–21 (ANA11, 16, and 20, respectively, in their studies) on the in vitro activation of human KISS1R using a LacZ reporter gene system in yeast. Orsini et al. (24) also investigated the binding and agonistic activity of ANA1–10 and ANA15–17 at the human KISS1R. We have also investigated the receptor binding affinity and the ability of these analogs to stimulate intracellular signaling pathways, although in our case by measuring phosphorylation of ERK1/2. In addition, we have investigated the in vivo activity of those analogs that bound relatively well to the KISS1R. Our data are generally in accord with those described by Nida et al. and Orsini et al. However, unlike Nida et al., although in accord with Orsini et al., we found that although ANA5 bound with higher affinity to the KISS1R than KP-10, it was not more potent than KP-10 in vitro. We have also presented data that suggests that ANA5 is less potent than KP-10 in vivo. Thus while ANA5 may act as a KISS1R super agonist in specific in vitro systems, it does not appear to have greater activity than KP-10 in vivo.

Rosner et al. (27) determined the binding and activity of a number of human KP-10 analogs at the human KISS1R in a series of studies to design an effective KISS1R antagonist. Their results also highlighted the importance of the five COOH-terminal amino acids to receptor activation. Because of the systematic design of these experiments, many of the analogs investigated incorporate a number of different substitutions. Other evidence suggests that the conformation of the Phe7-Gly6 peptide bond is important for bioactivity in human KP-10 (34). Specific amino acid substitutions at positions 1, 5, and 8 resulted in a high-affinity KISS1R antagonist, suggesting that these positions are important for the bioactivity of KP-10 (27).

Gutiérrez-Pascual et al. (8) investigated the rat analogs equivalent to the human analogs we designated ANA1–10, replacing sequential residues in the rat KP-10 molecule with alanine. Our findings are broadly in agreement with theirs. Their study also suggests that residues 9 and 10 are crucial for bioactivity of rat KP-10 at the rat Kissr. They suggest that the low agonist activity of ANA10 may reflect the importance of residue 10 in binding to the Kissr, but that this requires experimental confirmation. Binding data from our study and from Orsini et al. (24) strongly suggests that this is the case and that residue 10 of human KP-10 is critical for receptor binding.

It would be interesting to determine the effects of a rodent [dY]KP-10 analog on the HPG axis, and in particular whether such an analog would have greater bioactivity at the human KISS1R. Our studies did identify a novel potential super agonist for KISS1R. Interestingly, in the studies by Niida et al. (22), [dY]KP-10 did not demonstrate any activation of KISS1R. This may reflect the different reporter systems used. In our studies, [dY]KP-10 bound to the KISS1R with approximately fourfold lower affinity than KP-10. However, the increase in in vitro bioactivity exhibited by this peptide led to it being further

**Fig. 6.** Effect of ip administration of 0.15 mmol [dY]KP-10 and KP-10 on LH (A) and plasma total testosterone (B) in male mice at 20 and 60 min postinjection in vivo. ***P < 0.001 vs. saline, **** P < 0.001 vs. equivalent KP-10 (ANOVA with Post hoc Tukey's adjustment); n = 4–7.

**DISCUSSION**

Investigating the in vitro and in vivo bioactivity of a series of KP-10 analogs has identified [dY]KP-10 as a KISS1R agonist with greater in vivo bioactivity than KP-10. The kispeptins stimulate the HPG axis and thus offer a target for therapies to treat reproductive disorders. KP-10 has a similar effect in vitro to its longer counterpart, KP-54. However, KP-10 is less potent than KP-54 in vivo, possibly because it is broken down more quickly in the circulation (32). This limits the potential clinical utility of kispeptin-10 itself. KP-54 is a much longer peptide, and consequently more expensive to produce, making it less economically viable as a potential therapy. Rational modification of the KP-10 structure to increase bioactivity may produce a molecule with increased therapeutic potential.

Initial receptor binding studies identified the residues in KP-10 critical for receptor binding. The phenylalanine (F)
investigated in vivo. Despite its lower affinity, it demonstrated a similar potency in vitro and greater potency in vivo than KP-10. The difference in concentrations required for the effects may be due to the use of an artificial reporter system of CHO-K1S1R cells. [ΔVY]KP-10 appeared to have a more potent effect on LH and testosterone than KP-10 itself in vivo. No effect was seen on FSH, which may reflect that a higher dose was required. Endogenous kisspeptin has been reported to have an EC50 for its stimulatory effect on FSH ten times greater than that for its effect on LH (51).

Further experiments are required to determine whether the increased potency of [ΔVY]KP-10 in vivo reflects increased longevity of the molecule in circulation. [ΔVY]KP-10 had a lower affinity for the human KISS1R in vitro, suggesting that its increased effects may not be due to improved receptor binding. The tyrosine at position 1 of endogenous KP-10 may therefore be a target for proteolytic cleavage. Alternatively, altering the molecule at this point may alter its function in a more significant way at other sites. It may be possible to further increase the longevity of the effects of [ΔVY]KP-10 extending the molecule to convert it to an analog of KP-54. Although this would increase the cost of synthesis, the increased longevity may be sufficient to justify this cost. It is interesting that ip administration of KP-10 to male mice at doses of up to 0.15 nmol did not significantly increase LH levels at 20 min following injection. We have previously found that relatively high doses of human KP-10 are required to stimulate the HPG axis in rats following peripheral administration (32, 33). Mikkelson et al. (16) have compared the effects of ip administration of mouse and human KP-10 on testosterone in mice and suggest that mouse KP-10 is more potent than human KP-10 at stimulating testosterone release in this model. The lack of effect we observed may therefore reflect lower effectiveness of human KP-10 in mice. The utility of manipulating the kisspeptin system as a disease treatment is still to be demonstrated. Several groups have suggested that this approach may have clinical utility in the treatment of reproductive disorders, such as delayed puberty, prostatic cancer, and metastatic cancer (9, 22, 28, 35). Our studies have identified and tested a number of KP-10 analogs, one of which, [ΔVY]KP-10, has greater in vivo bioactivity than KP-10 itself. Further studies are required to explore the therapeutic potential of KISS1R super agonists.

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DISCLOSURES

The authors have nothing to disclose.

REFERENCES


Identification of the Hormone Kisspeptin in Amniotic Fluid

To the Editor:

Kisspeptin is the product of the KISS1 (KISS-1 metastasis-suppressor) gene and is the ligand for the G protein–coupled receptor, now known as the KISS1 receptor (KISS1R)1. Both kisspeptin and KISS1R play a crucial role in the regulation of reproduction and puberty.1 The KISS1 gene encodes a precursor peptide of 145 amino acid residues, which undergoes proteolytic processing to generate kisspeptins 10, 13, 14, and 54 (1). These peptides all share the common C-terminal decapeptide necessary for receptor activation (1). Inactivating mutations in the human KISS1R (KISS1 receptor) gene cause hypogonadotropic hypogonadism (1). During pregnancy, circulating plasma kisspeptin concentrations rise by

1 Human genes, KISS1, KISS-1 metastasis-suppressor, KISS1R, KISS1 receptor.
2 Nontandem abbreviations: KISS1, KISS1 receptor (previous symbol, GPR54); K, immunoreactivity.
7000-fold in the third trimester, compared with the concentrations in nonpregnant women (2). Plasma markers that are altered during pregnancy, such as huma

...nornal abnormalities or therapeutic amniocentesis for polyhydramnios. All volunteers were in their second or third trimester [mean (SD) gestational age, 17.9 (0.9) weeks] and the mean gestational age at partum was 39.2 (0.28) weeks. Exclusion criteria were marked comorbidity and an age <18 years or >45 years. Medical records were reviewed, and amniotic fluid analysis results, pregnancy complications, and pregnancy and birth outcomes were recorded. In all cases, the amniocentesis was uncomplicated, and the outcome of the pregnancy was a healthy baby with a typical karyotype.

We collected 2 mL of amniotic fluid into sterile containers containing 5000 kallikrein inhibitor units of aprotinin (0.2 mL Trasylol; Bayer). Samples were stored at −20°C until measurement of kispeptin immunoreactivity (IR) as previously described (3). To reduce preanalytical factors shown to influence RIA measurement of kispeptin (4), we collected and stored all samples in an identical fashion.

The peptide was extracted from amniotic fluid with Sep-Pak C18 cartridges (Waters) according to the manufacturer’s instructions, and kispeptin IR was characterized in amniotic fluid by fast protein liquid chromatography, as previously described (5).

The mean (SE) kispeptin IR in amniotic fluid was 95.9 (14) pmol/L. There was no correlation between gestational age and kispeptin concentration in amniotic fluid (P = 0.56; Fig. 1). Amniotic kispeptin concentrations for the two fetus sexes were similar [mean kispeptin IR, 95.2 (27.8) pmol/L and 105.9 (23.2) pmol/L for male and female fetuses, respectively; P = 0.77]. There was no correlation between kispeptin concentration in the amniotic fluid and either birth weight (P = 0.67) or gestational age at partum (P = 0.58). Kispeptin IR eluted as a single peak at a position consistent with the elution profile of kispeptin 54. The calculated mean chromatographic recovery was 46% (7%) (n = 3).

This report is the first to identify kispeptin in amniotic fluid. Kispeptin 10 has been shown to inhibit migration and invasion of trophoblast cells in placenta (2); thus, the concentration of kispeptin in amniotic fluid may be associated with pregnancy outcomes. The concentrations of amniotic fluid kispeptin (identified as IR in this study) were much lower than those reported for circulating maternal plasma (2). In contrast to maternal plasma concentrations, kispeptin in amniotic fluid was not observed to increase with gestational age. These findings are perhaps unsurprising, given that a number of amniotic fluid biomarkers display different concentrations during gestation, compared with the concentrations in maternal serum (5). It is possible that the lack of an observed correlation between the kispeptin concentration in amniotic fluid and gestational age reflects the small number of participants in this study. Amniocentesis is an invasive procedure with an associated risk of miscarriage and is most commonly performed during the second trimester; therefore, the majority of our samples were from women in the second trimester [mean gestational age, 17.9 (0.9) weeks]. Because of this risk, we performed no serial sampling of
amniotic fluid. The study therefore had a limited scope to investigate correlations between the concentration of kispeptin in amniotic fluid, the plasma kispeptin concentration, and gestational age.

In this study, the pregnancy outcome in all cases was a healthy baby with no chromosomal abnormalities identified. It would be interesting to investigate kispeptin concentrations in amniotic fluid samples from a wider cohort to investigate their utility in predicting pregnancy outcome. Given the high placental expression of KISS1, it is likely that the kispeptin IR detected in amniotic fluid was derived from the placenta (2). Thus, it would be interesting to examine any correlation between the kispeptin concentration in amniotic fluid and placental weight.

References

To the Editor:

Demethylase-Suppressed Corticotropin-Releasing Hormone-Stimulation Test Does Not Reliably Diagnose or Predict Recurrence of Cushing Disease

First-line treatment for Cushing disease is surgical removal of the adrenocorticotropin-secreting pituitary tumor. Because of the high risk of relapse, it is essential that patients receive long-term postoperative follow-up for disease recurrence through expert clinical evaluation and biochemical assessment of hypercortisolism, including the use of demethylas suppression (1). However, no gold-standard test has been shown to accurately predict recurrence (1). The demethylas suppression corticotropin-releasing hormone-stimulation (LEDEST-CRH) test was initially proposed to be more accurate in confirming hypercortisolism than the standard low-dose demethylas-suppression test.

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ORIGINAL ARTICLE

Subcutaneous oxyntomodulin analogue administration reduces body weight in lean and obese rodents

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Objective: To determine the efficacy of a long-acting oxyntomodulin (OXM) analogue, OXM6421, in inhibiting food intake and decreasing body weight in lean and diet-induced obese (DIO) rodents.

Research design and methods: The glucagon-like peptide-1 (GLP-1) receptor binding affinity and efficacy, sensitivity to enzymatic degradation in vitro and persistency in the circulation after peripheral administration were investigated for OXM6421 and compared with native OXM. The chronic effect of OXM6421 on food intake, body weight and energy expenditure was examined in lean rats, and its anti-obesity potential was evaluated in DIO mice.

Results: OXM6421 showed enhanced GLP-1 receptor binding affinity and cyclic adenosine monophosphate (cAMP) stimulation, and higher resistance to enzymatic degradation by dipeptidyl peptidase IV (DPP-IV) and neutral endopeptidase (NEP) compared with native OXM. OXM6421 persisted longer in the circulation than OXM after peripheral administration.

Acute administration of OXM6421 potently inhibited food intake in lean rodents, with cumulative effects lasting up to 24h. In lean rats, daily subcutaneous (s.c.) administration of OXM6421 caused greater weight loss than the pair-fed animals, and a higher rate of oxygen consumption than both the pair-fed and the saline controls. In DIO mice, continuous s.c. infusion of OXM6421 resulted in a significant weight loss, accompanied by an improvement in glucose homeostasis and an increase in circulating adiponectin levels. Once-daily s.c. administration of OXM6421 for 21 days caused sustained weight loss in DIO mice.

Conclusion: OXM6421 induces negative energy balance in both lean and obese rodents, suggesting that long-acting OXM analogues may represent a potential therapy for obesity.

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Keywords: oxyntomodulin analogue; food intake; body weight; energy expenditure

Introduction

Obesity is a global health problem, with its prevalence increasing at an alarming rate.1 It is associated with an increased early mortality rate because of many serious conditions, including type 2 diabetes, cardiovascular disease and various types of cancer.2,3 Thus, medical treatment of obesity has become a necessity. However, the currently available anti-obesity drugs are only moderately effective and all have associated side effects.4 At present, the most effective treatment for obesity is bariatric surgery,5 but its cost and associated mortality make it impractical to cope with the obesity epidemic.4 Thus, there is a vast unmet medical demand for a safe and effective anti-obesity therapy.

Recent evidence suggests that neural and endocrine signalling from the gut has an important physiological role in postprandial satiety and manipulation of the gut-brain axis may offer targets for future obesity therapies.6 Oxyntomodulin (OXM), a 37-amino-acid peptide, is a product of the proglucagon gene that is released postprandially from the L-cells of the small intestine in proportion to calorie intake.7 The appetite-suppressing and weight-reducing effects of OXM have been shown in both rodents and humans.8–12 OXM is thus a putative target for obesity therapy. However, its clinical utility is limited, mainly because of its short circulating half-life, which is estimated to be...
approximately 12 min. This is a major drawback, as multiple daily doses of large quantities of peptide would be required to sustain its effect, making it medically and economically unviable as an anti-obesity agent. It has been shown that renal clearance accounts for 35% of the peptide disappearance from the plasma after OXM administration in rats. In vitro protease degradation assays suggest that OXM may be susceptible to proteolysis by dipeptidyl peptidase IV (DPP-IV) and neutral endopeptidase 24.11 (NEP). To overcome the factors limiting OXM as a potential anti-obesity therapy, we have recently investigated the structure-function relationships and the degradation pathways of OXM, and identified the parts of the OXM sequence that are sensitive to degradation and/or involved in the binding to the glucagon-like peptide-1 (GLP-1) receptor. Several novel peptide analogues of OXM were synthesized, incorporating changes to the amino-acid sequence that were intended primarily to confer resistance against degradation by proteases (DPP-IV and NEP), and enhance binding affinity for the GLP-1 receptor. One OXM analogue, coded OXM6421, showed the greatest potential in initial feeding studies in lean rodents.

In this study, we characterized the long-acting OXM analogue OXM6421, investigating its binding affinity to the GLP-1 receptor and stimulation of cyclic adenosine monophosphate (cAMP) production, its effects on the release of hypothalamic neuropeptides and its sensitivity in vitro to degradation by DPP-IV and NEP. The rate of peptide disappearance from plasma following peripheral administration was also examined and compared with OXM. The chronic effects of OXM6421 on food intake, body weight and energy expenditure were investigated in lean rats. Its anti-obesity potential was evaluated in diet-induced obese (DIO) mice. The results suggest that OXM6421 may offer greater potential as an obesity therapy than native OXM.

Materials and methods

Materials

OXM analogue OXM6421 was synthesized by Bioled International LP (Exeter, UK). Human/mouse/rat OXM (all have the same sequence) was obtained from Itechem (St Helens, UK) and was used for all studies described below.

Animals

One hundred and fifty-five male Wistar rats (250–300 g, Charles River, UK) and fifty-four C57Bl/6 male mice (20–25 g, Harlan, UK) were singly housed and maintained at 21–23 °C under a 12:12 h light-dark cycle (light period 0700–1900 h) with free access to normal chow (RMi diet, Special Diet Services, Witham, UK) and water. For the DIO model, 6, 6-week-old C57Bl/6 male mice (Harlan, UK) were maintained on a high-fat diet (60% fat kcal; D12492, Research Diets, New Brunswick, NJ, USA) for 18 weeks. All animal procedures undertaken were approved by the British Home Office Animals (Scientific Procedures) Act 1986 (Project License 70/6402).

GLP-1 receptor binding assay

Rat lung has been shown to have high levels of GLP-1 receptor and studies have shown that the binding values using rat lung membrane are comparable to those from GLP-1 receptor over-expressing cell lines. Plasma membranes from rat lung tissue were prepared and used as previously described. Exendin-4 was used as label due to the instability of GLP-1. Membranes were incubated for 90 min with [125I]Exendin-4 (500 pM, 100 pmol) and unlabeled peptides (OXM, OXM6421) at 20 °C. Bound and free label were separated by centrifugation and bound radioactivity was measured using a gamma-counter. Specific binding was calculated as the difference between the amount of [125I]Exendin-4 bound in the absence and presence of unlabeled competing peptide.

cAMP assay

Chinese hamster lung fibroblast cells stably transfected with rat GLP-1 receptor (kind gift from Professor Bernard Thorens, University of Lausanne, Switzerland) were used for the cAMP bioassay, wherein cAMP production was measured using a cAMP Flaplate assay kit (PerkinElmer, Cambridge, UK). Confluent cells were harvested using 0.02% EDTA solution and re-suspended in stimulation buffer containing 3-isobutyl-1-methylxanthine, 1 µM (PerkinElmer) at a concentration of 5 × 10⁵ cells per 50 µL. Cell suspension (50 µL) was added to each well with varying concentrations of OXM6421 or OXM (concentration range 0.001 pm-500 nm). After 30 min incubation, 100 µL detection mix containing 0.74 kBq cAMP-[3H] tracer was added to each well. The plate was sealed and incubated for a further 2 h at room temperature, then counted using a 96-well microplate scintillation counter (Wallace Micrototra Trilux, Perkin-Elmer). The cAMP concentration was calculated using a non-linear plot (Prism, GraphPad software), and results quantified from a cAMP standard curve according to the manufacturer’s instructions.

Hypothalamic explant static incubation system

The effect of OXM6421 on the hypothalamic release of neuropeptides was determined as previously described. Briefly, hypothalamic explants from male Wistar rats were incubated with OXM6421 at 10, 100 and 1000 nm for 45 min in 600 µL of artificial cerebral spinal fluid after an initial 2-h equilibration and a 45-min basal incubation period. The viability of the tissue was verified by a final 45-min exposure to artificial cerebral spinal fluid containing 56 nm KCl. At the end of each period, the artificial cerebral spinal fluid was
collected and stored at −20 °C until the measurement of hypothalamic hormones. Levels of alpha-melanocyte-stimulating hormone (α-MSH), cocaine- and amphetamine-related transcript (CART), Agouti-related protein (AgRP) and neuropeptide Y (NPY) were measured using our in-house radioimmunoassays as previously described.12–25

**In vitro degradation assays**

DPP-IV protease assay. Digest buffer (100 mM TRIS-HCl, pH 8) containing 15 μM of peptide and 1 μg of porcine kidney DPP-IV (Sigma, Missouri, USA) was incubated at 37 °C. The reaction was terminated at the specified time point by addition of 5% 10% trifluoroacetic acid. The incubated samples were analysed by reverse-phase high-pressure liquid chromatography (Jasco HPLC system; Jasco Ltd., Great Dunmow, UK; Phenomenex, Phenomenex Ltd., Macclesfield, UK). Gemini C18 250 mm × 4.6 mm column, linear gradient of 27–31% acetonitrile in 0.05% trifluoroacetic acid/water in 0.05% trifluoroacetic acid over 50 min at 1 ml/min. Peptides and their degradation products were monitored by their absorbance at 214 nm. Percentage degradation was quantified by integration of peak areas related to undigested peptide peaks.14

NEP protease assay. The NEP protease assay was completed as in the DPP-IV assay, except for the following changes: digest buffer (50 mM TRIS-HCl, 50 mM NaCl, pH 7.5) and 200 ng of recombinant human NEP2 (R&D Systems, Wiesbaden, Germany). The incubated samples were run and analysed as above, but with a linear gradient of acetonitrile in 0.05% trifluoroacetic acid (15–60% acetonitrile over 30 min). Degradation products were monitored by their absorbance at 214 nm.15

**In vivo disappearance times of OXM6421 compared with OXM**

To investigate the longevity of detectable levels of OXM6421 and OXM in the circulation, rats were anaesthetized with an intraperitoneal administration of a mixture of hypnorm (trentalanyl citrate and fluanisone, Veta Pharma, Leeds, UK) and hyponol (midazolam hydrochloride, Roche, Herts, UK). The jugular vein was cannulated and blood samples were drawn immediately before (baseline), and at 5, 10, 20, 30, 60, 90 and 120 min after a subcutaneous (s.c.) administration of either 20 nmol kg⁻¹ OXM6421 or 2000 nmol kg⁻¹ OXM (these doses were approximately equivalent in their anorectic effect during the first 2 h after peptide administration in early dose-rangings studies). Plasma levels of OXM-like activity were measured using established in-house radioimmunoassays. For the OXM6421 assay, the antiserum raised in a rabbit immunized with OXM6421 conjugated to bovine serum albumin by carbodiimide cross-react 100% with OXM6421, <0.05% with OXM and extendin-4, and <0.01% with GLP-1 and glucagon. The OXM6421 label was prepared using the iodogen method and purified by high-pressure liquid chromatography. The specific activity of OXM6421 label was 53.8 Bq μmol⁻¹.

**Acute feeding study in fasted lean rats**

Rats were fasted for 24 h and received a s.c. administration of either OXM6421 (10 nmol kg⁻¹) or saline (maximum volume of 0.15 ml) between 0900 and 1000 hours. A pre-weighted amount of normal chow was provided to the rats immediately after the injection and food consumption was measured at 1, 2, 4, 6, 8 and 24 h after injection.

**Effects of daily peripheral injection of OXM6421 on food intake and body weight in lean rats**

As 10 nmol kg⁻¹ OXM6421 inhibited food intake significantly, 5 and 20 nmol kg⁻¹ doses of OXM6421 were chosen for the daily administration study. To test the effectiveness of these doses on acute feeding, rats were fasted overnight before receiving their first s.c. administration of 5 or 20 nmol kg⁻¹ of either OXM6421 or saline. Based on the pharmacokinetic characteristics of OXM6421, a washout period was not considered necessary before proceeding with the daily administration study. The animals continued to receive a daily s.c. administration of either saline or OXM6421 (5 or 20 nmol kg⁻¹) at between 0900 and 1000 hours for a further 13 days, with both food and body weight measured daily. Treatment was withdrawn on day 14 (first dose was given on day 0 and daily food intake and body weight measurements continued for a further 14 days (follow-up period).

**Effects of continuous s.c. infusion of OXM6421 on energy expenditure in lean rats**

To investigate the effect of OXM6421 on energy expenditure, a pair-feeding paradigm was first used to examine whether the reduction in weight gain in the OXM6421-treated rats was attributable solely to the reduction of food intake. This was followed by the measurement of oxygen consumption by indirect calorimetry with the Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH, USA), with the goal of assessing resting energy expenditure. Rats were acclimatized to the Comprehensive Laboratory Animal Monitoring System for 48 h before pump implantation. They were then randomized into three groups of approximately equal body weight (control, pair-fed and OXM6421). On the morning of the surgery, Alzet osmotic pumps (Durect; Model 2001; duration: 7 days) were implanted in the interscapular region under isoflurane anaesthesia. The mini-pumps for both control and pair-fed groups contained saline, whereas the OXM6421 group contained OXM6421 at an infuion rate of 30 pmol min⁻¹. This dose was selected on the basis of initial dose-ranging feeding studies with OXM and OXM analogues, in which we had found that a total daily infusion dose of approximately two-fold the acutely effective dose (i.e., 20 nmol kg⁻¹ s.c.) is required to effectively reduce food intake and body weight. After the surgery, rats were allowed to recover, returned to their home cage and their food and body weight measured daily at 0900-1000 hours.
Animals in the control and OXM6421 groups had ad libitum access to food. The pair-fed animals (pumps contained no OXM6421) were food restricted to receive the median daily food intake of the OXM6421-treated group from the previous day. On day 8, animals were transferred to the Comprehensive Laboratory Animal Monitoring System without food for a period of 6 h (0900–1500 hours). Oxygen consumption (ml kg\(^{-1}\) h\(^{-1}\)) was measured by indirect calorimetry at 24°C. Locomotor activity was measured simultaneously using optical beam sensors (Opto M3 animal activity meter; Columbus Instruments, Columbus, OH, USA).

**Acute feeding study in lean mice**

To test the effectiveness of OXM6421 in a second species, an acute feeding study was carried out in overnight fasted C57BL/6 mice and compared with OXM. Mice received an intraperitoneal administration (injection) with either OXM6421 (5 or 20 nmol kg\(^{-1}\)) or OXM (20 or 2000 nmol kg\(^{-1}\)) or saline at between 0900 and 1000 hours. Following injection, animals were returned to their home cages containing a pre-weighed amount of food, which was re-weighed at 1, 2, 4, 6 and 24 h post injection.

**Effects of continuous s.c. infusion of OXM6421 or OXM on food intake and body weight in DIO mice**

Male DIO mice were randomized by body weight into four groups, and were implanted with Alzet osmotic pumps (Durect; Model 1007D, DURECT Corporation, Cupertino, CA, USA) containing either OXM6421 at a delivery rate of 10 or 30 nmol kg\(^{-1}\) h\(^{-1}\) (a daily dose of 14.4 or 43.2 nmol kg\(^{-1}\)) or OXM at 2000 nmol kg\(^{-1}\) min\(^{-1}\) (a daily dose of approximately twofold of the acute effective dose for the inhibition of food intake), or saline. Food intake and body weight were monitored daily between 0900 and 1000 hours for 7 days. On day 8, mice were killed and blood collected by cardiac puncture. Blood glucose concentration was measured immediately with an Ascensia blood glucose meter (Bayer Healthcare, Newbury, UK). Plasma was stored at −20°C for the determination of insulin, leptin and adiponectin levels by radioimmunoassays (Linco Research, Billerica, MA, USA). The residual pump contents were extracted and stored at −20°C for analysis by high-pressure liquid chromatography.

**Effects of daily peripheral injection of OXM6421 on food intake and body weight in DIO mice**

To test the sensitivity of the DIO mice to an acute s.c. dose of OXM6421, they were fasted overnight before receiving their first injection on day 0, and food intake (high-fat chow) measured at 1, 2, 4, 6 and 24 h. Thereafter, DIO mice were daily subcutaneously administered either saline or OXM6421 (20 nmol kg\(^{-1}\)) for 23 days (starting from day 0) at 0900–1000 hours. The mice were maintained on the high-fat diet for the duration of the study with food intake and body weight monitored daily. Treatments were withdrawn on day 21. Daily food intake and body weight measurements were continued for a further 14 days.

**Statistical analysis**

Statistical advice was obtained from the Imperial College Statistical Advisory Service (Imperial College London). Results are shown as mean and s.e.m. Ex vivo data from hypothalamic static incubation experiments were analysed using a paired t-test to compare the basal and test periods for each explant. An unpaired t-test was used for the in vitro data and the disappearance times from plasma. Multiple data from acute feeding studies and plasma measurements were analysed using one-way statistical analysis of variance followed by post-hoc Dunnet’s test. In all cases, a P < 0.05 was considered to be statistically significant. Chronic studies were analysed using a non-parametric two-sample Wilcoxon rank-sum (Mann–Whitney) test comparing the treatment group with the control group. Generalized estimating equation was used to compare the control and treatment groups over the entire study period. Statistical analysis was performed using Stata 9 (StataCorp, College Station, TX, USA).

**Results**

**Comparison of (GLP-1) receptor binding affinity and cAMP production of OXM6421 with OXM**

OXM6421 showed a 30-fold greater affinity for the GLP-1 receptor than OXM as shown by the IC\(_{50}\) values (0.98 versus 29.1 nM; P < 0.001; n = 3–4; Table 1). The affinity of OXM for the GLP-1 receptor was similar to that found in our earlier studies comparing the IC\(_{50}\) of OXM, GLP-1, and exendin-4 (33.1, 0.34, and 0.27 nM, respectively).

In accord with these results, OXM6421 was 30-fold more potent at stimulating cAMP levels in Chinese hamster lung fibroblast cells stably transfected with rat GLP-1 receptor (P < 0.001; n = 3–4; Table 1).

**Effect of OXM6421 on ex vivo release of neuropeptides from rat hypothalamus**

OXM6421 at 100 and 1000 nM significantly increased alpha-melanocyte-stimulating hormone release from hypothalamic explants (161 and 177% versus their baseline values).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>GLP-1 receptor binding activity, cAMP production and sensitivity to produce degradation of OXM6421 and oxyntomodulin (OXM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OXM</td>
</tr>
<tr>
<td>GLP-1 receptor affinity (IC_{50}) (nM)</td>
<td>29.1 ± 4.4</td>
</tr>
<tr>
<td>cAMP production (EC_{50}) (nM)</td>
<td>0.65 ± 0.08</td>
</tr>
<tr>
<td>DPP-4 degradation at 24 h (%)</td>
<td>100 ± 0.0</td>
</tr>
<tr>
<td>Neuropeptide degradation at 24 h (%)</td>
<td>77.5 ± 5.0</td>
</tr>
</tbody>
</table>

Abbreviations: cAMP, cyclic adenosine monophosphate; DPP-4, dipeptidyl peptidase IV; GLP-1, glucagon-like peptide-1; OXM, oxyntomodulin. Results are means ± s.e.m., n = 3–4. **P < 0.01, ***P < 0.001 versus OXM.
values, respectively; *P < 0.05; n = 7–9). However, OXM6421 had no significant effect on cocaine-and amphetamine-regulated transcript, Agouti-related protein and neuropeptide-Y release (Figure 1).

In vitro degradation of OXM6421 compared with OXM

When peptides were incubated with DPP-IV (1 μg) for a period of 2 h, no degradation of OXM6421 was observed, whereas no OXM was detectable after 2 h incubation. Degradation of OXM6421 by NEP (200 ng) was also markedly reduced compared with OXM (64 and 77% degradation for OXM6421 and OXM, respectively; n = 3; Table 1).

In vivo disappearance time of OXM6421 versus OXM

Both OXM6421 and OXM reached peak plasma concentration by approximately 10 min post s.c. administration (n = 5). However, plasma levels of OXM declined sharply by 30 min post administration, whereas plasma levels of OXM6421 remained elevated throughout the whole study duration (2 h; Figure 2).

Effects of a single dose of OXM6421 on food intake in fasted lean rats

OXM6421 (10 nmol·kg⁻¹) caused a significant, 54% reduction in cumulative food intake over 8 h (P < 0.0001) compared with the saline controls. At 24 h post injection, cumulative food intake of the OXM6421 group was 13% less than that of the saline controls (P < 0.001; Figure 3). However, there were signs of the anorectic effect waning between 8 and 24 h.

Effects of chronic daily administration of OXM6421 on food intake and body weight gain in lean rats

Based on the results of the acute feeding study in rats, two doses (5 and 20 nmol·kg⁻¹) were chosen for the chronic study assessing the effects of OXM6421 in reducing food intake and body weight gain in rats. The 5 nmol·kg⁻¹ daily dose of OXM6421 had no significant effect on cumulative food...
intake or body weight change. However, at 20nmol·kg⁻¹, OXM6421 caused a 15% reduction in total food intake over the 14-day treatment period (P<0.01 versus saline; n=8-9; Figures 4a and b). Daily food intake during the post-treatment period was similar between the groups (Figure 4b).

From day 4, body weight was significantly lower in the OXM6421 20nmol·kg⁻¹ group (P<0.05). At the end of the 14-day treatment period, body weight in the OXM6421-treated rats was 9% less than that of the controls (P=0.01). The significant weight reduction was sustained for the period studied after withdrawal of the treatment (Figure 4c).

Energy expenditure following continuous s.c. infusion of OXM6421, compared with pair-fed and control lean rats
Cumulative food intake over the 7-day period was reduced by 36% in the OXM6421-treated (30pmol·kg⁻¹·min⁻¹) compared with the saline controls (P<0.001; Figure 5a). Body weight was decreased in both OXM6421 and pair-fed groups (P<0.001 versus saline control), and the weight loss in the OXM6421 group was significantly greater than that in the pair-fed group (P<0.05 versus pair-fed; n=12; Figure 5b). During subsequent monitoring in the Comprehensive Laboratory Animal Monitoring System, there was a significant difference in oxygen consumption between the control and the OXM6421-treated (P<0.0001; generalized estimating equation), and between the pair-fed and the OXM6421-treated (P<0.0001; generalized estimating equation), but not between the control and the pair-fed animals (Figure 5c).

OXM6421 increased the mean oxygen consumption compared with both the control and the pair-fed groups (ml/kg⁰⁷·h⁻¹·min⁻¹: 240±34 for the control; 2812±48 for the OXM6421-treated; 2530±45 for the pair-fed; P<0.001; Figure 5d). The mean body weights on the morning of the VO₂ measurement were: 345, 303 and 311g for the control, OXM6421 and the pair-fed groups, respectively. Locomotor activity was not affected by OXM6421 treatment (Figure 5e).
Acute effect of OXM6421 on food intake compared with OXM in fasted lean mice.
Both OXM6421 (5 and 20 nmol/kg⁻¹) and OXM (20 and 2000 nmol/kg⁻¹) reduced food intake in the first hour compared with the saline control (P<0.01 versus saline; n = 10-12; Figure 5), but the effect was lost by 2 h with OXM. In contrast, both doses of OXM6421 significantly reduced 24 h food intake compared with the saline controls (P<0.01; Figure 6).

Effects of continuous s.c. infusion of OXM6421 or OXM on food intake and body weight and plasma hormones in DIO mice.
OXM at 2000 pmol/kg⁻¹ min⁻¹ and OXM6421 at 30 pmol/kg⁻¹ min⁻¹ decreased food intake for the first 1 or 2 days, respectively (P<0.01 versus control). Cumulative food intake over the 7-day period was similar between OXM6421 10 pmol/kg⁻¹ min⁻¹ and OXM 2000 pmol/kg⁻¹ min⁻¹ groups (15 and 13% reduction, respectively, versus saline controls). At 30 pmol/kg⁻¹ min⁻¹, OXM6421 caused a significant, 34% reduction in the cumulative food intake (P<0.001 versus saline controls; n = 9-10; Figure 7a). Weight loss from their initial body weight after 7 days was 9.0±1.1% in the OXM6421 30 pmol/kg⁻¹ min⁻¹ group compared with...
Figure 7: Effects of continuous subcutaneous infusion of OXM6421 10 or 30 pmol·kg⁻¹·min⁻¹, or OXM 2000 pmol·kg⁻¹·min⁻¹, (a) on total food intake; (b) body weight change; white circle = saline; white square = OXM6421 10 pmol·kg⁻¹·min⁻¹; black circle = OXM6421 30 pmol·kg⁻¹·min⁻¹; black triangle = OXM 2000 pmol·kg⁻¹·min⁻¹; (c) blood glucose; (d) plasma insulin; (e) adiponectin and (f) leptin in male diet-induced obese mice. Results are mean±s.e.m. (n = 7-10). *p < 0.05; **p < 0.01; ***p < 0.001 compared to saline.

1.6 ± 0.8% in the control group (p < 0.01; Figure 7b). High-pressure liquid chromatography analysis of post-study pump contents revealed that there was no significant peptide degradation in any of the treatment groups (data not shown). In addition, 7-day infusion of OXM6421 at 30 pmol·kg⁻¹·min⁻¹ resulted in a significant decrease in
blood glucose level \(P < 0.05\), and a non-significant, 31% decrease in plasma insulin (Figures 7c and d). There was also a significant increase in adiponectin in the OXM6421 30 nmol kg\(^{-1}\) min\(^{-1}\) group \((P < 0.05)\), and a 34% reduction in plasma leptin level \((P = 0.08)\) (Figures 7e and f).

Effects of daily s.c. administration of OXM6421 on food intake and body weight in DIO mice.

OXM6421 20 nmol kg\(^{-1}\) showed potent inhibition of food intake following the first administration, with a significant, 57% reduction in cumulative food intake in the first 24 h (OXM6421: 1.53 ± 0.22 g versus saline: 3.58 ± 0.25; \(P < 0.0001\); \(n = 8\)). After daily administration, cumulative food intake was significantly lower in the OXM6421-treated DIO mice than the most profound inhibition seen in the first week \((P = 0.0047)\); generalized estimating equation; Figure 8a). Mice receiving OXM6421 lost weight during the treatment period, whereas the control group gained weight. At the end of the 21-day treatment, body weight in the OXM6421-treated mice was 11% lower than that of the saline controls \((P < 0.01);\) Figure 8b). Withdrawal of the treatment resulted in weight gain in both groups, despite this, the difference between the two groups in cumulative body weight change remained significant over the entire study period (Figure 8b). All animals appeared healthy with no obvious behavioural abnormalities.

Discussion

Our results showed for the first time that a long-acting OXM analogue, termed OXM6421, effectively reduced body weight in two rodent species. In the DIO mice (a model that shares a number of traits with human obesity), once-daily s.c. administration of OXM6421 for 21 days produced approximately 11% weight loss compared with the saline controls, indicating its potential as an anti-obesity therapy. Although earlier studies have shown the potential of OXM to reduce body weight, clinical usage as an obesity therapy is limited because of its rapid clearance and short circulating half-life. Both DPP-IV and NPE have been shown to degrade OXM in vitro.\(^{5,17}\) Evidence of the involvement of DPP-IV in the breakdown of OXM in vivo came from our recent study that co-administration of OXM and a DPP-IV inhibitor resulted in a greater reduction of food intake.\(^{16}\) The resistance of OXM6421 to degradation by the enzymes DPP-IV and NPE observed in the present study is likely responsible for its prolonged anorectic effect. The longer circulating time compared with native OXM following peripheral administration indicates that OXM6421 has an improved pharmacokinetics, which is consistent with its long-lasting inhibition of food intake. It has been suggested that the anorectic effect of OXM may be mediated by the GLP-1 receptor, as the effects were blocked by the GLP-1 receptor antagonist exendin-4\(^{-29}\), and were absent in the GLP-1 receptor knockout mouse.\(^{26}\) The marked enhancement of GLP-1 binding and cAMP production with OXM6421 may account for its increased potency in the acute feeding study in mice.

In lean Wistar rats, chronic treatment with a relatively small daily dose of OXM6421 (20 nmol kg\(^{-1}\)) significantly decreased cumulative food intake, and caused a 9% reduction in weight gain after 14-day treatment compared with the controls. The results of the pair-feeding study suggested that OXM6421-induced negative energy balance by affecting both energy intake and expenditure. The OXM6421-treated rats lost significantly more weight than that of the pair-fed group, indicating that inhibition of food intake was not solely responsible for the weight loss. The effect on energy expenditure was then confirmed by studies in the Comprehensive Laboratory Animal Monitoring System. Resting energy expenditure (measured as oxygen consumption in

Figure 8 (a) Reduction in food intake and (b) body weight in male diet-induced obese mice administered saline (white circles) or OXM6421 20 nmol kg\(^{-1}\) (black circles) once daily (subcutaneously) for 21 days. First dose was given on day 0. Results are means ± s.e.m. \((n = 8)\). \(* P < 0.05; ** P < 0.01; *** P < 0.001\) versus saline.
the light-phase) was significantly higher in the OXMM6421-treated than both the pair-fed and the control rats. Interestingly, it has been shown that OXM did not affect basal resting energy expenditure in humans, and the observed increase in total energy expenditure was because of an increase in activity during the ‘free-living’ period. Further studies are required to clarify whether these discrepancies reflect differences between species or pathways activated.

The precise mechanism whereby OXMM6421 induces negative energy balance is not known at present. The central satiety action of native OXM is thought to mediate through the hypothalamus and brainstem. An increase in alpha-melanocytestimulating hormone has been reported earlier with OXM.50 In the present study, OXMM6421 was also found to increase alpha-melanocyte-stimulating hormone release from rat hypothalamus, without significant effects on neuropeptide-Y, Agouti-related protein and cocaine- and amphetamine-regulated transcript release. It is well established that the melanocortin system has an important role in the regulation of energy homeostasis.50 In addition to its anorectic action, alpha-melanocyte-stimulating hormone has been shown to activate the brown adipose tissue through its sympathetic innervations and increase resting energy expenditure. Thus, it is possible that OXMM6421 may induce negative energy balance, at least in part, through the melanocortin system. Further studies are required before a definitive conclusion can be made with regard to the mechanism of action of OXMM6421. Interestingly, it has recently been shown that GLP-1 and glucagon receptors’ dual agonism can reverse obesity in rodents,51,52 which further highlights the important role of the peptides from the pre-proglucagon family in the modulation of energy balance.

The anti-obesity potential of OXMM6421 was determined in DIO mice, a well-characterized rodent model that shows many of the metabolic consequences of human obesity (including insulin resistance, glucose intolerance, leptin resistance and increased expression of inflammatory markers). Continuous infusion of OXMM6421 dose-dependently reduced cumulative food intake and, at 30 pmol kg⁻¹ min⁻¹, caused a significant weight loss over a 7-day infusion period. This weight loss was accompanied by a significant increase in plasma adiponectin levels and a reduction in plasma leptin levels (although this did not reach statistical significance), suggesting that the weight loss was at least partly due to the reduction in adipose tissue. Moreover, blood glucose level was also significantly reduced, together with a 31% reduction in plasma insulin level, indicating that there may be an improvement in insulin sensitivity, probably as a result of the weight loss. Whether OXMM6421 has any direct effect on glucose homeostasis remains to be investigated. However, a recent study has shown that OXM did not affect insulin action on glucose disposal and production during a hyperinsulinaemic euglycaemic clamp study in DIO mice.53 Furthermore, in DIO mice, the weight loss after 21 days daily s.c. administration was of similar magnitude to that observed in the lean rats, and there was no evidence of decreasing sensitivity to OXMM6421 over time. Tachyphylaxis may be a problem with gut hormone-based therapy than with other weight-reducing agents, because they are released daily throughout life, and are chronically elevated in certain endocrine tumours and in gut diseases associated with thinness, as well as after bariatric bypass surgery—where there is no evidence of escape. However, results from the current study do not provide further information regarding the endogenous functions of OXM, which have been well-documented elsewhere. Although there is no evidence to date suggesting that obese subjects may have a lower level of circulating OXM, elevated OXM levels have been found in patients who have had jejunoileal bypass surgery,54,55 which is believed to be at least partly responsible for the successful weight reduction in these patients. In summary, these studies show that OXMM6421, an OXM analogue, shows favourable pharmacological characteristics. Its enhanced potency and longevity in suppressing food intake, and the consistent efficacy in reducing body weight at relatively low doses in both lean and obese rodent models suggests that it may have the potential to be an anti-obesity therapy. However, whether these findings in rodents predict an anti-obesity effect in humans remains to be investigated.

Conflict of interest

YLL, HF, JSM, BCT, JCS, JB and KGM have nothing to declare. MAB and MJD receive royalties from and SRR is an inventor of United Kingdom patent application no. PCT/GB02/04082 and PCT/GB04/00617. SRR is a consultant from Thiakis, a subsidiary of Wyeth Pharmaceuticals (Filher). The company will reveal the structure of the oxyntomodulin analogue and give legitimate investigators the compound for experimental use under the condition that they sign a confidentiality agreement.

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