Molecular studies on Neuropeptide Y receptors involved in the regulation of feeding behaviour

A thesis submitted to the University of Glasgow
for the degree of Doctor of Philosophy

by
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PREFACE

I hereby declare that this thesis embodies the results of my own work and includes nothing which is the result of work done in collaboration.

E.J.Grant
November 1998

Publications resulting from the work presented in this thesis:

E.J.Grant, M.D. Fidock and J.M. Allen.
Isolation and identification of a rat NPY Y5 receptor genomic clone. Abstract-4th International NPY Conference, Regulatory Peptides 71 (3) 220, (1997).

E.J.Grant, M.D. Fidock and J.M. Allen.
Distribution of the Neuropeptide Y Y5 receptor mRNA in peripheral tissues.
Society for Neuroscience 27th Annual Meeting Abstract, Volume 23(1)964.

Summary

The regulation of energy and nutrient homeostasis is a complex procedure involving interactions between numerous neuropeptides, neurotransmitters and hormones implicated in the control of this fundamental behaviour. The PP-fold peptide family member Neuropeptide Y (NPY) is a well documented stimulator of food intake and is believed to play a role in the physiological regulation of ingestive behaviour.

At the start of the work presented in this thesis the NPY receptor subtype responsible for mediating the characteristic augmentation in feeding was thought to be a novel, previously unidentified receptor subtype, as the pharmacology of the cloned NPY receptor subtypes failed to mimic the pharmacology known to stimulate NPY-elicited eating. The therapeutic implications for the development of an anti-obesity drug based on an antagonist to the "feeding" receptor for NPY made the cloning of this receptor subtype a rapidly evolving and highly competitive field. Initial efforts were therefore directed at cloning the feeding receptor for NPY using various novel receptor cloning strategies.

The structural diversity exhibited by the members of the NPY receptor gene family made cloning using a homology screening strategy difficult, as demonstrated by the difficulties encountered in designing degenerate primers pairs for PCR or Genetrapper™ technology. As a result, functional expression cloning strategies were employed as an alternative strategy to clone novel NPY receptor gene family members, in particular the feeding receptor for NPY. Two different COS cell expression cloning strategies were used to identify NPY receptor gene family members following ligand binding and screening for cells expressing the desired cDNA by different detection methods.

During the course of this work the structure of the NPY Y5 receptor was reported using an expression cloning strategy identical to one described in this thesis, and this initial report proposed that this receptor was the feeding receptor for NPY. However, subsequent reports describing a role for the NPY Y1 receptor in the regulation of NPY-induced feeding have generated considerable controversy regarding the identity of the NPY receptor subtype involved in feeding behaviour regulation. The cloning of the NPY Y5 receptor enabled further studies to be directed toward examining the regulation of the Y5 gene, with a view to elucidating further the role of both the Y1 and Y5 receptors in the control of feeding behaviour. Hybridisation screening of a rat genomic library permitted the identification of a partial rat Y5 genomic clone, and enabled further efforts to characterise the promoter region to be carried out. Additional studies were carried out to investigate the regulation of Y1 and Y5 receptors by examining changes in the levels of receptor mRNA expression.

ACKNOWLEDGEMENTS

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I would also like to acknowledge all my friends; the Dundee girls, especially Lisa, Paula and Laura, for being great friends and providing me with an excellent distraction. Louise for her support, friendship and evil humour, Liz for her encouragement, support and much appreciated humour, Pam for her thoughtfulness, like-mindedness and constant amusement, David (the funniest person I know) for helping me solve the worlds problems and for his encouragement, and Stuart for his friendship over the years and for keeping my feet on the ground.

I would also like to thank Ben for all his support and encouragement. Thanks for having faith in me and for making this thesis that bit easier. Your endless patience never ceases to amaze me!

Finally, I am very grateful to my family (Mum, David and Matthew) for their love, support and encouragement throughout this thesis, and everything else both past and present.

DEDICATION

For my mum, whose strength of character and selflessness have inspired me always.
I couldn't have achieved this without you.

Table of contents

CHAPTER 5: NOVEL RECEPTOR CLONING : COS CELL EXPRESSION.

LIST OF TABLES

CHAPTER 3

CHAPTER 4

ABBREVIATIONS

3'UT	3' untranslated sequence
5-HT	5-hydroxytryptamine
5'UT	5' untranslated sequence
5'UTR	5' untranslated region
ACTH	adrenocorticotrophic hormone
ADH	Anti-diuretic hormone
ARC	Arcuate nucleus
BAT	Brown adipose tissue
βGal	β Galactosidase
bp	base pair
bPP	bovine pancreatic polypeptide
BSA	Bovine Serum Albumin
^{o}C	Degrees celsius
cAMP	Cyclic adenosine monophosphate
CART	Cocaine and Amphetamine Regulated Transcript
CAT	Chloramphenicol acetyltransferase
CCK	Cholecystokinin
cDNA	Complementary deoxyribonucleic acid
cfu	colony forming unit
CGRP	Calcitonin Gene Related Peptide
CNS	Central Nervous System
CORT	Corticosterone
CRE	Cyclic adenosine monophosphate Response Element
CRF	Corticotrophin Releasing Factor
dA	deoxyadenosine
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbeccos Modified Eagle Medium
DMSO	dimethyl Sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dpm	disintegrations per minute
ds	double stranded

dT	deoxythymidine
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
g	rtelative centrifugal force
G3PDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GABA	γ-amino butyric acid
GLP-1	Glucagon-like peptide 1
G-protein	Guanosine nucleotide binding protein
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
HEK cells	Human Endothelial Kidney cells
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hPP	human pancreatic polypeptide
icv	intracerebroventricular
IPTG	isopropyl-β-D-thio-galactoside
Kb	Kilo base pair
KDa	Kilodalton
LB	Luria-Bertani
LPL	Lipoprotein Lipase
MCH	Melanin Concentrating Hormone
MEM	Modified Essential Medium
mRNA	Messenger ribonucleic acid
MSH	Melanin-Stimulating Hormone
NA	Noradrenaline
NFM	Non-fat milk
NH_4OAc	Ammonium acetate
NPY	Neuropeptide Y
OFR	open reading frame
PBS	Phosphate Buffered Saline
PDE	Phosphodiesterase
PCR	Polymerase Chain Reaction
PFH	Perifornical hypothalamus
pfu	plaque forming unit
PI	Phosphatidyl inositol
PKA	cAMP-dependent Protein Kinase
PKC	Protein Kinase C
PNS	Peripheral Nervous System

PP	Pancreatic Polypeptide
PVN	Paraventricular nucleus
PY	Peptide Y
PYY	Peptide YY
RAMP	Receptor Activity Modifying Protein
RNA	Ribonucleic acid
RNase	Ribonulease
rpm	revolutions per minute
RQ	Respiratory quotient
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium Dodecyl Sulphate
SM	Storage medium
ss	single stranded
SV40	Simian Virus 40
T4PNK	T4 Polynucleotide Kinase
T_D	Dissociation temperature
TdT	Terminal deoxynucleotidyl transferase
tRNA	Transfer ribonucleic acid
UCP	Uncoupling Protein
UV	Ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Chapter 1

Introduction

Chapter 1

Introduction

Appetite, energy balance and body weight gain are modulated by diverse neurochemical and neuroendocrine signals originating from different organs in the body and diverse regions in the brain. Specific brain regions perform important integrative functions in this process, acting through a variety of systems to co-ordinate interactions between nutrients, amines, neuropeptides and hormones. These systems underlie normal nutrient intake and metabolism, and are thought to be responsible for regulating circadian cycle feeding behaviour and also gender and age related patterns of feeding behaviour in several species. Alterations in these normal neurochemical -neuroendocrine control systems may be associated with abnormal eating patterns and conditions such as obesity. Therefore, an understanding of the systems that control eating behaviour may provide a foundation for the treatment and possible prevention of such disorders.

1.1 Homeostatic control of feeding behaviour

Most species exhibit a strong circadian pattern of feeding behaviour, principally controlled by extensive central and peripheral entities involved in the control of food intake. Definitive brain structures and neurotransmitters have been allocated primary roles in the regulation of ingestive behaviour, and allied with behavioural and physiological regulation, these form the complex system responsible for the homeostatic control of food intake.

Maintenance of bodyweight is achieved by an intricate balance between energy intake (food consumption) and energy expenditure (basal metabolic rate and activity). This energy homeostasis is ultimately governed by the brain, where a variety of afferent signals reflect the nutritional state of the animal and its external environment, particularly food availability. These signals are integrated in order to moderate efferent pathways which control feeding behaviour and energy expenditure. Several hypotheses ascribing pivotal roles for macronutrients in the maintenance of body weight have been described since the initial concept of body weight homeostasis was first introduced.

A role for glucose in the control of such energy homeostasis was outlined in the 1950's by means of the glucostatic hypothesis, which highlighted the role of blood glucose as fuel, and stated that animals would be hungry when blood glucose was low and satiated when blood glucose was high (Mayer, 1955). A similar function was attributed to fat regulation by Kennedy, as described in the lipostatic hypothesis. Kennedy suggested that an increase in

body fat is sensed by a central "adipostat" resulting in compensatory responses such as a reduction in food intake being enforced in order to return the body to its previous adipose steady state. Conversely, declining fat stores would prompt feeding when food was available (Kennedy, 1952). The identification of such a "lipostat" factor in the form of leptin, an adipocyte-derived hormone, lends further support to the role of lipostatic mechanisms governing energy balance (Coleman, 1973 and Zhang, 1994).

1.2 Set Point Theory of food intake and body weight regulation.

The tendency of an individual to return to a certain body weight suggests that there is a biological set point for body weight much like the set points which form the basis of any negative feedback biological control system (Harris, 1990). Therefore when an individual's body weight is stable, and fat is neither being gained nor lost, energy acquisition must equal energy expenditure. Regardless of baseline body weight, energy conserving mechanisms are called upon when an intervention such as caloric restriction (resulting in low energy stores), results in a decrease in fat mass to below the steady state value and a concomitant decrease in daily energy expenditure. The resulting augmentation in food intake is stimulated via biological signals such as glucostatic and lipostatic signals, until energy stores are replenished, and the set point value is once again established (Figure 1.1). Conversely, if an individual is caused to gain fat mass in excess of the steady state value, energy expenditure increases until the set point value is re-established. Like any biological control system, if the set point were to be increased, body weight would increase accordingly to meet this new value (Leibel, 1995).

It is widely accepted that the regulation of human feeding is extremely complex, and that physiological variables play a crucial role alongside the numerous other aspects involved in the regulation of ingestive behaviour. A cognitive set point model, outlined by Booth in 1980, delineates the role which the environment (culture, socio-economic class etc.) has on body weight. Figure 1.2 illustrates that relative to a personally selected "ideal" body weight set point, as individuals we are constantly receiving a variety of cognitive signals about how we look, body weight, clothing size and health concerns (Booth, 1980). Adaptive changes of the larger human brain, whereby improved procurement of food is achieved by a complex of interacting food-related systems argues further the complexity of feeding behaviour as dependant on physiological and learned and cognitive aspects. Another important consideration in

Figure 1.1 Set-point model of bodyweight control whereby feeding behaviour is regulated via glucostatic and lipostatic signals relayed to control centres in the brain.

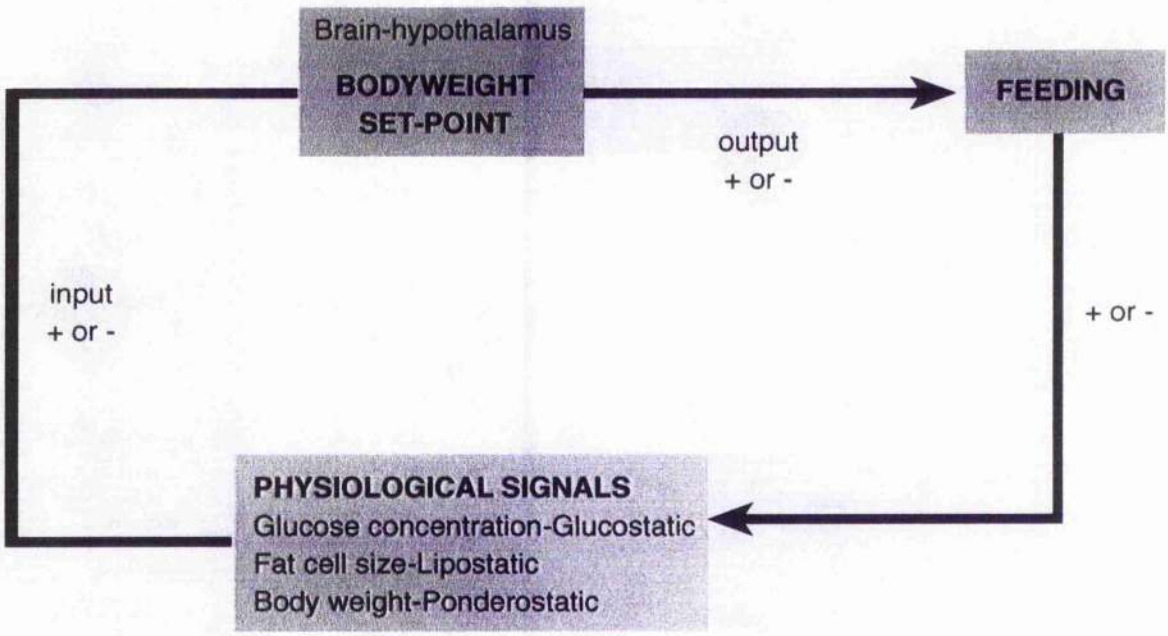
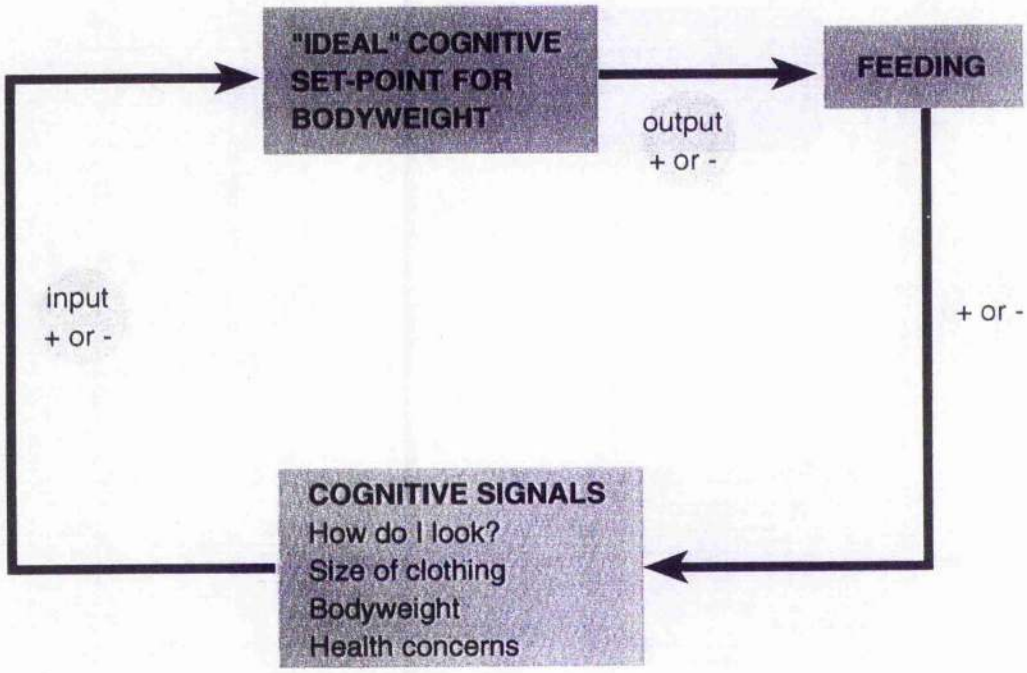

Brain-hypothalamus
BODYWEIGHT SET-POINT
FEEDING
output
+ or -
input
+ or -
+ or -
PHYSIOLOGICAL SIGNALS
Glucose concentration-Glucostatic
Fat cell size-Lipostatic
Body weight-Ponderostatic

Figure 1.2 Cognitive set-point model of bodyweight control whereby feeding behaviour is regulated by various cognitive signals.


"IDEAL" COGNITIVE SET-POINT FOR BODYWEIGHT
FEEDING
output
+ or -
input
+ or -
+ or -
COGNITIVE SIGNALS
How do I look?
Size of clothing
Bodyweight
Health concerns

humans in favour of the complexity of feeding behaviour is that the type, amount and frequency of food intake is often dictated by a particular culture (Rowland, 1996)

1.3 Homeostasis and obesity

The central nervous system monitors body composition and ensures that energy acquisition and energy expenditure are co-ordinated and precisely regulated so that fat mass remains constant over long periods of time, despite variable food intake and activity. Failure to maintain a balance between these two processes results in the complex disorder of obesity, a disorder which remains a dilemma in Western society, with an increasing prevalence and affiliation with numerous associated health risks including cardiovascular disease, hypertension and non-insulin dependant diabetes. Obesity is believed to be of both genetic and environmental origin, implicating accountable factors such as excess caloric intake, decreased physical activity, social and economic forces, and metabolic and endocrine abnormalities (Weiser, 1997).

Although evidence exists in support of these potential causative factors, a definitive mechanism for obesity remains to be defined. Contributing environmental factors include increased food intake in the form of high fat foods which are highly palatable, and more efficiently converted to body fat than carbohydrate or protein. Coupled with poor regulation of dietary fat, and a sedentary lifestyle, these factors promote the likelihood of development of obesity.

Evidence for genes which control body weight and composition in humans has come from sources such as adoption, twin and family studies. A convincing argument in favour of a genetic contribution predisposing obesity has come from results of these studies. Significant correlations were observed between the bodyweights of adoptees and their biological parents, upon comparing bodyweights of adoptees and both biological and adoptive parents. The lack of correlation observed between the adoptees and adoptive parents, who share a domestic environment, emphasises the role genetic factors may play in obesity (Naggert, 1997).

Early observations have led to the proposals that genes involved in body weight homeostasis act by sensing food intake, available energy stores in adipose tissue, and activity levels. These sensory inputs are then relayed to central regulatory sites, where the information is integrated and a response initiated relevant to the animal's nutritional status. Genetic variations in the signalling molecules involved in this regulatory system may therefore account for the heritable component of body fat content.

Several genes involved in the complex regulatory network responsible for maintaining a balance between food intake and energy expenditure, have recently been cloned and characterised (For a review see Weigle, 1996, Naggert, 1997). The precise role of such genes

in the regulation of feeding behaviour and their role in obesity remains to be completely elucidated. However, by examining the result of various mutations in these "obesity" genes, the role of individual genetic factors may be illuminated.

It is unlikely that either single genes or single brain structures exert stronger or predominant control over a process such as feeding, and in reality multiple and probably redundant genes, neural pathways, hormones and transmitters are involved. Therefore, an appreciation of the forces which act ordinarily to regulate body composition is necessary in order to fully understand the mechanisms by which disruption of specific genes may contribute to obesity.

1.3.1 Identification of obesity genes

An "obesity gene" could be any gene encoding a protein involved in body weight regulation. The polygenic nature of human obesity, coupled with the impact of environmental variables on body composition, make it difficult to directly identify novel obesity genes in humans.
Rapid progress has been facilitated by familiarity with rodent models of obesity and a detailed knowledge of the analogies between mouse and human genomic maps. Remarkable advances have been achieved by the isolation, identification and characterisation of the genes mutated in five monogenic mouse models of obesity. These mutations, which essentially predispose obesity in mice, have been positionally cloned by conventional methods, and are widely used as models for human obesity. The mutated genes for all five models provide definitive genetic evidence for specific pathways directly regulating bodyweight and adiposity. Figure 1.3 summarises the genetic defects responsible for the five main mouse models of obesity. As the table outlines, each of these genes function at some level in one of the feedback pathways which ultimately link energy intake and energy expenditure to total adipose mass. Future research should establish which genes are involved in prevalent neurobiological pathways regulating ingestive behaviour, and in which tissue the altered gene product must be expressed to produce the obese phenotype. Deeper understanding of the complex control of feeding may ultimately provide potential new therapeutic options for human obesity. For a comprehensive review of human obesity and the genetic and environmental implications see Science 1998 Vol 280, pp 1364-1390.

Figure 1.3 Summary of the genes involved in the five mouse models of genetic obesity.

Name	Normal protein function	Mutation	Effect of mutation	Phenotype	Date cloned
Obese (ob)	Leptin-circulating satiety signal	Nonsense at Arg 105	Defective leptin production	•hyperphagia severe obesity •diabetes •reproductive deficiency •stunted growth	1994
Diabetes (db)	Leptin receptor	Splicing error	Loss of receptor function	Similar to (ob)	1996
Agouti (A^y)	High affinity antagonist of MSH at its receptor	Ubiquitous promoter	Unregulated expression of agouti proteins	•all yellow coat •hyerinsulinema •insulin resistance •development of tumours	1992
Tubby (tub)	Putative PDE expressed in retina and brain	Unspliced intron	Carboxy terminal deletion of protein	•Late-onset obesity •insulin resistance •retinal degeneration •deafness	1996
Fat (fat)	Carboxypeptidase E enzyme involved in processing neuropeptides involved in feeding	Missense mutation	Lack of CPE activity resulting in defective processing of hypothalamic peptides involved in feeding	•Progressive adult onset obesity •hyperinsulinema •infertilty	1995

1.4 Neurobiology of feeding

Neurochemical and neuroendocrine systems control macronutrient intake and metabolism. Diverse signals from the periphery and brain are co-ordinated to ensure effective modulation of energy balance, appetite and bodyweight gain.

1.4.1 Integration of diverse signals that determine energy and nutrient balance.

As the regulation and maintenance of bodyweight is undoubtedly complex, integrative, interdisciplinary approaches have been used to obtain information about multiple neurochemical and neuroendocrine determinants of energy balance, nutrient stores and bodyweight. Diverse signals involved include:

1)Simple nutrients in the blood e.g. glucose, fatty acids, amino acids.
2)Classical neurotransmitter molecules for rapid short-term communication.
3)Larger neuropeptides for slower, more long-term action.
4)Hormones for both neuromodulatory and metabolic processes.

These signals derive from different peripheral organs and different areas of the central nervous system and contribute in co-ordinating physiological and behavioural regulation of eating behaviour. The main components of these diverse control systems are discussed below.

1.4.2 Peripheral neurochemical-neuroendocrine systems

In the periphery a variety of substances have been identified and attributed major roles in the regulation of energy and nutrient homeostasis. These include gastro-intestinal signals in the form of various peptides, which upon release into the gastro-intestinal tract after a meal, are involved in co-ordinating several aspects of digestion, absorption and metabolism. Information from these peripherally released peptides can also be transmitted, via the vagus nerve to the brain, where behavioural processes related to feeding termination and satiety are instigated.

1.4.3 Neurochemical-neuroendocrine systems in the hypothalamus

The integration of metabolic information from the periphery with signals within the central nervous system requires the involvement and specialised functions of multiple brain areas. Several distinct brain regions serve to regulate specific aspects of feeding behaviour, in a

pathway of events encompassing the various afferent signals. These include the lower brainstem which relays and integrates neural information between peripheral autonomic endocrine organs and forebrain structures, the midbrain and thalamic areas which interpret this information in relation to signals generated by the sensory properties of foods, and forebrain structures such as the nucleus accumbens, amygdala and frontal cortex, which perform the task of integrating this incoming information with various cognitive factors related to the rewarding or aversive aspects of food (Leibowitz, 1992). Figure 1.4 illustrates the regulation of energy balance by the central nervous system, in particular the hypothalamus.

1.4.4 The hypothalamus and the neurobiology of feeding

A major function in this sequence of events is performed by the hypothalamus, which plays a central role in the integrated control of feeding and energy homeostasis. This structure exhibits extensive vascularity and neural projections from the lower brainstem, and thus remains closely linked to both circulating nutrients and hormones and neural signals from the periphery (Leibowitz; 1986).

The belief that the hypothalamus plays a crucial part in the regulation of energy homeostasis was originally based upon results of brain lesions. Evidence from disruption of specific hypothalamic regions gave rise to the consensus that the hypothalamus acted as a centre for both satiety and feeding regulation. Lesions of the ventromedial hypothalamus resulted in the formation of hyperphagic obesity, whereas lesions of the lateral hypothalamus elicited severe hypophagia and weight loss. From these findings, the existence of a ventromedial hypothalamic satiety centre and a contrasting lateral hypothalamic feeding centre were postulated (see Figure 1.5) (Luiten, 1987).

The identification of a number of neurotransmitter and neuromodulator systems within the hypothalamus involved in the control of appetite and body weight homeostasis, allowed further examination of the neurochemical-neuroendocrine system underlying the complex regulation of these systems. By examining responses to hypothalamic injection of various neurochemicals implicated in feeding behaviour, the effects of neurotransmitters such as 5-hydroxytryptamine, noradrenaline, dopamine and various neuropeptides including neuropeptide Y, galanin and corticotrophin releasing hormone were elucidated.

Figure 1.4 Regulation of energy balance by the central nervous system, in particular the hypothalamus which integrates signals indicating the nutritional state, together with hedonic and cognitive signals to control food intake and metabolism (Adapted from Wilding, 1997).

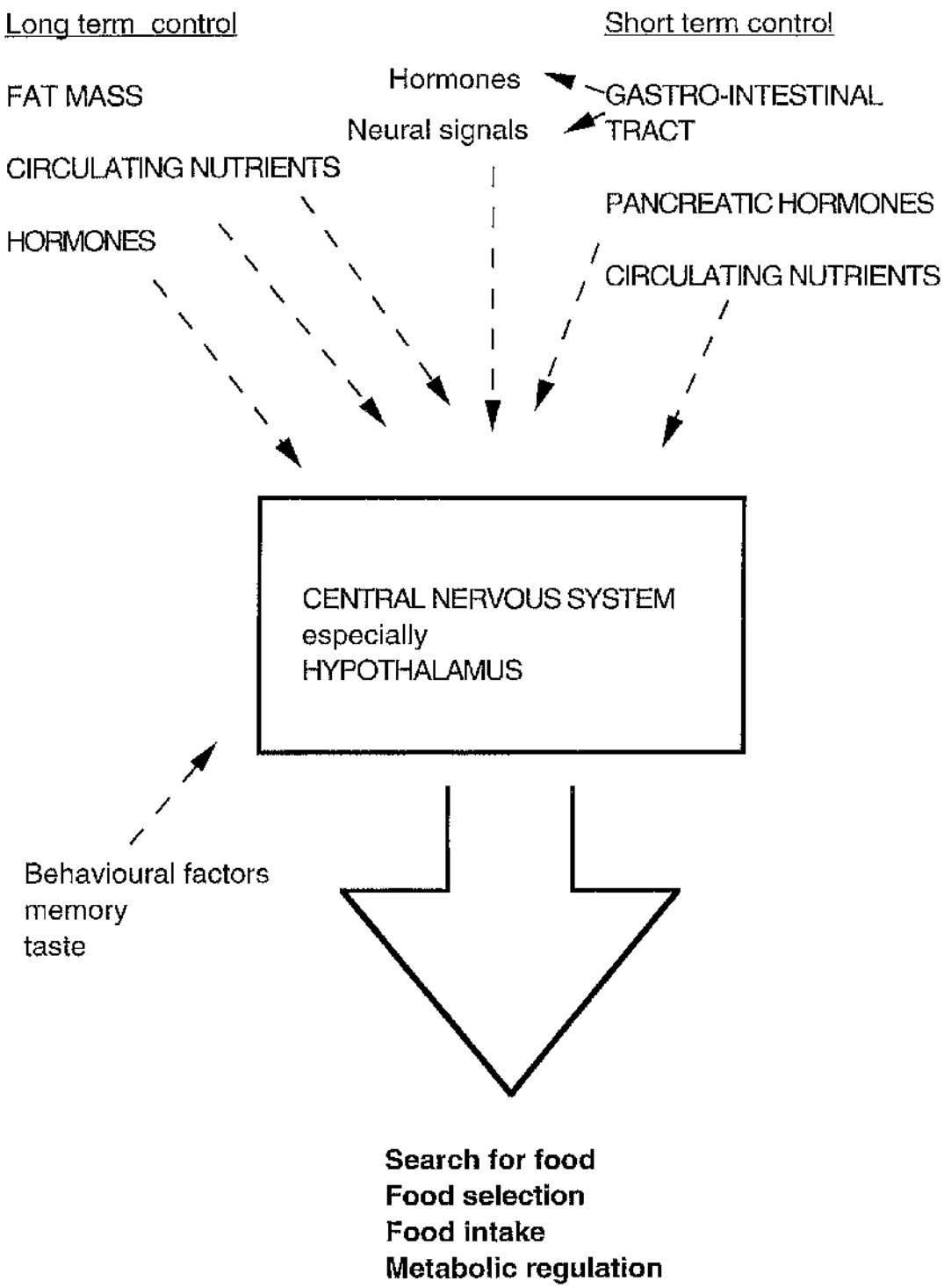
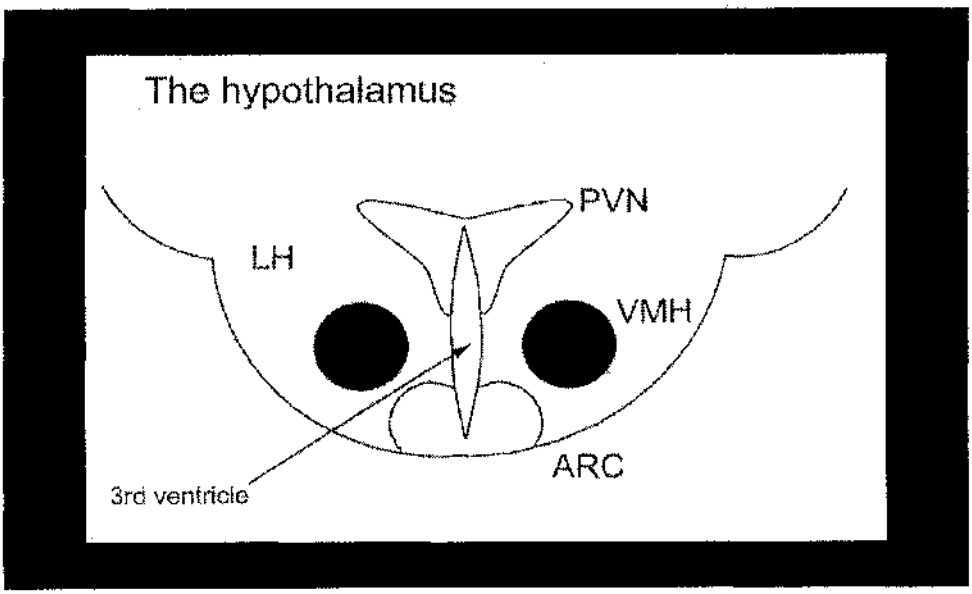

Long term control
Short term control
FAT MASS
Hormones
GASTRO-INTESTINAL
Neural signals
TRACT
CIRCULATING NUTRIENTS
PANCREATIC HORMONES
HORMONES
CIRCULATING NUTRIENTS
CENTRAL NERVOUS SYSTEM
especially
HYPOTHALAMUS
Behavioural factors
memory
taste
Search for food
Food selection
Food intake
Metabolic regulation

Figure 1.5 Anatomy of the hypothalamus. This figure shows a schematic section through the rat hypothalamus. The LH (lateral hypothalamus) and VMH (ventromedial hypothalamus) have been traditionally regarded as the feeding and satiety centre respectively. This simplistic view is now outdated and the PVN (paraventricular nucleus) and ARC (arcuate nucleus) are now thought to be equally important.


The hypothalamus
PVN
LH
VMH
ARC
3rd ventricle

1.4.5 Endogenous starvation and satiety signals

From these studies, it was clear that two distinct categories of neurotransmitter or neuropeptide existed; namely those whose actions augmented the feeding response, and those whose actions resulted in a marked attenuation of ingestive behaviour. The presence of such neurochemical systems within the hypothalamus, and their demonstrated dramatic effects on appetite regulation, would suggest these neurotransmitters and neuropeptides are present endogenously, where they serve to control feeding and maintain a balanced nutritional status. A more precise function for these neurochemicals is more resolutely defined by closer examination of the levels present in various nutritional states, such as fasted or fed models. Significant elevation of neurochemicals known to stimulate feeding behaviour have been observed in starved state models, therefore depicting a role for these neurochemicals in response to negative energy balance whereby an increase in food intake is physiologically desirable and tantamount to the survival of the animal. These neurotransmitters, neuropeptides or hormones may therefore be examples of endogenous starvation signals, present inherently to ensure survival. Examples of such endogenous starvation signals include: the neuropeptides neuropeptide Y, galanin, orexin and opioid peptides, the neurotransmitters noradrenaline and gamma-amino butyric acid (GABA), and the hormones growth hormone releasing hormone and melanin concentrating hormone (Clark, 1984; Kyrkouli, 1990; Sakurai, 1998; Stanley 1988; Grossman, 1960; Feizfel, 1994 and Qu, 1996). Conversely, markedly elevated levels of other specific neurochemicals have been found in fed state models, where they act to signal satiety and consequently a cessation of feeding. These include the peptides glucagon-like peptide-1, urocortin and CART (cocaine and amphetamine regulated transcript), the neurotransmitter 5-hydroxytryptamine, the hormones leptin, cholecystokinin, corticotrophin releasing factor and oxytocin (Turton, 1996; Spina, 1996; Kristensen, 1998; Pollock, 1981; Zhang, 1994; Gibbs, 1973; Arase, 1988 and Verbalis, 1986). The opposing effects of these endogenous systems are functionally significant, as the hypothalamus behaves as both a starvation and satiety centre, and these neurotransmitters and neuropeptides play a part in the overall hypothalamic modulation of ingestive behaviour (For a review see Leibowitz, 1992 and Wilding, 1997).

Another interesting observation is the striking circadian rhythms displayed by these hypothalamic neurochemicals. These neurochemicals and their receptors are not uniformly active across the circadian cycle, with activities of stimulatory signals peaking sharply at the beginning of the natural feeding period, when the body's glycogen stores and blood glucose are low (Tempel; 1989b, Leibowitz, 1990).

Macronutrient selectivity is another phenomenon observed, whereby specific sets of neurochemicals have a role in stimulating the ingestion of a particular food group.

1.4.6 Carbohydrate balance

One such system involved in potentiating carbohydrate intake in response to a decrease in carbohydrate utilisation, functions through the amino acid gamma amino butyric acid, the amine noradrenaline, and the peptide neuropeptide Y, in association with corticosterone and circulating blood glucose. A primary site of action lies in the medial region of the hypothalamus, in particular the paraventricular nucleus (PVN), where the neurochemicals and receptors are co-localised. The nature of macronutrient specificity was revealed by studies employing a diet selection paradigm, which allowed selection of pure macronutrient sources following PVN administration of the aforementioned agents. Carbohydrate ingestion was specifically potentiated compared to protein or fat ingestion, in addition to an increase in the size and duration of carbohydrate rich meals.

These neurochemicals are not uniformly active across the circadian cycle, with activities peaking at the beginning of the natural feeding period, consistent with a strong preference in both rats and humans, for this macronutrient during the early hours of the feeding cycle (Tempel, 1989b.).

1.4.7 Fat balance

Fat balance is also regulated by a similar system, whereby a different set of substances in the PVN of the hypothalamus specifically regulate the ingestion of fat. These neurochemicals include the neuropeptide galanin, the opioid peptides and the mineralocorticoid aldosterone. Like neurochemicals involved in the potentiation of carbohydrate intake, the substances which stimulate fat consumption are not consistently active throughout the circadian cycle, with highest potency during the late hours of the circadian cycle. In humans and rats, appetite for fat progressively rises over the course of the natural feeding cycle, and this appetite shift may be attributed to the increased action of these neurochemicals.

1.4.8 Protein balance

In rats and humans, appetite for protein increases gradually over the course of the active circadian cycle. Opioid peptides have been shown to potentiate protein intake, and also the peptide growth hormone releasing factor which stimulates protein intake upon hypothalamic injection.

From these observations, it is clear that these endogenous satiety and starvation signals play a major part in maintaining a balanced nutritional status. The specific hypothalamic pathways traversed by these neurochemicals, and the belief that specific receptor subtypes within the hypothalamus mediate these dramatic effects, allows further exploitation of each neurotransmitter or neuropeptide system in an attempt to underpin the exact role of any one neurochemical in the regulation of feeding behaviour.

1.5 Neuropeptide Y

Neurons produce a variety of peptides, secreted from axons and dendrites and collectively known as neuropeptides. These peptides arise from larger precursors that are processed enzymatically, yielding the mature neuropeptides. These neuropeptides are believed to serve as both neurotransmitters and neuromodulators by their interactions at cell membrane receptors. Neuropeptide Y is a neuronally derived peptide which belongs to a peptide family often referred to as the pancreatic polypeptide (PP) family, which also includes the endocrine peptides, peptide YY (PYY), pancreatic polypeptide (PP) and the non-mammalian (fish) pancreatic peptide Y(PY) (Tatemoto, 1982a; Kimmel, 1975 and Andrews, 1985).

1.5.1 Discovery of Neuropeptide Y

Neuropeptide Y (NPY) was isolated from porcine brain in 1982 by Tatemoto *et al* using a chemical method employed to identify polypeptides exhibiting an amide structure at their carboxy terminal (Tatemoto, 1982b). Sequence analysis revealed that NPY comprised 36 amino acids, and had a tyrosine residue at its amino-terminus and a tyrosine amide at its carboxy-terminus (Figure 1.6) (Tatemoto, 1982c). The nomenclature of this family of peptides arose as the abbreviation for tyrosine using the single letter amino acid code is Y, and when discovered these peptides had no attributable functions, and were named according to their organ of origin, hence pancreatic polypeptide and neuropeptide Y. Evolution of the PP-fold peptides has been studied in detail, and it is believed that a common ancestral NPY/PYY sequence diverged early in evolution to give rise to the respective peptides (Larhammar, 1996a). NPY is considered to be one of the most conserved peptide sequences known, exhibiting very few differences in sequence across a wide variety of species, and retaining a high percentage identity between species separated in evolution by millions of years (Larhammar, 1996a, Blomqvist, 1992). This species conservation is evident from the predicted human and rat mature NPY amino acid sequence which are identical, and differ from the porcine sequence only by a single amino acid substitution at position 17, where a leucine residue is replaced by a methionine residue (Larhammar, 1993, 1996a).

Figure 1.6 Primary structure of neuropeptide Y (porcine).

1 5 10
Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-
15 20
Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-
25 30 35
Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-

NH_2

All three peptides are 36 amino acids in length, and a striking conservation of amino acid sequence is maintained between this family of peptides, particularly those amino acids involved in the formation of the distinct tertiary structure shared by these peptides.

1.5.2 NPY structure

The structure of the PP family was deduced initially by x-ray crystallography analysis of the avian pancreatic polypeptide (Blundell, 1981; Glover, 1985). From these studies, it was revealed that NPY and the other PP family members all feature a compact tertiary structure comprising an N-terminal polyproline helix, encompassed by residues 1-8 and an amphiphilic α-helix encompassed by residues 15-30. The helices lie anti-parallel, and are connected with a ß-turn, creating a hair-pin loop often referred to as the PP-fold (Blundell, 1981). This fold is followed by a carboxy-terminal tail of six amino acids which project away from the α-helix (figure 1.7). Computer aided molecular modelling has lent support to this configuration, whereby the tertiary structure is preserved by extensive hydrophobic interactions between the helices (Allen *et al* , 1987). These intramolecular contacts confer spatial proximity between the N and C termini, which is believed to be essential for biological activity, as the epitope that binds to the NPY Y1 receptor is composed of residues present in the combined C- and N-termini of the molecule (Wahlestedt, 1986). Therefore, the tertiary structure of NPY appears to be essential for both the biological activity of the peptide and for receptor selectivity.

1.5.3 Neuropeptide Y distribution

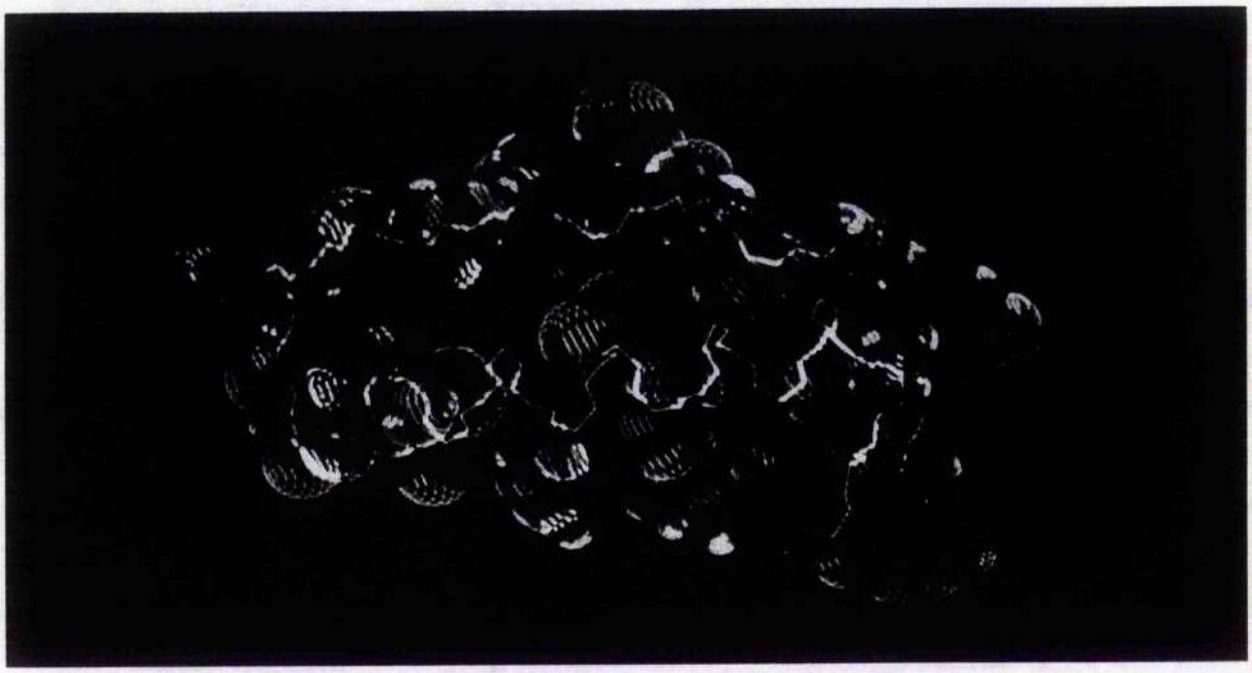
1.5.3.1 NPY in the central nervous system

Since the initial discovery of NPY by Tatemoto, it has been shown that the concentration of NPY in certain brain regions is greater than other putative neurotransmitters. Immunocytochemistry and radioimmunoassay studies with antibodies to NPY revealed a massive neuronal system for NPY, where NPY was shown to be the most abundant neuropeptide found in rat brain (Allen *et al*, 1983). Neurones containing NPY-like immunoreactivity have been shown to be abundant in the central nervous system, and are notably most prevalent in the limbic system, hypothalamus, basal ganglia and cortex, with virtually no immunoreactivity found in the cerebellum (Allen *et al* , 1983).
Broadly, two basic types of neurons have been shown to be immunoreactive for NPY; namely interneurons which give rise to short axons and local connections, and long-projecting neurons whose axons can traverse a long distance from the cell body, innervating structures in other brain regions. Within the central nervous system, NPY is co-localised with a wide variety of

Figure 1.7 Computer constructed three-dimensional tertiary structure of NPY.

N-terminal polyproline helix denoted numerically as residues 1-8
Amphiphilic α-helix denoted numerically as residues 15-30

I - 8



15 - 30

other neurotransmitters, in a distinct pattern dependent on anatomical location of the NPY-containing neurones.

1.5.3.2 NPY in the peripheral nervous system

Peripherally NPY is present in many post-ganglionic sympathetic nerves, especially around blood vessels supplying numerous tissues including heart, kidney, lungs, intestinal tract, pancreas, uterus and urinary bladder. NPY has also been detected in enteric, cardiac and noradrenergic perivascular nerves, and in a subset of parasympathetic nerves (Grundemar & Hakansen, 1994).

The widespread distribution displayed by NPY in numerous nerve terminals and cell bodies, lends support to the role of NPY in a number of diverse functions ranging from somatic, sensory and cognitive brain functions to endocrine and cardiovascular regulation. A modulatory role for NPY is also suggested by the observation that NPY is co-localised and co-released with other neurotransmitters such as noradrenaline, gamma-amino butyric acid (GABA) and somatostatin (Grundemar & Hakanson, 1994).

1.5.4 Biological Effects of the NPY family of peptides

NPY exerts potent biological effects on many targets both in the central and peripheral nervous systems, either involving NPY per se, or as a result of modulatory interactions with other agents. The aforementioned evolutionary conservation of NPY implies a role for NPY as an important neurotransmitter.

1.5.4.1 NPY actions in the central nervous system

The widespread distribution attributed to central NPY suggests that NPY may serve as a multi-functional neuropeptide involved in numerous processes and involved in a variety of regulatory functions. The role of NPY in the central nervous system has been explored by direct injection of the peptide and peptide analogues and subsequent evaluation of the behavioural, endocrine and autonomic consequences. Central NPY has been associated with modulation of autonomic functions including cardiovascular and respiratory activity (Fuxe, 1983), regulation of behavioural aspects such as circadian rhythm (Albers & Ferris, 1985) and ingestive behaviour (Stanley, 1984), and has been implicated in the pathophysiology of the affective disorders depression and anxiety (Heilig, 1993). NPY also serves to help govern memory processing (Flood, 1989), neuronal excitability (Erickson, 1996), neuroendocrine function via the release of hypothalamic and pituitary hormones (Kerkerian, 1985) and sexual behaviour via an effect on luetenising hormone releasing hormone (McDonald, 1990).

1.5.4.2 NPY actions in the peripheral nervous system

The role of neuropeptide Y in the peripheral nervous system has been determined by administering NPY to tissue preparations or by intravenous application of NPY. From these studies, NPY gained recognition as a well characterised vasoconstrictor, where the characteristic response to peripheral administration of NPY was potent and prolonged vasoconstriction. Intravenous application of NPY was shown to elicit a sustained elevation in blood pressure in numerous different species including man, an effect particularly prominent in the renal and coronary vasculature.

The most established role of NPY in the periphery is associated with the actions exerted by NPY at the sympathetic neuro-effector junction, where it is co-localised and co-released with the sympathetic nerve neurotransmitter noradrenaline (NA), and where it acts in a co-operative manner (Wahlestedt, 1990a). Indeed, a major portion of the NPY-immunoreactive nerves in peripheral organs represents classical sympathetic fibres where NPY co-exists with NA. The three main effects of NPY at the sympathetic neuro-effector junction are:

1) A direct post-junctional response i.e vasoconstriction manifested in certain vascular beds.

2) A post-junctional potentiating effect on NA evoked vasoconstriction, suggesting a modulatory role for NPY (Ekblad, 1984).

3)A pre-junctional suppression of stimulated NA release (Wahelstedt, 1990b).

Two main receptor subtypes were defined at the sympathetic neuro-effector junction, namely, Y1 and Y2 (Wahlestedt, 1986). At this junction, the Y1 receptor mediated the postsynaptic vasoconstrictor activity of NPY, whereas the Y2 receptor mediated the presynaptic effects of NPY on noradrenaline release. In this way the Y1 and Y2 receptors can be considered as analogous to the α1 and α2 adrenoceptors where different receptor subtypes mediate different responses to the ligand. However, as for the α adrenoceptors, the site of expression of the Y1 and Y2 receptors at the synapse is not rigid and it is now known that there are Y1 receptors at some presynaptic sites and Y2 receptors can occur postsynaptically. Subsequent studies with Y1 receptor antagonists have confirmed the role the Y1 receptor plays in mediating the vasoconstrictor action of NPY (Malmstrom, 1995 and Serradeil-Le Gal, 1997).

1.6 Receptors mediating the actions of NPY

1.6.1 Historical aspects of receptor activation

The initial proposal of NPY receptor heterogeneity arose as a result of observations that the entire NPY molecule was necessary to evoke vasoconstriction, whereas C-terminal fragments of NPY were effective in suppressing sympathetic nerve activity (Wahlestedt, 1986). The subsequent principal subdivision of NPY receptors into the NPY Y1 and Y2 subtypes has survived the test of time and is further supported by additional data such as the development of selective subtype analogues, synthesis of receptor antagonists, and cDNA and gene cloning technology (Michel, 1997).

1.6.2 Signal Transduction of NPY receptors

All known NPY receptors belong to the large superfamily of G-protein coupled heptahelical receptors. They appear to use similar signal transduction pathways, as illustrated by findings that in almost every cell type studied, NPY receptors demonstrate a preferential coupling to pertussis toxin sensitive G-proteins, namely members of the Gi and Go family (Limbrid, 1988).

The signalling responses of NPY receptors are typically under the control of these G-proteins, in that inhibition of adenylyl cyclase is found in almost every tissue, and cell type investigated. However, adenylyl cyclase inhibition probably fails to explain many of the functional responses obtained following stimulation of NPY receptors, and additional signalling responses are observed in restricted cell types. These include inhibition of calcium channels, and either activation or inhibition of potassium channels.

1.6.3 Present definition of NPY receptors

To date five distinct NPY receptors have been cloned. A surprising phenomenon arising from sequence analysis of these cloned receptors, is the apparent lack of sequence homology exhibited between the different subtypes. However, even with the low sequence homology displayed amongst members, there appears to be a distinct clustering of amino acid sequence similarity between certain subtypes, with sequence alignments depicting a Y1, Y4 and Y6 grouping, within which, these receptors appear to be more closely related to one another than the Y2 and Y5 receptors which clearly define another family (Larhammar, 1996b). Varying degrees of conservation are observed across species, with the Y1 receptor displaying tight conservation across a variety of species, in contrast with the Y4 receptor which appears to be

one of the most rapidly evolving receptors, possessing low sequence identity even between rat and.human sequences (Larhammar, 1996b).

1.6.4 Characteristics of receptor subtypes

1.6.4.1 Y1 receptor

The NPY Y1 receptor is the best characterised receptor for NPY . The cloning in 1990 of a rat cDNA encoding a G-protein coupled orphan receptor (Eva, 1990), which was subsequently shown to encode a Y1 type receptor, marked the advent of NPY receptor subtype identification. Subsequently, species homologues from mice (Eva, 1992), man (Larhammar, 1992) and *Xenopus laevis* (Blomqvist, 1995) were identified for this receptor which mediates many of the well established functions of NPY in the periphery. The genomic organisation of the gene encoding this Y1 subtype has also been determined in humans and mice, with the human Y1 gene being located on chromosome 4q (Eva, 1992; Herzog, 1993).

The human Y1 receptor protein is 384 amino acids in length, contains the seven transmembrane motif characteristic of G-protein coupled receptors, and exhibits a striking degree of sequence conservation, with the rat and human gene being highly orthologous, displaying 94% amino acid identity (Larhammar, 1996b). This receptor can effectively couple to both phosphatidylinositol (PI) hydrolysis, and the inhibition of adenylyl cyclase activity, an effect dependent upon the cell type expressing the receptor (Herzog, 1992). The importance of this dual coupling ability in normal cell function is unknown, although it has been demonstrated that the Y1 receptor can couple to either second messenger system in tissue preparations or cell lines naturally expressing the receptor (Aakerlund, 1990 and Hinson, 1988). Three splice variants of the first exon have been identified in the 5' region of the human Y1 receptor which are expressed in tissue specific patterns (Ball, 1995). The murine Y1 receptor possesses two splice variants, both binding NPY, but differing in their efficiency of coupling to subsequent signal transduction procedures (Nakamura, 1995).

The pharmacology displayed by this receptor is characterised by the following order of potency, where:

NPY = PYY _ > Pro^{34}-substituted analogue >> C-terminal NPY fragments.

As the order of potency for this receptor suggests, the whole NPY molecule is required for activation of the Y1 receptor, as removal of either the N- or C-termini results in a loss of affinity for the Y1 receptor. Originally this receptor was described as possessing micromolar affinity for C-terminal fragments of NPY such as NPY 13-36, but interacted with the full length NPY and PYY molecules with nanomolar affinity (Wahlestedt, 1986). A selective Y1 agonist was reported in 1990, in the form of the substituted NPY analogue $Leu^{31}Pro^{34}$ NPY, which demonstrated Y1 receptor selectivity (Fuhlendorf, 1990). Subsequent studies revealed

that the substituted analogue Pro^{34} NPY was sufficient to display selectivity for the Y1 receptor (Potter,1991).

The selectivity of this substituted NPY analogue for the Y1 receptor has been exploited and used as a means of elucidating the distribution of the receptor (Gehlert, 1992). With the aid of radio-labelled Pro^{34} NPY, the Y1 receptor has been shown to be predominant in brain regions such as cerebral cortex, thalamus and certain nuclei of the amygdala, and consistent with a postsynaptic localisation, the mRNA for the Y1 receptor displays a similar distribution (Eva, 1990). Messenger RNA for the Y1 receptor has also been detected in a variety of peripheral tissues, including heart, kidney and gastro-intestinal tract (Wharton, 1993). Screening of neuroblastoma cell lines using mono-iodinated NPY identified SK-N-MC cells as a human cell line containing an apparently homogenous population of Y1 receptors (Sheikh, 1989).
The effects associated with activation of Y1 receptors are shown in figure 1.8.

Relative progress has been made in the design of Y1 antagonists. Peptide based compounds such as 1229U91(GW11229), which is a symmetrical dimeric peptide joined in an anti-parallel fashion, is a compound with an extremely high affinity for the Y1 receptor, and is capable of blocking NPY-induced second messenger responses via this receptor (Daniels, 1995). However this compound also has a high affinity for Y4 receptors, where it can behave as either an agonist or antagonist (Gehlert, 1996b). A second molecule, BIBP3226, whose design was based on the C-terminal region of the NPY molecule and modified into a non-peptide structure, exhibits low nanomolar affinity for the Y1 receptor, and appears to share overlapping binding sites with NPY (Doods, 1996 and Sautel, 1996). However, this compound has been shown to exhibit nanomolar affinity at other reported subtypes (Gerald, 1996), and unfortunately, limited aqueous solubility coupled with poor oral absorption and central nervous system penetration, limit the use of this compound for testing further the role of the Y1 receptor. A third Y1 antagonist (SR120819) has been described which has nanomolar affinity and is capable of Y1 inhibition in second messenger studies and tissue preparations (Serradeil-Le Gal, 1995). More recently, a series of novel benzimidazoles have been evaluated as Y1 selective antagonists (Zarrinmayeh, 1998).

1.6.4.2 Y2 receptor

Like the Y1 receptor, this NPY receptor subtype was delineated using vascular preparations (Wahlestedt, 1986). Pharmacological evidence had detailed the existence of this subtype, which was initially cloned in 1995 from human SMS-KAN cells using an expression cloning technique (Rose, 1995). Subsequent independent groups also cloned this receptor from a variety of sources, namely: human brain cDNA libraries (Gehlert, 1996a and Gerald, 1996) and the human neuroblastoma cell line KAN-TX (Rimland, 1996).

The human Y2 receptor protein is 381 amino acids, exhibits low homology with other known receptors for the PP-fold peptides, and displays a high degree of species conservation; the rat and human gene share 98% amino acid identity (Larhammer, 1996b). Like the Y1 receptor, this receptor is coupled to the inhibition of adenylyl cyclase, although some studies have demonstrated a lack of pertussis toxin sensitivity (Foucart, 1989; Colmers, 1989) in certain systems. Therefore, like the Y1 receptor, the Y2 receptor may display differential coupling to second messengers.

The pharmacology displayed by this receptor is characterised by the following order of potency:

NPY = PYY > C -terminal fragment NPY^{13-36} > Pro^{34} NPY > PP.

As the order of potency suggests, the Y2 receptor is capable of recognising carboxy- terminal fragments of NPY, and appears not to require the whole NPY molecule for activation, thereby creating a basis for pharmacological distinction between the Y1 and Y2 receptors. The C-terminal region of the peptide is of crucial importance for recognition by Y2 receptors, as demonstrated by the inactivity of the substituted analogue Pro^{34}NPY at Y2 receptors (Fuhlendorf, 1990) and the selective Y2 agonist activity of the C-terminal fragment, NPY 13-36 (Wahlestedt, 1986).

The Y2 receptor is located in a variety of brain tissues, with particular abundance displayed in the hippocampus, substantia nigra, thalamus, hypothalamus and brainstem (Dumont, 1993 and Gehlert, 1992). In the periphery, Y2 mRNA is less abundant, with Y2 receptors distributed in the peripheral nervous system, particularly in sympathetic, parasympathetic and sensory neurones (Gehlert, 1998). SMS-KAN cells are a human cell line containing a homogenous population of Y2 receptors (Wieland, 1995)

The effects associated with activation of Y2 receptors are illustrated in figure1.8.

Several agonists for this receptor have been described since the initial identification of this subtype using the C-terminal fragment NPY^{13-36} (Wahlestedt, 1986). A modified, cyclic dodecapeptide analogue of NPY has been shown to be the smallest full agonist at the human Y2 receptor, and has aided modelling of the bioactive conformation of NPY at the Y2 receptor (Rist, 1996). A competitive peptide antagonist based on the design of a cyclic molecule containing four attached COOH terminal fragments of NPY, and termed T4-[NPY(33-36)]4, has been described as a selective Y2 antagonist, and represents the first selective Y2 receptor antagonist (Grouzmann, 1997).

1.6.4.3 Y3 receptor

The Y3 receptor is distinguished from other members of the NPY receptor family in that it displays a high affinity for NPY, while demonstrating a relatively low affinity for PYY. This receptor is best characterised in rat brainstem, in the region of the nucleus tractus solitarius, where application of NPY elicits a dose dependent attenuation of blood pressure and heart rate (Grundemar, 1991a, 1991b). A brainstem Y3 receptor has also been identified by electrophysiology technology (Glaum, 1997). However, the expression of Y1, Y2 and other PP family receptors in this area make pharmacological evaluation difficult, as a variety of receptor subtypes may play a role in cardiovascular regulation via the brainstem. Several other model systems such as rat colon (Dumont, 1994), rat lung (Hirabayashi, 1996) and rat and bovine adrenals (Bernet, 1994; Norenberg, 1995) also display the ligand binding profile which is characteristic of the Y3 receptor. In bovine adrenal chromaffin cells, the Y3 receptor is coupled to an influx of calcium and does not appear to affect adenylyl cyclase activity (Wahlestedt, 1992).

The pharmacology displayed by this receptor is characterised by the following order of potency:

NPY >>Pro^{34}NPY >NPY^{13-36} >>PYY, PP.

The effects associated with activation of the Y3 receptor are illustrated in figure 1.8.

As this receptor has not been cloned, and no specific agonists or antagonists have been described, the current consensus holds that the evidence is insufficient to grant the Y3 site receptor status. Recommendations outlined by the International Union of Pharmacology have designated nomenclature for such binding sites and responses where NPY is considerably more potent than PYY as "putative" NPY receptors (Michel, 1997).

1.6.4.4 Y4 receptor

A homology cloning strategy was responsible for the cloning of the Y4 receptor in 1995 (Lundell, 1995). The gene was initially cloned from a human genomic library and originally designated PP1, while another group identified the clone and named it Y4 (Bard, 1995).

Rat (Lundell, 1996, Yan, 1996) and murine (Gregor, 1996a) homologues have subsequently been cloned, and there appears to be considerable sequence divergence exhibited when human and rat Y4 sequences are compared, in contrast to the apparent species conservation displayed by the Y1 and Y2 receptors. Only 75% amino acid identity is observed, making this one of the most poorly conserved G-protein coupled receptors known (Lundell, 1996). Like other receptors in this family, agonist stimulation of Y4 results in an inhibition of adenylyl cyclase activity as well as stimulation of PI hydrolysis and mobilisation of intracellular calcium (Gehlert, 1998).

A unique feature of the Y4 receptor is evident in the unusual pharmacology displayed, the predominant characteristic being the extremely high affinity for PP of the same species.
The pharmacology displayed by this receptor is characterised by the following order of potency (Gehlert, 1996b):
PP >>Leu^{31} Pro^{34}NPY > PYY > NPY > NPY^{13-36}
This pharmacological order of potency suggests that like the Y1 receptor, both the C- and N-termini are required for full potency, implying that the binding domain is similar in these two subtypes, as both the Y1 and Y4 receptors fail to recognise C-terminal fragments of NPY. It is noteworthy that structure-activity analysis has revealed differing binding profiles between rat and human Y4 receptors. The rat Y4 receptor appears to possess a more selective receptor interaction with rat PP vs NPY or PYY, whereas the human Y4 receptor exhibits a less selective interaction with rat PP vs NPY or PYY, and a greater dependence on N-terminal PP residues, relative to rat (Walker, 1997).

The distribution of Y4 mRNA differs between species, with human Y4 mRNA levels being prevalent in colon, small intestine and pancreas, while other peripheral tissues appear to lack message, and various CNS regions display low expression levels (Lundell, 1995). In contrast, rat Y4 mRNA levels are abundant only in the testis and lung (Lundell, 1995). Additional heterogeneity has been suggested as a result of findings that in rat brain, high levels of ^{125}I bPP binding are found principally in two structures, namely the area postrema and the interpeduncular nucleus. As the high level of binding in the interpeduncular nucleus is not observed using a radioligand with high affinity for Y1 and Y4 receptors, such as ^{125}I Leu^{31} Pro^{34}-PYY, another subtype of PP receptor may be involved (Gehlert, 1997a).

The functions attributed to the Y4 receptor are likely to mediate some of the actions elicited by PP in the digestive tract such as, inhibition or stimulation of gastric secretion, inhibition of pancreatic secretion, decrease in gall bladder activity and a reduction in intestinal motility, as outlined in figure 1.8 (For a review see Hazelwood, 1993). Centrally administered PP produces a modest increase in feeding (Clark, 1984), which may be mediated by centrally located subtypes, but which remains unclear to date (Gehlert, 1997a).

Among the NPY antagonists, the Y1 antagonist BIBP3226 has a very low affinity for the human Y4 receptor, whereas the other Y1 antagonist 1229U91 behaves as a high affinity agonist at the Y4 receptor (Schober, 1998). PP is a useful tool for identifying and defining Y4 sites, as it exhibits low affinity for both Y1 and Y2 receptors. There are currently no selective Y4 antagonists described.

1.6.4.5 Y5 receptor

In 1996 the cloning of a fifth member of the NPY receptor family was described. The NPY Y5 receptor was cloned using an expression cloning technique with libraries derived from rat hypothalamus (Gerald, 1996), and was shown to encode a 456 amino acid protein which displays relatively low identity (<35%) with other members of the PP-fold receptor family. Another group reported the cloning of a shorter 445 amino acid version, which may be the result of alternative splicing (Hu 1996). The human orthologue was subsequently cloned, and shown to exhibit 87% overall amino acid identity and 99% identity in the transmembrane domains. The human Y5 receptor displays an interesting genomic arrangement in that it appears to reside on human chromosome 4q i.e. the same location as the human Y1 receptor gene but in the opposite orientation (Gerald, 1996 and Hu, 1996). A mouse homologue was isolated from a mouse brain cDNA library, and analysis of the predicted amino acid sequence indicates that the polypeptide encoded by this cDNA is 89% and 97% identical to the human and rat Y5 receptors respectively (Nakamura, 1997). When expressed in mammalian cells, this receptor, like the other NPY receptor family members, also couples to the inhibition of adenylyl cyclase (Gerald, 1996).

The pharmacology of this receptor is characterised by the following order of potency:

NPY > PYY= Pro^{34}NPY = NPY^{2-36} = PYY^{3-36} >>NPY^{13-36}.

The rat clone for this receptor exhibits an unusual pharmacology in that the receptor displays highest affinity for NPY and PYY, with substantially lower affinity for rat PP. However, human PP has a very high affinity for the rat Y5 receptor (Gerald, 1996) and provides a potential tool for determining the physiological function of this receptor in the rat. Substituted analogues such as Pro^{34}NPY exhibit a high affinity for this subtype, as do longer C-terminal fragments of NPY and PYY such as NPY^{2-36}, which has a similar potency when compared to the native peptide. The most selective peptide for this receptor is [d-Trp^{32}]NPY, which displays low nanomolar affinity for the Y5 receptor, while exhibiting micromolar affinity for the other NPY receptor subtypes (Gerald, 1996).

The distribution of the Y5 receptor has been elucidated from Northern blot analysis and *in situ* hybridisation studies, with mRNA for this subtype being most prevalent in several brain areas, in particular within various hypothalamic structures, as well as a peripheral location in the testis (Gerald, 1996 and Hu, 1996). Y5 mRNA distribution is similar in humans, differing only in the amount of Y5 mRNA present, where levels are more abundant in rat brain (Jacques, 1997). Phenomenal interest has mounted surrounding this NPY receptor subtype, as the pharmacology of this receptor demonstrates great similarity to the pharmacology observed to mimic NPY-stimulated enhancement of food intake (Gerald, 1996). This receptor may therefore be responsible for mediating the characteristic effects that NPY exerts on feeding

behaviour, and may represent the previously referred to "Y1-like" or "feeding receptor". Further evidence in support of the role of this receptor in food intake regulation is derived from the distribution of this subtype which is predominant in areas of the brain believed to be important in co-ordinating the regulation of food intake and energy balance (Gerald, 1996). The Y5 receptor may also be involved in mediating the NPY-stimulated enhancement of renal sodium excretion (Bischoff, 1997), as the order of potency for this receptor also mimics this response. Figure 1.8 summarises the effects of activation at theY5 receptor.

No selective Y5 antagonists have been described in the literature to date. However, the ability of proposed Y1 selective antagonists to block NPY stimulation of feeding behaviour in several studies, has shed controversy upon the role of the Y5 receptor in NPY induced feeding (Kanatani, 1996). Further research will reveal which receptor subtype(s) are involved in mediating this effect of hypothalamic NPY.

1.6.4.6 y6 Receptor

In 1996 an additional receptor subtype was cloned from mouse genomic DNA, which exhibited Y1-like pharmacology, encoded a 371 amino acid protein and was initially designated Y5 (Weinberg, 1996). Subsequent reports described the same clone as a high affinity PP receptor (PP2) (Gregor, 1996b) and a C-terminal peptide preferring Y2-like receptor (Y2b) (Matsumoto, 1996). Homologues have also been cloned from mice, rabbits, primates and humans. In primates and humans, the gene is a pseudogene, and therefore the recommended designated nomenclature for this receptor is denoted in lowercase as y6. Gene distribution studies have shown that the y6 gene is present in human, monkey, mouse, dog, cow, rabbit, and chicken, but completely absent in rat (Burkhoff, 1998). The y6 receptor exhibits very limited sequence homology to the other NPY receptor family members, and appears to be most closely related to the Y1 receptor.

The human and primate sequences differ from those in mice and rabbits, as they contain a deletion of a single nucleotide, resulting in a frame shift mutation located in the putative third intracellular loop of the receptor. As a result, the human and primate y6 receptor sequences contain a termination codon in a position that results in a 290 amino acid truncated protein, and lacks the characteristic 7 transmembrane domain. The resulting receptor fails to bind PP-fold peptides as the protein encoding the receptor is not expressed. *In vitro* mutagenesis of the pseudogene to restore the frame results in a protein with the predicted 7 transmembrane domain topology, but still fails to bind members of the PP family of peptides. In comparison the murine or rabbit clones readily express functional receptor proteins, suggesting that the y6 gene in primates may have become a non-functional pseudogene during evolution (Rose, 1997).

This receptor has proved difficult to categorise in terms of pharmacology as various groups have observed differing results, with the receptor appearing to exhibit diverse pharmacology. The pharmacology of this NPY receptor therefore remains controversial (Weinberg, 1996; Gregor, 1996b and Matsumoto, 1996).

The mRNA for this receptor is unusually long at 9.8Kb in the mouse, and identification and subsequent distribution analysis has been accomplished by *in situ* hybridisation as opposed to Northern blot analysis which failed to detect y6 mRNA in murine and rabbit brain. Expression of the y6 receptor in murine brain was initially identified in the hypothalamus (Weinberg, 1996), and was identified using RT-PCR in rabbit brain areas including hippocampus and hypothalamus, and in small intestine and adrenals (Matsumoto, 1996). The human y6 gene has been localised to human chromosome 5 (Gregor, 1996), and in contrast, mRNA for the human receptor has been readily detected using Northern blot analysis, and shown to be widespread in heart and skeletal muscle (Gregor, 1996b and Matsumoto, 1996).

This receptor remains in an experimental context, as it is unlikely that it plays any role in human physiology, and its unusual pharmacology needs to be characterised before an understanding of the role it may play, with regard to actions of the PP-fold peptides, can be delineated in other species expressing the full length receptor.

1.6.4.7 Additional sites

Additional receptor subtypes for the PP-fold family have been suggested from reports outlining a preferential PYY response in several systems. The PYY-preferring receptor has been identified in several systems, is distinguished by exhibiting a modest preference for PYY over NPY and was first described in the rat small intestine as having a 5-to 10-fold higher affinity for PYY over NPY (Laburthe, 1986). Subsequent reports have described this receptor in adipocytes (Castan, 1993) and in a kidney proximal tubule cell line (Voisin, 1993). This receptor behaves like the other PP-fold receptor family members in that it appears to be coupled to the inhibition of adenylyl cyclase via a pertussis toxin sensitive G-protein (Voisin, 1993). The receptor(s) responsible for this pharmacology have not been successfully cloned to date, and it has been suggested that the pharmacology may be the result of Y1 receptor expression in a different tissue (Holliday, 1997). Critical to the further identification of this receptor will be the identification of specific clones for the receptor, resulting in a greater understanding of its physiological role.

A receptor belonging to the NPY receptor family has also been cloned from *Drosophila melanogaster* (Li, 1992), and more recently multiple NPY receptor subtypes have been cloned from the Zebrafish (*Danio rerio*), which appear to be distinct from all known mammalian

Figure 1.8 Summary of the physiological effects observed upon activation of the NPY receptor subtypes.

Receptor subtype	*Effect*	*Target*
Y1	Vasoconstriction and potentiation of vasoconstriction	Blood vessels
	Anxiolysis and sedation	Amygdala
	Stimulation of feeding behaviour	Hypothalamus
Y2	Vasoconstriction	Certain blood vessels
	Suppression of transmitter release	Sympathetic and parasympathetic nerve fibres
	Enhanced memory retention	Hippocampus
	Suppression of glutamate release	Hippocampal neurons
	Suppression of noradrenaline release	Locus coruleus
Y3	Centrally mediated increase and decrease in arterial blood pressure and bradycardia	Nucleus tractus solitarius
	Inhibition of glutamate responsiveness and baroceptor reflex	Nucleus tractus solitarius
	Inhibition of catecholamine release	Adrenal medulla
Y4	Inhibition of pancreatic secretion	Pancreas
	Inhibition or stimulation of gastric secretion	Gastro-intestinal tract
	Reduction in gall bladder activity	Gall bladder
	Stimulation of feeding behaviour	Hypothalamus
Y5	Stimulation of feeding behaviour	Hypothalamus
	Stimulation of renal sodium excretion	Kidney

subtypes (Lundell, 1997 and Ringvall, 1997). It remains to be determined whether mammalian homologues exist, and more detailed studies are required in order to determine the role these subtypes play in the large family of NPY/PP receptors

1.7 The role of Neuropeptide Y in feeding behaviour

As detailed above in section 1.4, since the 1950's, the hypothalamus has been implicated in the control of feeding behaviour and energy homeostasis, with the pursuit of hypothalamic signals specifically encoding the sensation and experience of hunger, being highly competitive and of widespread interest. The past decade has witnessed numerous revelations implicating hypothalamic NPY as the long sought after transducer of appetite.

1.7.1 Historical aspects of the role of NPY in feeding behaviour

The wealth of interest surrounding NPY, with respect to obtaining new insight into the complex neural network underlying consummatory behaviour, can be attributed to the discovery of the orexigenic (from the Greek word orexus meaning appetite) properties of NPY in the rat. In 1984 the first studies were published describing stimulation of ingestive behaviour upon intracerebroventricular (icv) injection of NPY (Clark, 1984). Clark *et al* demonstrated that injection of a 2μg dose of NPY resulted in significant stimulation of food intake in treated rats when compared with control treated rats. From these initial studies, NPY appeared to induce satiated animals to eat a normal sized meal at low doses, while at higher doses, NPY was capable of inducing eating behaviour of unparalleled proportions. These dramatic effects suggested that NPY and other structurally related members of the PP-fold family, may play a role in regulating naturally occurring feeding behaviour (Clark, 1984). As a result of these findings, Clark concluded that NPY or an NPY-like peptide abundant in the rat brain may be the neurotransmitter or neuromodulator responsible for the physiological regulation of feeding behaviour.

Subsequent experiments demonstrated in a number of species including pigs (Parrot, 1986), mice (Morley, 1987a), and sheep (Miner, 1989), that central administration of NPY stimulates feeding and enhances the on-going pattern of feeding in a dose related manner. In the same year, Levine *et al* also described the ability of NPY to stimulate feeding, and proposed additionally that NPY could alter the receptor conformation of other compounds which act to augment ingestive behaviour for example dopamine and opioids, resulting in an enhanced effect due to cooperativity between these entities and NPY (Levine, 1984). NPY was shown to increase total ingestion time (Kalra, 1987), and repeated NPY injection effectively postponed the satiety which normally follows a single injection (Morley, 1987b), suggesting that NPY stimulates ingestive behaviour by a mechanism involving reduced satiety signals. Detailed

analysis of ingestive patterns (Lynch, 1994), confirmed attenuation of satiety as a contributing mechanism by which NPY elicits its characteristic effects on feeding behaviour.

Evidence also suggested that NPY, as well as attenuating satiety, could stimulate ingestive behaviour by increasing the motivation to eat. Using a selection of behavioural paradigms designed to assess motivation, Flood *et al* implicated increased motivation to eat as a mechanism by which NPY augments food intake (Flood, 1991). The potent stimulatory effect of NPY on feeding is evident from findings that NPY enhances feeding and drinking during nocturnal feeding, following a 24 hour food deprivation period, and during the first half of the light cycle, a period when rats normally refrain from eating (Kalra,1996).

It is noteworthy that the pattern of feeding elicited by neuropeptide Y is of a discontinuous nature, resembling the normal nocturnal feeding pattern in the rat (Kalra, 1988), with NPY release in relevant brain sites activated at the onset of the dark phase, where it serves to initiate and sustain nocturnal feeding (Clark, 1984, 1985).

1.7.2 Anatomical Pattern of Neuropeptide Y induced feeding

As previously discussed in section 1.4.4 the hypothalamus is a brain region with paramount importance in the co-ordination of food intake and energy balance, and which also harbours the highest NPY concentration of any brain region (Allen *et al* , 1983). With this in mind, experiments were performed in order to delineate the role of the hypothalamus in NPY-elicited eating.

The paraventricular nucleus (PVN) of the hypothalamus is richly innervated with this peptide, containing one of the densest supplies of NPY-containing presynaptic terminals in the brain, and also housing a close functional relationship between NA and NPY. As a result of existing evidence detailing a stimulation of feeding behaviour by PVN injection of exogenous NA, as well as by release of endogenous NA, it was suggested that NPY in the PVN may act similarly in its effects on feeding behaviour. In 1984, Stanley and Leibowitz demonstrated that injection of NPY into the PVN of satiated, brain cannulated rats, elicited a strong, dose-dependent increase in food intake, at picomolar doses (Stanley, 1984). This robust feeding response suggested an important role for hypothalamic NPY in the control of feeding behaviour.

The following year witnessed the advent of NPY as a powerful stimulant of ingestive behaviour in its own right, when previous hypotheses outlining a possible interaction between NPY and NA in the hypothalamic control of feeding were dispelled, and NPY was shown to elicit a robust increase in food intake without functional interaction with NA (Stanley, 1985a). Subsequent studies demonstrated the ability of NPY to elicit sustained hyperphagia and obesity

in rats, upon PVN injection, and postulated that disturbances in NPY function may play a role in disorders of eating behaviour and bodyweight regulation (Stanley, 1986).

Immunocytochemistry studies documented the presence of NPY in the PVN, and revealed a pattern of NPY distribution in exclusive axon terminals, where NPY forms a variety of conventional synaptic contacts (Sawchenko, 1988). Studies detailing immediate early gene expression in various brain sites in response to intracerebroventricular injection of NPY, also confirmed the presence of NPY target cells in the PVN and surrounding sites implicated in feeding (Li, 1994).

More recent studies proposed that an additional hypothalamic site is involved in NPY elicited eating. A cannula mapping study of the hypothalamus revealed that NPY is most effective in stimulating feeding when injected into the perifornical hypothalamus (PFH), an area lateral to the PVN (Stanley, 1993). The multiple sites of action regarding feeding and NPY in the hypothalamus, suggests that NPY may have distinct functions in the PVN and PFH. NPY in the PFH has been implicated in mediating eating behaviour, while the PVN has additional roles in regulating metabolic and endocrine effects associated with NPY elicited eating behaviour (see sections 1.7.6.1 and 1.7.6.2) (Stanley, 1993).

1.7.3 Hypothalamic NPY and feeding-The pathway involved

Various studies have illustrated that two populations of NPY producing-neurones, the intrahypothalamic group clustered in the arcuate nucleus (ARC), and the extrahypothalamic group in the brainstem, project to various hypothalamic sites implicated in the control of ingestive behaviour; namely the PVN, PFH, dorsomedial nucleus, ventromedial nucleus and arcuate nucleus (Sahu, 1988b and Kalra, 1996). Projections of NPY-containing cells in the arcuate nucleus are postulated to be the primary source of NPY involved in the regulation of feeding, as almost complete elimination of NPY from the brainstem failed to alter the normal ingestive behaviour pattern in rats (Sahu, 1988b). Therefore, a specific pathway encompassing cell bodies in the ARC, projecting principally to terminals in the PVN, was proposed to mediate the augmentation of feeding behaviour elicited by hypothalamic NPY (Mjhanwar-Uniyal, 1993). Experimentally induced reduction in the NPY supply to the PVN, either by interruption of NPY production in the ARC by anti-sense oligodeoxynucleotides (Akabayashi, 1994), or by immunologic impairment of ARC NPY, resulted in a significant suppression of food intake (Burlet, 1995), highlighting further the importance of this pathway in the NPY mediated stimulation of feeding.

1.7.4 Evidence for the role of NPY as an endogenous mediator of food intake

Evidence in favour of NPY as a physiological neurochemical signal involved in the regulation of food intake has come from observations that a single dose of NPY administered during the dark phase, when rats normally eat, rapidly and reliably enhances the ongoing rate of food intake for four hours (Clark, 1985), and that continuous intravenous NPY infusion produces sustained hyperphagia and obesity resembling that elicited by either hypothalamic lesions or genetic alterations (Stanley, 1986). Further support for the hypothesis that NPY may function as a physiological promoter of food intake, came from studies aimed at investigating NPY neuronal function in response to food intake and food deprivation. Fasting and feeding were shown to induce reciprocal changes in the concentration of NPY in the PVN of the rat, with food deprivation producing a dramatic accumulation of NPY in this hypothalamic region (Kalra, 1988). The reciprocal responses evoked by sequential food deprivation and satiety, indicate that under normal circumstances NPY in the PVN may act primarily to stimulate food intake. Perturbations in daily patterns of food intake, e.g. after a period of food deprivation, have been shown to promote a steady increase in NPY levels in the ARC, representing an enhanced rate of NPY synthesis, and further implicating NPY in the control of food intake (Kalra, 1988 and Sahu, 1988a). Subsequent studies demonstrated changes in PVN neuropeptide Y levels in an experimental paradigm which reliably reflects appetite in the rat, where sequential changes in appetite reflected alterations in NPY release in the PVN. NPY release in the PVN was shown to be a potent orexigenic signal for periodic eating behaviour, as NPY concentrations were shown to be elevated before the introduction of food, and thereafter, levels decreased significantly during the course of eating (Kalra, 1991). Intense exercise has also been shown to elicit increased hypothalamic NPY levels, suggesting that negative energy balance, whether caused by decreased energy intake, or increased energy expenditure, results in stimulated hypothalamic NPYergic activity (Lewis, 1993). Physiological situations such as lactation, which demand increased food consumption, also induce upregulation of the ARC-PVN NPYergic system (Malabu, 1994).

Further evidence supporting the role of NPY as an endogenous hypothalamic modulator of food intake, has come from studies where hypothalamic NPY expression has been shown to be elevated following food deprivation, with a specific increase in preproNPY (the NPY precursor) mRNA localised to the ARC of the hypothalamus. This supports the existence of a specific ARC-PVN NPY pathway regulating food intake, with increased levels of preproNPY mRNA in the ARC resulting in a concomitant increase in NPY synthesis, reflected by augmented levels of the peptide in terminals in the PVN (White, 1990). More recent findings have reported that low levels of hypothalamic NPY may also result in

hyperphagia and obesity, and have attributed this to an increased receptor sensitivity to NPY (Kalra, 1997). The NPY receptor involved in the modulation of feeding behaviour will be discussed in detail in section 1.8.

Increased secretion of NPY in the PVN has been observed in animal models of hyperphagia and obesity, and also in diabetic animals, where hyperphagia coincides with increased release of NPY in the PVN (Sahu, 1992), with a concomitant activation of NPY synthesis in the ARC in response to the elevated demand imposed by NPY hypersecretion (White, 1990).
NPY has also been implicated in the pathogenesis of eating disorders such as bulimia and anorexia. Cerebrospinal fluid NPY concentrations are significantly elevated in underweight anorexic patients, and in many anorexic patients studied at intervals after weight restoration, but are normalised following restoration of body weight in long term weight restored anorexics (Kaye, 1990). Similarly the related peptide family member PYY is implicated in the pathogenesis of bulimia, as PYY is significantly elevated in the cerebrospinal fluid of bulimic patients (Kaye, 1990).

The use of anti-sense technology as a means of evaluating the effects of NPY on feeding behaviour remains controversial, with different groups reporting conflicting data. Initial studies using anti-NPY oligonucleotides to determine the effects of this treatment on food intake, feeding behaviour and bodyweight, described a significant decrease in cumulative food intake, meal size and duration, and bodyweight after anti-sense treatment (Hulsey, 1995). More recently, findings have been presented which describe a lack of specificity, coupled with non-selective effects on food intake and bodyweight, in rats treated with an oligonucleotide against NPY (Dryden, 1998). Therefore, the validity of this approach in determining the physiological part NPY may play in the regulation of appetite, remains questionable.

Another strategy employed to assess the function of NPY with regard to ingestive behaviour, is the use of genetically deficient NPY mice. The use of NPY-knockout mice theoretically permits the study of the role performed by NPY in the long-term regulation of feeding and adiposity. Erickson *et al* in 1996 described the effects of NPY removal on bodyweight, and surprisingly, despite the absence of NPY, the knockout mice maintained normal bodyweight, appearing normal in every aspect, with the exception of a propensity for seizure development (Erickson, 1996). NPY deficient mice displayed a normal hyperphagic response, and rapidly gained weight following a fast. Consistent with these findings, the neuroendocrine response to fasting did not appear to be impaired in NPY deficient mice (Erickson, 1997). Potential explanations for the lack of detectable effects of NPY deficiency on feeding behaviour, is that unique adaptations to congenital NPY deficiency are capable of functionally compensating for the absence of the peptide, or that redundant mechanisms exist. As food intake is a

fundamental physiological process, essential to survival, it is exceedingly likely that such mechanisms are called into play when an endogenous mediator of food intake is removed during development of the organism.

1.7.5 Natural dietary preferences and NPY

NPY appears to have a role in controlling the ingestion of specific macronutrients, as revealed by studies employing self-selection paradigms, where rats had access to diets containing pure macronutrient (Stanley, 1985b). Hypothalamic NPY acts to preferentially augment carbohydrate ingestion, while having little or no effect on fat or protein consumption (Stanley, 1985b). It has been postulated that the highly favoured carbohydrate ingestion elicited by NPY (and PYY), could be related to the fact that this diet provides a rapid source of usable energy (Steffens, 1969).
The pathway thought to mediate the actions of NPY on food intake has also been associated with specific ingestion of carbohydrate. This was demonstrated when NPY projections from the arcuate nucleus to the parvocellular division of the PVN were shown to be uniquely involved in the selective consumption of carbohydrate elicited by NPY (Jhanwar-Uniyal, 1993).

1.7.6 Metabolic and endocrine effects of NPY in the regulation of feeding behaviour

The role of NPY in the regulation of nutrient and energy balance is not confined solely to an effect on feeding behaviour, as in addition, endocrine systems and metabolism are also crucially important factors which may be influenced by NPY.

1.7.6.1 Endocrine systems

NPY modulates the release of certain hormones which have the ability to influence energy metabolism and food intake. These include corticosterone (CORT), adrenocorticotrophin hormone (ACTH), insulin and anti-diuretic hormone (ADH). The neuroendocrine actions of NPY involve the PVN, which has a primary function in controlling the release of these hormones. The neuroendocrine effects of NPY are stimulatory in nature, with PVN injection eliciting enhanced release of CORT (Wahlestedt, 1987 and Stanley, 1989), insulin (Akabayashi, 1994) and ADH (Luiten, 1987). The effects of NPY on these endocrine systems is functionally significant, as these hormones influence nutrient metabolism. CORT promotes increased ingestion and metabolism of carbohydrate and synthesis and deposition of fat, insulin favours net energy gain to be stored as triglycerides, and ADH also elicits prominent effects on carbohydrate metabolism. The stimulatory effect of NPY on the release

of CORT, ACTH, insulin and ADH is consistent with the proposed role of NPY in the control of carbohydrate intake and metabolism (Leibowitz, 1994).

1.7.6.2 Metabolic processes

Regulation of metabolism by paraventricular NPY was first described as a result of observations that NPY specifically enhanced carbohydrate intake (Stanley, 1984) and preferentially increased fat deposition (Stanley, 1986). In the PVN, NPY elicits a dose-dependent increase in respiratory quotient (RQ), with a resulting diversion of metabolism toward carbohydrate utilisation and fat synthesis (Menendez, 1990). The selective augmentation in carbohydrate consumption elicited by NPY may therefore occur as a result of this metabolic diversion. Intracerebroventricular injection of NPY has two effects on metabolism in addition to increased carbohydrate consumption; namely a reduction in brown fat thermogenesis, and an augmentation in white fat lipoprotein lipase (LPL) enzymatic activity, resulting in a decrease in energy expenditure in brown fat, and an increase in energy storage in white fat. The stimulation of LPL activity is consistent with the effects of NPY on respiratory quotient, as an increase in RQ promotes carbohydrate oxidation which in turn favours fat storage by promoting lipogenesis (Billington, 1994). NPY decreases brown fat thermogenesis by reducing gene expression for uncoupling protein (UCP) which is present in brown adipose tissue and serves to uncouple oxidative phosphorylation, thereby regulating energy balance (Billington, 1994). NPY also inhibits the sympathetic outflow from the hypothalamus, which drives thermogenesis in brown adipose tissue (BAT), and in doing so reduces energy expenditure (Egawa, 1990 and Bing, 1998).

The aforementioned information accumulated in recent years provides compelling evidence for regarding NPY as a potent orexigenic signal, and depicts a physiological role for NPY as an endogenous appetite-transducing signal. Via its effects on ingestive behaviour, endocrine systems and metabolism, NPY acts as a starvation signal, ensuring energy homeostasis is maintained. The dramatic effects elicited by NPY on feeding behaviour and body weight, have given rise to tremendous interest regarding how this peptide executes its characteristic effect. As a result, a rapidly evolving and highly competitive field of research has emerged, with a view to elucidating further the NPY receptor subtype involved in transducing these effects, and to understanding more comprehensibly a constituent of the complex network which regulates feeding behaviour and energy homeostasis.

1.8 Evidence for a distinct receptor subtype mediating NPY induced feeding

The NPY receptor responsible for mediating NPY induced eating attracted universal interest, as the cloning of this receptor subtype would permit further progress in the development of an anti-obesity agent. The pharmacology of the feeding response characterised by NPY was determined by studies using NPY fragments to reveal which aspects of the NPY molecule were important for stimulation of feeding, and selective NPY receptor agonists to determine the receptor subtype involved.

These studies revealed that both N- and C-terminal regions of the NPY molecule, as well as the α-helix, contribute to NPY elicited eating, as alterations of either terminal region significantly modified NPYs' potency (Kalra, 1991; McLaughlin, 1991 and Stanley, 1992). A structure-function analysis strategy was subsequently employed in order to determine the pharmacological profile of the feeding response, with respect to the role of the then cloned receptors, Y1 and Y2 . With the aid of selective receptor agonists and fragments of the NPY molecule, the pharmacology of the feeding response elicited by NPY was delineated.

In 1990 Kalra and co-workers demonstrated an augmentation in ingestive behaviour after icv or PVN injection of NPY, the Y1 selective agonist $Leu^{31}Pro^{34}NPY$, and NPY^{2-36} an N-terminal tyrosine deleted fragment, which proved to be consistently more effective than the whole NPY molecule in the stimulation of feeding at each dose tested. The Y2 selective agonist NPY^{13-36} appeared to be completely ineffective in eliciting a response. The ability of the Y1 selective agonist to evoke a response implicated the Y1 receptor in the NPY induced feeding response (Kalra, 1991). Additional studies confirmed the pharmacological profile shown to mediate NPY elicited eating, demonstrating the markedly greater effectiveness of the Y1 selective agonist when compared with the Y2 selective agonist, and earmarking the Y1 receptor as the NPY receptor involved in transducing the effects on appetite (Stanley, 1992). However, the indisputable potency of NPY^{2-36}, which is uncharacteristic of Y1-mediated effects, suggested that the receptor involved may be a variant of this subtype (Stanley, 1992). Another difficulty in establishing the Y1 receptor as the entity that mediates the feeding response, is the apparent absence of substantial Y1 binding and Y1 mRNA (Eva, 1990) in paraventricular and perifornical areas of the hypothalamus (Dumont, 1993).

As the pharmacology of the feeding response failed to resemble that required to activate Y1 or Y2 receptors (Kalra, 1991 and Stanley, 1994), and NPY^{2-36} was able to fully stimulate feeding, but not reduce body temperature like the whole NPY molecule (Jolicoeur, 1991),

this led to the consensus that the NPY receptor responsible for eliciting NPY induced eating was a novel NPY receptor subtype with "Y1 -like" properties. This theory was further supported by other studies describing evidence for a heterogeneous population of pharmacologically different NPY receptors mediating the central actions of NPY (Bouali, 1994).

The location of this postulated "feeding receptor" was believed to be the hypothalamus, as existing evidence had illustrated the role of the hypothalamus in co-ordinating energy homeostasis and food intake. The dramatic effects on feeding behaviour observed upon hypothalamic injection of NPY also supported a hypothalamic distribution (For more detail refer to section 1.4 and 1.7).
The ability of an NPY antagonist PYX-2 to block spontaneous carbohydrate intake at the onset of the dark cycle in freely feeding rats, and to inhibit the stimulatory action of exogenously administered PVN NPY, provided evidence for the existence of endogenous hypothalamic NPY receptors responsible for mediating the action of NPY in the PVN (Leibowitz, 1992).

1.8.1 Cloning of the Y5 receptor

The search for the "feeding receptor" finally yielded in 1996, when the cloning of an NPY receptor subtype involved in NPY -induced food intake was reported in *Nature*. An expression cloning strategy had been employed to clone the Y5 receptor from rat hypothalamus (Gerald, 1996). The mRNA for this receptor was distributed primarily in the central nervous system, including the PVN of the hypothalamus, the proposed location of the feeding receptor (Gerald, 1996). The distribution of Y5 mRNA in midline thalamic nuclei, which project heavily to the amygdala, suggested another potential role for the Y5 receptor in the regulation of the emotional aspect of appetitive behaviours (Gerald, 1996). The mRNA for this receptor is rare, as illustrated by the extremely low level of abundance of the Y5 receptor in the hypothalamic library used to clone the receptor, which contained only one Y5 receptor clone (Gerald, 1996). Use of the peptide analogue demonstrated that the pharmacological profile of the cloned Y5 receptor appeared to be consistent with that for the *in vivo* pharmacology of feeding in rodents (Clark, 1984; Kalra, 1991 and Stanley, 1992), with C-terminal fragments of NPY and PYY; namely NPY^{2-36} and PYY^{3-36} eliciting a robust stimulation of food intake (Gerald, 1996).

The Y1 receptor was dismissed as the feeding receptor in this study; the evidence in favour of the Y5 receptor reinforced by findings that the Y1 selective antagonist BIBP3226 failed to reduce food intake induced by NPY (Gerald, 1996). The distribution of Y5 mRNA in critical areas of the hypothalamus known to regulate food intake, together with an *in vitro*

pharmacology profile consistent with the *in vivo* feeding data, strongly suggested that the Y5 receptor was the primary mediator of NPY induced feeding. In the same year, another group reported the cloning of the Y5 receptor from rat hypothalamus, which shared a similar pattern of mRNA distribution, exhibited an identical pharmacological profile, and differed only in amino acid sequence, containing 11 fewer N-terminal residues than the previously reported Y5 receptor (Hu, 1996).

Anti-sense oligonucleotides targeted to the Y5 receptor were used to assess the functional importance of this receptor *in vivo*. Intracerebroventricular injections of Y5 anti-sense prevented fasting-induced food intake in rats, significantly decreased basal food intake, and inhibited the increase in food intake after icv NPY injection (Schaffhauser, 1997). The development of a non-peptide, high affinity Y5 selective antagonist also fuelled the argument in favour of the Y5 receptor as the feeding receptor, as *in vivo* studies using this antagonist (CGP71683A) revealed a dose dependent inhibition of NPY induced feeding in satiated rats, a reduction in meal size and number, and a concomitant decrease in bodyweight after 28 days administration (Hofbauer, 1997; Criscione, 1997). However, the apparently clear-cut role postulated for the Y5 receptor in mediating NPY-elicited eating was tainted, when a study describing the inability of a Y5 selective antagonist to inhibit NPY induced feeding was reported (Kanatani, 1997).

1.8.2 Feeding receptor controversy

The inability of the selective Y5 antagonist to inhibit NPY induced feeding (Kanatani, 1997) sparked considerable controversy regarding the identity of the feeding receptor, and coupled with recent findings strongly implicating the NPY Y1 receptor as the appetite transducing agent, the literature at present remains controversial and contentious. Various groups have reported contradicting findings, implicating either the Y5 receptor, the Y1 receptor, a novel NPY receptor, or a functionally co-operative relationship between Y1 and Y5 receptors, as the means by which NPY elicits eating.

The evidence incriminating the Y1 receptor in the control of feeding, has arisen primarily from studies demonstrating the ability of Y1 selective antagonists to inhibit food intake. The peptide antagonist 1229U91, which has previously been described as a potent and selective Y1 receptor antagonist (Daniels, 1995), was shown to block both NPY induced and natural feeding in rats (Kanatani, 1996). In the same study, radiolabelled ^{125}I 1229U91 detected high affinity binding sites in rat hypothalamic membranes, signifying the presence of Y1 receptors in the hypothalamus, and further supporting the proposed role for Y1 receptors in mediating ingestive behaviour (Kanatani, 1996, 1998).

Subsequent studies with Y1 selective antagonists have reinforced the argument in favour of the Y1 receptors' role in feeding. The di-peptide Y1 selective antagonist GI 264879A also rendered a long lasting dose dependent decrease in food intake and bodyweight in rats, stimulated by an increase in either endogenous or exogenous NPY (Matthews, 1997). More recently, a novel NPY Y1 receptor selective antagonist BIB3304 has also been shown to inhibit feeding in rodents (Wieland, 1998). Spontaneous food intake in Zucker fatty rats (who lack an enzyme required for effective processing of hypothalamic peptides) was also shown to be suppressed by the Y1 selective antagonist 1229U91, suggesting NPY is involved in feeding behaviour in these animals, via the Y1 receptor subtype (Ishihara, 1998).

Immunohistochemistry and *in situ* hybridisation studies revealed a decline in the number of Y1 receptors and Y1 mRNA levels in the ARC of fasted rats, thereby endorsing the postulated role outlined for Y1 receptors in the feeding response to NPY (Cheng, 1998). An antisense strategy against the Y1 receptor also implicated hypothalamic Y1 receptor involvement in the feeding elicited by NPY (Lopezvalpuesta, 1996). However, other studies employing anti-sense technology have ruled out a role for Y1 receptors in mediating this response, as food intake was shown to be paradoxically increased after Y1 anti-sense treatment, in studies carried out in rats (Heilig, 1996).

1.8.2.1 Y1 and Y5 receptor involvement in the feeding response to NPY

New evidence has emerged recently from studies with Y1 and Y5 knockout mice, which suggests that both of these NPY receptors are involved in feeding behaviour. Like NPY knockout mice (Erickson, 1996), Y1 and Y5 deficient mice do not display overt major disturbances in bodyweight and daily food intake. This isn't entirely surprising considering the central control of feeding behaviour is highly redundant, ensuring this fundamental function is unaffected.

However, fasting induced feeding is severely affected in Y1 -deficient mice, in contrast with NPY induced feeding which appeared relatively unaltered when compared with wild-type controls. This data indicates that the Y1 receptor participates in the stimulation of food intake, but that other types of NPY receptors are also involved (Pedrazzini, 1998).

In Y5 deficient animals, food intake induced by icv injection of high doses of NPY and PYY^{3-36}, was significantly decreased compared with wild-type controls. Feeding induced by lower doses of NPY and related peptides was similar in both Y5 deficient and wild-type animals, suggesting that the Y5 receptor is partially responsible for the ability of NPY and PYY^{3-36} to stimulate food intake in mice, and implicates the involvement of another NPY receptor subtype (Marsh, 1998). The concurrent administration of the Y1 selective

antagonist 1229U91 completely abolished the orexigenic effects of NPY in Y5 deficient mice, suggesting that Y1 or Y1-like receptors, together with Y5 receptors mediate the response to centrally administered NPY (Marsh, 1998).

A role for both Y1 and Y5 receptors in the regulation of NPY-induced feeding is substantiated by findings that the Y5 selective antagonist L152804 is ineffective at inhibiting NPY elicited eating, but can effectively attenuate bPP induced feeding. This implicates the Y5 receptor in the regulation of bPP but not NPY induced feeding. Conversely, the Y1 selective antagonist 1229U91 was shown to inhibit NPY induced feeding, but was ineffective at inhibiting bPP evoked feeding, indicating that the Y1 receptor is involved in NPY induced feeding (Kanatani, 1997). These findings are corroborated by the aforementioned knockout studies, where hPP stimulated food intake was completely abolished in Y5 deficient mice, compared with wild-type controls, inferring that hPP may exert its actions via the Y5 receptor (Marsh, 1998).

As the Y1 and Y5 receptor genes are under the transcriptional control of a common promoter region on chromosome four, it remains to be ascertained whether this is of relevance to the function of the Y1 and Y5 receptors in the feeding response (Herzog, 1997). Expression and characterisation of the Y5 receptor in rat brain has further perpetuated the argument in favour of multiple NPY receptor subtypes mediating feeding (Dumont, 1998). The low densities of Y5 binding detected in rat hypothalamus, implies that Y5 receptor protein is expressed and translated by a small percentage of hypothalamic neurones, and that the effect of NPY on ingestive behaviour is likely to be mediated by more than one class of NPY receptor (Dumont, 1998).

1.8.2.2 A Novel NPY receptor involved in feeding behaviour

An additional theory adding to the existing controversy, has arisen from reports that a receptor distinct from any of those currently cloned, mediates NPY induced feeding. In a study by O'Shea and co-workers, dose-response relationships of the five cloned NPY receptors with various NPY analogues and C-terminal fragments revealed that NPY elicited eating in the rat appeared to be mediated by a functionally distinct receptor (O'Shea, 1997). Studies performed with Y1 selective antagonists 1229U91 and BIBP3226, have suggested that the Y1 receptor does not participate in the NPY feeding response, as 1229U91 failed to block NPY stimulated feeding in CD-1 mice, and the inhibition of ingestive behaviour exhibited by BIBP3226 was shown to be non specific regarding the Y1 receptor, as the stereoisomer of this compound BIBP3435, which has negligible affinity for the Y1 receptor, evoked a similar blockade of NPY induced feeding (Li, 1997). As a result of these findings, it was proposed that an undefined subtype of the Y1 receptor, or indeed a unique NPY

receptor may transduce the effects of NPY elicited eating (Li, 1997). These claims were further substantiated by reports that novel, high affinity Y1 selective antagonists LY353485 and LY357879, were capable of attenuating NPY induced food intake *in vivo* after icv administration of these compounds in CD-1 mice. As the actions of these antagonists differed from those exhibited by the Y1 antagonists 1229U91 and BIBP3226, this data suggests that a subtype of the Y1 receptor, or a novel NPY receptor may mediate ingestive behaviour (Ivengar, 1997).

To date, the identity of the receptor involved in mediating the NPY induced feeding response remains controversial with conflicting findings described in the literature. The advent of delineating the precise mechanics attributed to NPY elicited feeding remains to be fully embraced, and until such a date, the understanding of this peptide's role in regulating the physiology of feeding remains incomplete.

1.9 Functional relationships between NPY and other starvation and satiety signals

As the regulation of appetite and energy homeostasis, is accomplished by a complex network of neurotransmitters, neuropeptides and hormones, the interactions between the NPY pathway, and other components of the circuitry involved in the maintenance of energy homeostasis are of paramount importance.

1.9. 1 NPY -Leptin interaction.

The adipocyte derived hormone leptin has been implicated in the control of energy homeostasis, where it is proposed to act as a negative feedback signal to the hypothalamus, limiting obesity in times of nutritional abundance (Zhang, 1994; Campfield, 1995). Dramatic regulation of the leptin transcript and protein have also been observed in response to short term alteration in food intake, with fasting eliciting a prominent down-regulation, and excessive caloric intake resulting in up-regulation (Frederich, 1995; Maffei, 1995). Leptin is secreted by fat cells in response to nutritional status where it acts on the brain to inhibit feeding, increase thermogenesis, and decrease bodyweight (effects which act in opposition to those observed with hypothalamic NPY). The actions of leptin in suppressing food intake and stimulating thermogenesis are proposed to be mediated in part by inhibiting NPY neurones in the hypothalamus (Stephens, 1995). Leptin appears to reduce levels of NPY in the ARC and PVN, implying concomitant inhibition of synthesis and transport (Stephens, 1995; Wang, 1997). An additional role for leptin influence on NPY signalling has come from studies detailing inhibiton of NPY in the PVN after leptin administration, while UCP gene expression increases, stimulating thermogenesis (Kotz, 1998). As repletion of NPY in

the PVN effectively reversed the feeding inhibitory and thermogenic effects of leptin administration, these results provide functional evidence for leptin modulation of the ARC-PVN NPYergic pathway (Kotz, 1998). Leptin may also have postsynaptic effects, reducing the activity of released NPY in that the hyperphagia induced by injections of NPY into the hypothalamus is attenuated by leptin administration.

1.9.2 NPY -5-HT interaction

The monoamine neurotransmitter 5-hydroxytryptamine (5-HT), is a well established suppressant of food intake. The inhibitory actions on food intake have been localised to the hypothalamus specifically the PVN, where injection of 5-HT attenuates feeding in free feeding and food deprived rats (Shor-Posner, 1986). Pharmacological analysis of the 5-HT elicited reduction in food intake has demonstrated that the effect is mediated by post-synaptic 5-HT_{1B} receptors (Grignashi, 1995). In rats, selective 5-HT reuptake blocking drugs such as fluoxetine are effective in blocking food intake, as are drugs which block both 5-HT reuptake and stimulate 5-HT release, such as fenfluramine (Gibson, 1993). Recent evidence has suggested that the hypophagic actions of 5-HT may be mediated, in part , through the NPY pathway. The 5-HT antagonist methysergide, which stimulates feeding, increases NPY concentrations in the ARC and PVN of the hypothalamus (Dryden, 1993). Opposing these effects, selective 5-HT_1 agonists which reduce food intake, significantly reduce NPY concentrations in the PVN (Dryden, 1996) , and in addition, fenfluramine injections, which reduce food intake by raising synaptic concentrations of 5-HT, reduce levels of NPY in the hypothalamus (Rogers, 1991). The current consensus suggests that NPY and 5-HT have opposing effects in the hypothalamus and that 5-HT may produce its hypophagic activity by directly inhibiting the firing of NPYergic neurones in the ARC leading to reduced NPY release from the PVN (Wilding, 1997).

1.9.3 NPY-Glucagon-like peptide-1(GLP-1) interaction

The gut peptide Glucagon-like peptide-1 (GLP-1), an incretin hormone, is synthesised within the rat brain and is effective at reducing food intake when injected into the 3rd ventricle in the rat, following both food deprivation and icv injection of NPY (Turton, 1996). GLP-1 receptors are present in appetite regulating areas of the brain, such as the PVN of the hypothalamus and the central nucleus of the amygdala. Injection of exendin 9-39, a selective GLP-1 antagonist, increases food intake in the rat and potentiates the appetite stimulating effect of NPY. Assessment of hypothalamic NPY mRNA levels after administration of GLP-1 , revealed no change in NPY mRNA levels , and suggested that GLP-1 does not act by altering hypothalamic NPY synthesis (Turton, 1996). The precise interaction between

GLP-1 and NPY requires further investigation in order to fully appreciate the function of these centrally acting regulators.

1.9.4 NPY-Corticotrophin releasing factor (CRF) interactions

CRF is expressed in the PVN and is an important regulator of adrenocorticotrophin hormone (ACTH) secretion. CRF injected into the PVN elicits weight loss, by reducing appetite and stimulating thermogenesis. It has been proposed that CRF and NPY act as opposing influences in the regulation of energy balance, with CRF mRNA levels altered in the opposite direction to NPY in conditions of energy deficit, such as starvation. There is evidence of synaptic connections between NPY and CRF neurones in the PVN (Mercer, 1996), and CRF may normally inhibit NPY mediated feeding systems, as putative damage to the CRF-containing cells by prior injection of an immunotargeted toxin for CRF into the PVN of rats, has been shown to increase food intake elicited by NPY (Menzaghi, 1993).

1.9.5 NPY-Cocaine and amphetamine regulated transcript (CART) interactions

The centrally-located peptide CART is a satiety factor whose actions are closely related to hypothalamic levels of NPY and leptin. Food deprived animals display a pronounced decrease in expression of CART mRNA in the ARC, and in animal models of obesity with disrupted leptin signalling, CART mRNA is almost absent from the ARC. ICV injection of CART inhibits normal and starvation induced feeding, and injection of an antibody to CART peptide stimulates feeding, suggesting that endogenous CART may exert an inhibitory tone on feeding (Lambert, 1998). Central injection of CART completely blocks the feeding response induced by NPY in a dose dependent manner, and immunostaining for CART revealed a distribution pattern in terminals of the PVN, in particular CART positive cell bodies have been observed around NPY positive varicosities, suggesting a functional interaction between CART and NPY (Lambert, 1998). It is proposed that CART may play a role in regulating the suppression of food intake mediated by leptin, as a balanced reduction and induction of NPY and CART respectively, may be the mechanism of action utilised by leptin (Kristensen, 1998).

1.9.6 NPY-Cholecystokinin (CCK) interactions

CCK is a well studied satiety signal, first described over 20 years ago and now widely regarded as a physiological mediator of satiety (Gibbs, 1973). CCK is synthesised within the gut wall and released into the circulation in response to the presence of nutrients, in particular fatty acids. In rats, injection of CCK peripherally or into the CNS reduces food intake, decreasing meal size by hastening the normal behaviour sequence associated with

satiation, an effect which is blocked by vagotomy. CCK is known to act via 2 different receptors, the CCK-A receptor and the CCK-B receptor, which are found in the gastrointestinal tract and in the CNS (Wank, 1995). The development of specific antagonists at these receptor subtypes has permitted the role of these receptors to be clarified regarding the role they may play in the regulation of food intake. Blockade of CCK-A receptors increases food intake by delaying the onset of satiety in rats, an effect thought to be predominantly mediated by peripheral CCK-A receptors. Administration of CCK-B receptor antagonists blocks the effect of peripheral and centrally administered CCK, and also increases food intake (Dourish, 1989). CCK stimulates receptors on the vagus nerve, which passes signals to the brainstem and thus to other appetite regulating areas, such as the hypothalamic PVN, where CCK appears to act as a neurotransmitter. CCK has been shown to elicit a marked satiety effect in NPY-treated rats, suggesting that NPY induced feeding may be modulated by the satiating action of prandially released CCK (Rowland, 1988).

1.9.7 NPY-Opioid interactions

Intracerebroventricular injection of β-endorphin has been shown to stimulate feeding, albeit to a lesser extent than NPY (Gosnell, 1986; Levine, 1989). Chronic administration of opioid antagonists have been shown to decrease food intake and bodyweight in rats (Mann, 1988), and blockade of endogenous dynorphin with antibodies or pharmacological antagonists reduces food intake in rats after food deprivation, suggesting a physiological role for opioid peptides in feeding behaviour (Lambert, 1993). A close anatomical relationship exists between opioid and NPY networks in the hypothalamus (Horvarth, 1992), and an interaction between NPY and opioid peptides has been suggested by findings that opiate receptor antagonists effectively attenuated NPY induced feeding and increased NPY concentrations in the hypothalamus (Levine, 1989 and Lambert, 1994). NPY has also been shown to stimulate β-endorphin release in the hypothalamus (Kalra, 1995), and it has been postulated that the potent orexigenic effects of NPY may include an augmentation of β-endorphin release evoked by NPY.

1.9.8 NPY -Galanin interactions

Galanin, a 29 amino acid neuropeptide which is widely distributed in both the central and peripheral nervous systems, is another potent orexigenic peptide densely concentrated in the hypothalamus, where it serves to increase preferentially fat ingestion and enhance fat deposition via a reduction in energy expenditure (Krykouli, 1990; Tempel, 1990 and Menendez, 1992). Galanin operates through neurones in the PVN and medial preoptic area, and galanin gene expression and synthesis are strongly activated under conditions associated with increased fat intake and fat deposition, such as the mid to late hours of the active feeding

cycle in the rat. The actions of galanin are mediated through distinct G protein coupled receptors, of which the GalR2 receptor is the hypothalamic receptor believed to mediate the ingestive functions of galanin (Howard, 1997). Galanin, like NPY is believed to be a physiological mediator of nutrient and energy balance, via its effects on food ingestion, circulating hormones, and metabolism, and is thought to constitute a distinct hypothalamic circuit in the complex network of systems involved in regulation of feeding behaviour. Synaptic links between NPY and galanin have been observed in a number of hypothalamic sites including the ARC and PVN, suggesting a possible interaction between these neuropeptides.

1.9.9 NPY-Melanin concentrating hormone interactions

Melanin concentrating hormone (MCH), is a cyclic 19 amino acid neuropeptide which is located primarily in the lateral hypothalamus, with mRNA for MCH being confined to this area of the brain. The observation that MCH mRNA is overexpressed in the hypothalamus of ob/ob mice led to further investigation into the role of this peptide in relation to regulation of bodyweight. Administration of MCH into the hypothalamus of rats was shown to stimulate food intake, highlighting a role for this peptide as a potent orexigenic signal (Qu, 1996). A potential relationship exists between MCH and melanin-stimulating hormone (MSH). MSH is a satiety signal whose actions are antagonised in the agouti mouse, resulting in obesity. The MSH receptors MC1 and MC4 are blocked by the overexpressed agouti protein, and it has been suggested that the obesity which develops could be due to a mimicking of the action of MCH by the agouti protein (Qu, 1996). The MC4 receptor is restricted to the brain, and it is believed that antagonism at this receptor elicits the characteristic obesity development. Recent findings have reported that the orexigenic effects of an MC4 antagonist are mediated by NPY, as administration of the Y1 selective antagonist 1229U91 significantly attenuated the orexigenic effects of HS014 an MC4 antagonist (Kask, 1998).

1.9.10 NPY-Orexin interactions

The orexin peptides are two recently discovered neuropeptides, which are derived from the same precursor by proteolytic processing. These peptides, termed orexin A-and B- have been localised to neurones in and around the lateral and posterior hypothalamus of rat brain. Central administration of these peptides elicits a stimulation in food consumption, and coupled with findings that prepro-orexin mRNA levels are up-regulated upon fasting, a role for these peptides in the regulation of feeding behaviour has been postulated (Sakurai, 1998).

Possible interactions between these peptides, NPY, and other constituents of the complex circuitry which regulates ingestive behaviour remain to be elucidated.

Recent findings have presented evidence detailing interactions between leptin, and potential targets of leptin signalling in the hypothalamus. Central administration of leptin was associated with a decrease in hypothalamic galanin, melanin concentrating hormone, proopiomelanocortin and NPY gene expression, as well as decreased food intake and body weight gain (Sahu, 1998). This study has provided a more detailed insight into signalling in the hypothalamus, and suggest that leptin's action on food intake and bodyweight gain is most likely mediated by inhibiting excitatory signals in the feeding circuitry. These findings support the complex circuitry regulation of feeding behaviour, and suggest that other unidentified neural signals may also have a role to play in modulating this intricate physiological function.
Figure 1.9 illustrates the effects of NPY on feeding behaviour, endocrine systems and metabolism and highlights the putative interactions between NPY and the aforementioned mediators of feeding.

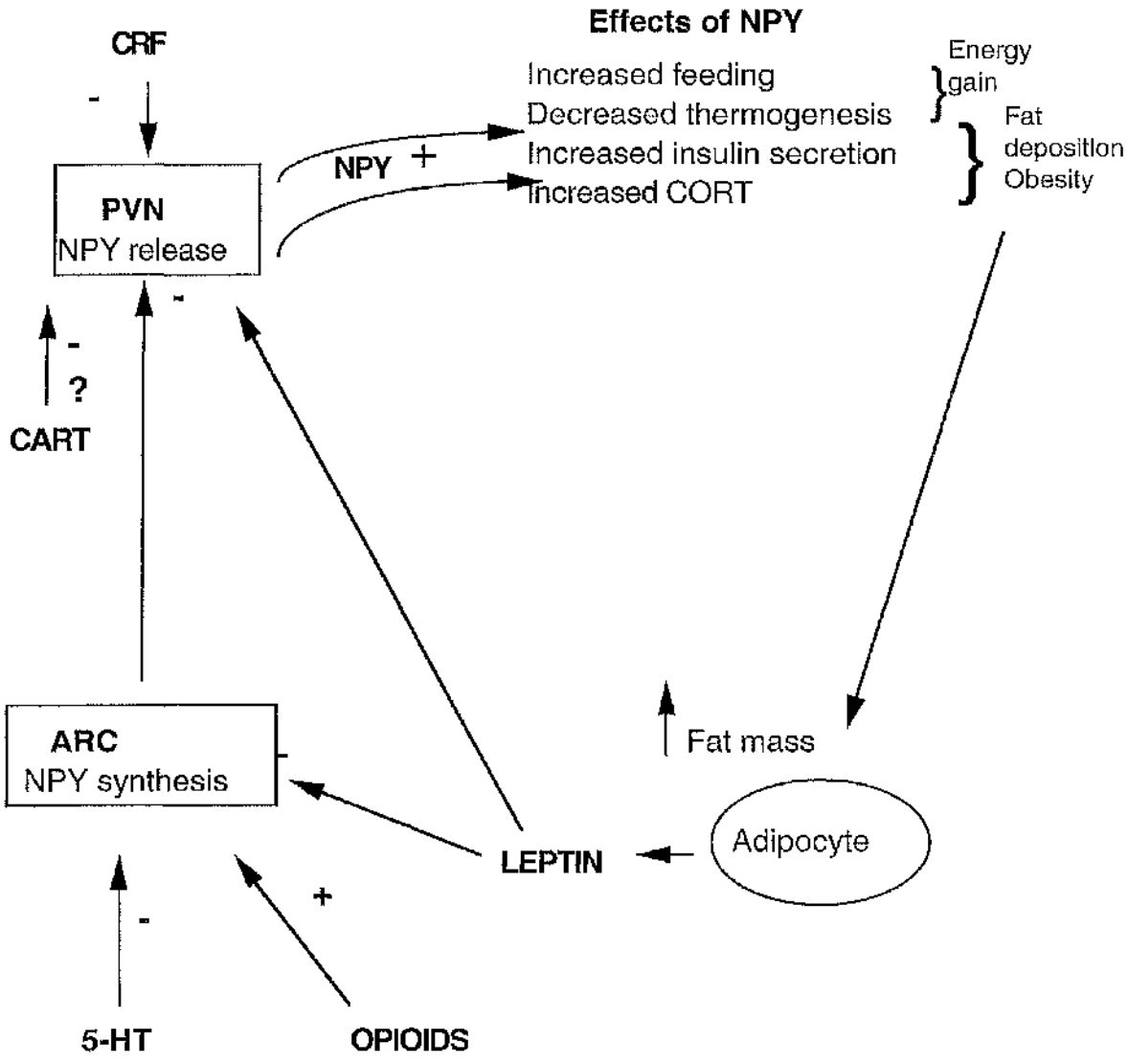
1.10 Scope of this thesis

The potential role of NPY in the regulation of feeding behaviour generated considerable interest in characterising the receptor (s) responsible for mediating this response. The therapeutic implications regarding the potential development of an anti-obesity agent make this field of research highly evolving and extremely competitive.

The main aim of this thesis was to characterise the nature of the feeding NPY receptor(s) involved in the hypothalamic regulation of feeding behaviour. At the start of the work presented in this thesis, the available pharmacological data suggested that the NPY receptor responsible for the regulation of feeding behaviour was distinct. Initial efforts were therefore directed at using different strategies to clone this receptor. During the course of this work, the structure of the NPY Y5 receptor was reported, and this initial report proposed that this receptor was the feeding receptor for NPY.

However, two years after the cloning of this receptor, the identity of the receptor subtype involved in mediating NPY induced feeding is highly controversial, and a role for the NPY Y1 receptor subtype in the regulation of NPY elicited eating has been suggested.
Additional work was performed to explore the differential regulation of both the Y1 and Y5 receptor genes with a view to elucidating further the role of these receptors in the control of feeding behaviour.

Figure 1.9 The arcuato-paraventricular projection of NPY neuronal activity, showing the principal effects of NPY injected into the PVN, and outlining the role of other neural mediators on NPY activity.


Effects of NPY
CRF
-
PVN
NPY release
NPY +
Increased feeding
Decreased thermogenesis
Increased insulin secretion
Increased CORT
Energy gain
Fat deposition
Obesity
-
-
?
CART
ARC
NPY synthesis
Fat mass
Adipocyte
LEPTIN
+
-
5-HT
OPIOIDS

Chapter 2

NPY receptor gene family analysis: Attempts at homology screening as a cloning strategy.

Chapter 2

2.1 Introduction

Neuropeptide Y has numerous actions within the central nervous system including the well characterised effect on feeding behaviour. Centrally administered NPY has been shown to significantly augment ingestive behaviour (Clark, 1984), an effect attributed to the hypothalamus, where injection of NPY elicits the most potent stimulation of feeding described to date (Stanley, 1985). Hypothalamic NPY levels are markedly elevated following a period of food deprivation, where NPY serves to promote feeding and reduce energy expenditure via effects on ingestive behaviour, endocrine systems and metabolism (Stanley, 1985., Wahlestedt, 1987., Stanley, 1989., Menendez, 1990). There is mounting evidence in favour of NPY as an endogenous mediator of food intake, as fasting and feeding have been demonstrated to elicit reciprocal changes in NPY concentration, with food deprivation producing a dramatic accumulation of NPY in the hypothalamus, with levels decreasing significantly during the course of eating (Kalra, 1988).

The paraventricular nucleus (PVN) of the hypothalamus harbours a dense supply of NPY-containing presynaptic terminals, and studies have implicated this hypothalamic region in mediating NPY elicited eating behaviour (Stanley, 1984., 1985). NPY is believed to mediate its physiological effects via an interaction with distinct receptor subtypes. Pharmacological studies with fragments of the NPY molecule and various analogues of NPY demonstrated the existence of multiple receptor subtypes (Wahlestedt, 1986). To date, five distinct NPY receptor subtypes have been cloned (Y1, Y2, Y4, Y5 and y6), all of which belong to the large superfamily of G-protein coupled receptors, where they appear to use similar signal transduction pathways, demonstrating a preferential coupling to pertussis toxin sensitive G-proteins, namely members of the Gi/Go family. The subsequent signalling response observed upon activation of receptor and G-protein is typical of receptors under the control of these G-proteins, in that inhibition of adenylyl cyclase activity is found in most cell types and tissues investigated. As outlined in Chapter 1 section 1.6, each NPY receptor subtype exhibits a characteristic pharmacology, differing from the other NPY receptor family members and allowing distribution and physiological effects of receptor stimulation to be determined (see figure 1.8).

The feeding response to NPY failed to correlate with the known pharmacology described for the cloned family members. Studies revealed that Y1 selective agonists such as $Leu^{31}Pro^{34}NPY$ were capable of stimulating ingestive behaviour as effectively as the whole NPY molecule, as were Y2 selective agonists such as C-terminal fragments of NPY; namely NPY^{2-36} and NPY^{3-36} but not NPY^{13-36}. The markedly greater effectiveness of the Y1

selective agonist to stimulate feeding when compared with the Y2 selective agonist, initially implicated the Y1 receptor in the NPY induced feeding response. However, the ability of NPY^{2-36} to consistently stimulate feeding more effectively than the whole NPY molecule, suggested that a distinct NPY receptor subtype may be responsible for mediating the profound effects on feeding behaviour elicited by NPY, as this compound is not an effective agonist at Y1 receptors.

As a result of these findings the receptor involved in mediating the feeding response to NPY was believed to be pharmacologically distinct from any of the NPY receptor subtypes previously described. A hypothalamic pattern of distribution was proposed for this "feeding receptor", as the hypothalamus is a critical brain region involved in maintaining energy and nutrient balance, housing a significant concentration of NPY and responsible for the dramatic effects of NPY on food intake.

As obesity is highly prevalent particularly in Western society, and associated with numerous related health risks including hypertension and diabetes mellitus, the cloning of a receptor involved in the physiological regulation of eating behaviour, would undoubtedly prove invaluable, as development of a selective antagonist at this receptor would theoretically represent a potential treatment for disorders such as obesity.

When the project of this thesis started, the primary structure of the human Y1, Y2 and Y4 receptors had been defined, as these receptors had been cloned by orphan receptor homology cloning (Eva, 1990), expression cloning (Gerald, 1995) and homology cloning (Lundell , 1995) respectively. These sequences were aligned in order to identify regions of homology to design degenerate oligonucleotide primers, for use in a PCR cloning strategy aimed at cloning the receptor involved in NPY elicited eating. The cloning of the Y5 receptor in 1996 permitted a more specific PCR cloning strategy to be employed, as oligonucleotide primers homologous to the Y5 receptor cDNA were able to be designed.

2.2 Experimental strategy

2.2.1 Degenerate PCR strategy

A degenerate PCR approach was employed in this study, as the sequences of three NPY receptor family members had been elucidated, and a homology screening approach to cloning this receptor was permitted by aligning these sequences and designing oligonucleotide primers from conserved regions of these receptors. The rationale behind this strategy was that the NPY receptor involved in mediating the feeding response would likely share regions of homology with the other members of the NPY receptor gene family, thereby enabling use of a degenerate

PCR approach to identify this receptor. Figure 2.1 represents a schematic approach to the degenerate PCR strategy employed.

2.2.2 Human and Rat Y5 PCR strategy

The cloning in 1996 of the Y5 receptor (Gerald, 1996) prompted further sequence alignments and permitted a more specific PCR strategy to be employed, as it enabled oligonucleotide primers homologous to the cDNA of this receptor to be designed and subsequently used in a PCR reaction with cDNA from various brain regions, including the hypothalamus. The rationale behind this experimental strategy was that the generation of a full length or partial clone would provide DNA which could be used in future experiments such as Northern and Southern blot analyses. Figure 2.2 illustrates the cloning strategy employed for human and rat Y5 receptor cloning.

2.2.3 Tissue distribution of the human Y5 receptor

Distribution of the human Y5 receptor was investigated by Northern blot analysis. A human RNA master blot containing poly A^+ RNA from numerous tissues, was probed with a DNA fragment (obtained by PCR) encompassing a region of the human Y5 open reading frame in order to determine the distribution pattern exhibited by this receptor.

2.3 Chapter specific methods

2.3.1. Oligonucleotide design and synthesis

Degenerate oligonucleotide primers were designed from conserved regions of the Y1, Y2 and Y4 receptors (Figure 2.3). The sequences of the primers were derived from conserved regions of the first extracellular loop and seventh transmembrane domain (see Appendix II for primer sequences).

The oligonucleotides used in these experiments were synthesised commercially by Dr V. Math (Division of Biochemistry and Molecular Biology, Institute of Life Sciences, University of Glasgow), on an Applied Biosystems Model 280A DNA synthesiser using phosphoramide technology. Oligonucleotides were supplied in 35% NH_4OH, purified by ethanol precipitation and dissolved in distilled water. The concentration of the purified oligonucleotide was then determined by measuring the absorbance at 260nm (see section 2.3.1.6)

Figure 2.1 Cloning strategy used for degenerate PCR:

PCR fragments obtained by degenerate PCR were cloned into a plasmid vector by the method depicted in the flowchart.

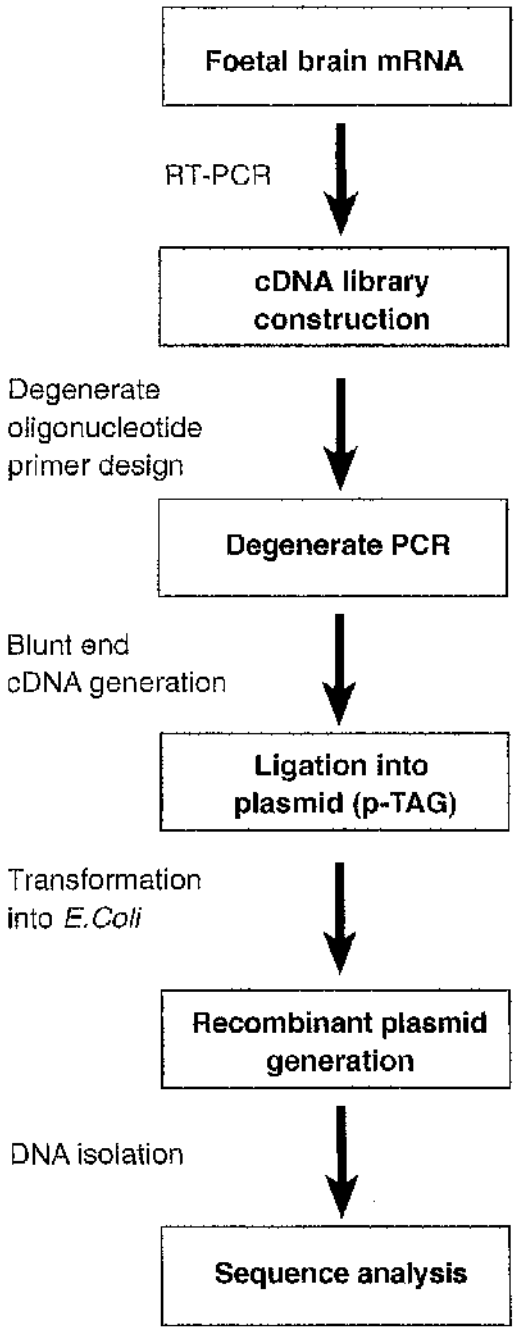

Foetal brain mRNA
RT-PCR
cDNA library construction
Degenerate oligonucleotide primer design
Degenerate PCR
Blunt end cDNA generation
Ligation into plasmid (p-TAG)
Transformation into E.Coli
Recombinant plasmid generation
DNA isolation
Sequence analysis

Figure 2.2 Cloning strategy used for human and rat Y5 PCR.

PCR fragments obtained using oligonucleotide primers homologous to the Y5 receptor were cloned into a plasmid vector by the method depicted in the flowchart.

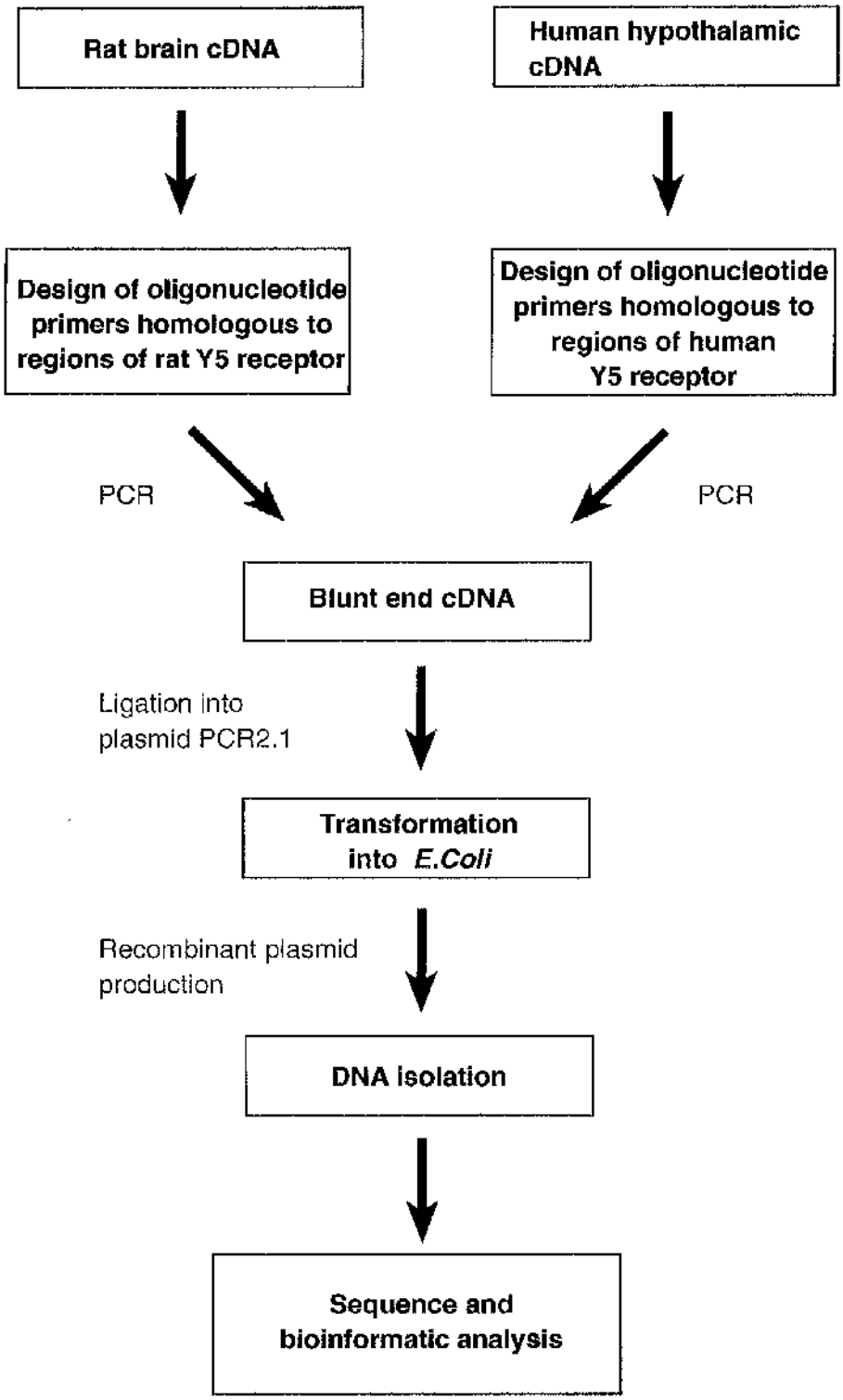
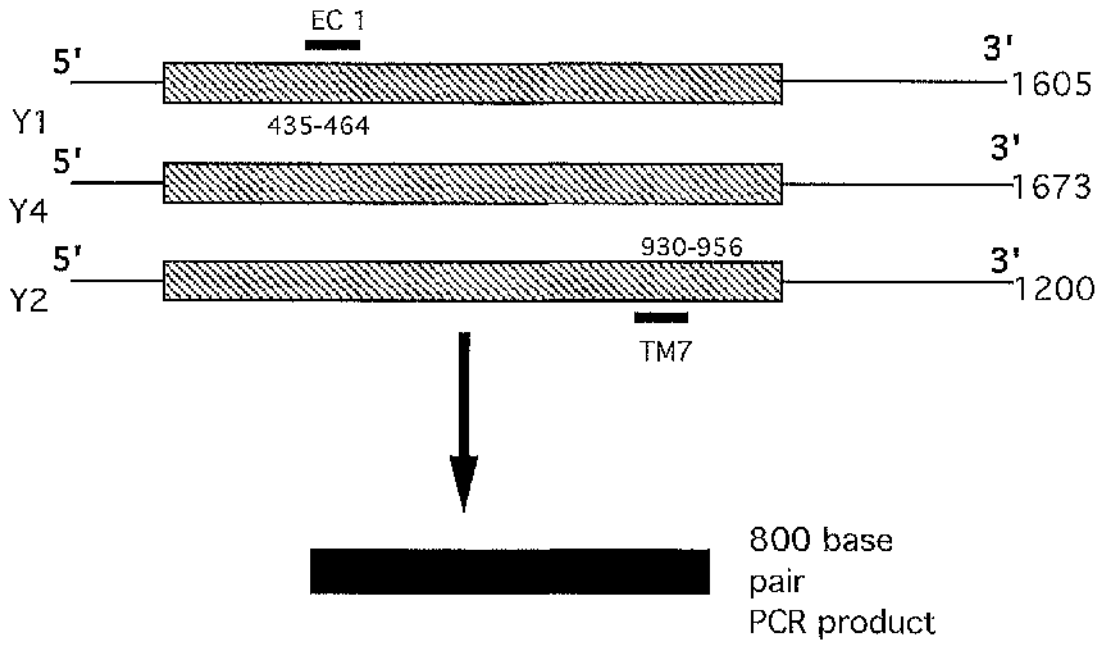

Rat brain cDNA
Human hypothalamic cDNA
Design of oligonucleotide primers homologous to regions of rat Y5 receptor
Design of oligonucleotide primers homologous to regions of human Y5 receptor
PCR
PCR
Blunt end cDNA
Ligation into plasmid PCR2.1
Transformation into E.Coli
Recombinant plasmid production
DNA isolation
Sequence and bioinformatic analysis

Figure 2.3 Degenerate oligonucleotide primers schematic.

Schematic of cDNA sequences and the degenerate oligonucleotide primers designed for PCR purposes. Hatched regions on the cDNA correspond to translated sequnces, with wide bars representing the oligonucleotide primers used in the PCR reaction.
EC1 = extracellular domain 1
TM7 = transmembrane domain 7
PCR fragment = 800bp


EC 1
5'
3'
1605
Y1
435-464
5'
3'
1673
Y4
930-956
5'
3'
1200
Y2
TM7
800 base
pair
PCR product

2.3.1.1 Human foetal brain PCR

1µg human foetal brain cDNA library constructed in the expression vector CDM was used in a PCR reaction with 0.1µM of forward and reverse degenerate oligonucleotide primers. Reaction buffer 10 X (Promega Corp), 25mM $MgCl_2$ (Promega Corp), 150µM dNTP mix (containing equal concentrations of dATP, dCTP, dGTP and dTTP) and 2.5 units *Taq* polymerase (Promega Corp) were added and the reaction volume was increased to 100µl. The reaction was carried out in a thin walled 0.5ml microcentrifuge tube, and after gentle mixing, 40µl of mineral oil were layered on top of the reaction mix to prevent evaporation during amplification. After an initial denaturation step at 94°C for 3 minutes, the reaction was allowed to cycle 30 times through a sequence of temperatures: 1) denaturation of template: 94°C for 45 seconds, 2) primer annealing at 35°C for 3 minutes, 3) DNA polymerisation at 72°C for 2 minutes. A final elongation step at 72°C for 10 minutes was also performed. The annealing temperature used in the reaction was relatively low, in order to maximise the generation of products obtained with these degenerate oligonucleotide primers. *Taq* DNA polymerase was used to amplify cDNA fragments homologous to the NPY receptors, with the aim of isolating novel members of the NPY receptor family. This enzyme was used in preference to other DNA polymerases, as the ability of this polymerase to generate 3' deoxyadenosine overhangs (Clark, 1988., Myers, 1991) permits the use of vectors containing complementary 5' deoxythymidine overhangs and enables convenient subcloning of PCR products obtained. Reaction mixtures containing no template DNA were used as PCR negative control reactions.

2.3.1.2 Recombinant plasmid generation

The PCR products generated using the thermostable *Taq* DNA polymerase were cloned into the p-TAG cloning vector using the LigATor kit (R&D Systems) following manufacturer instructions.

2.3.1.3 Ligation

T4 DNA ligase (R&D Systems) was used to catalyse the formation of a phosphodiester bond between adjacent 3'-hydroxyl groups and 5'-phosphate termini in DNA (Weiss, 1986). A p-TAG vector (R&D systems) containing 5' deoxythymidine overhangs was used as the recipient vector for the PCR fragment generated in the above reaction. A 10µl reaction volume contained 10mM ATP, 100mM DTT, 200ng PCR product, 10X ligase buffer, 50ng p-TAG vector and 10 units of enzyme. The reaction was allowed to proceed overnight at 16°C.

2.3.1.4 Transformation of bacteria with DNA

A 20µl aliquot of competent cells (supplied) was thawed on ice for each sample of DNA to be transformed. 1µl of DNA containing between 10 to 100ng DNA was added to the suspension of competent bacteria, and after gentle mixing, was incubated on ice for 30 minutes. The bacteria/DNA mixture was then subjected to "heat shock" by incubation at 42°C for 40 seconds followed by incubation on ice for a further 2 minutes. Eighty microlitres of SOC media was then added and the mixture incubated at 37°C for 1 hour. A 50µl aliquot of this mixture was plated onto LB-ampicillin agar plates containing IPTG/X-Gal. Control transformation reactions using circular non-recombinant plasmid vector were performed alongside experimental samples to assess transformation frequency. Reactions eliminating plasmid DNA served as negative controls. Recombinant transformants, which have an interrupted β– Galactosidase gene, grew as white colonies while non-recombinant colonies were stained blue due to reaction of β– Galactosidase with X-Gal.

2.3.1.5 Isolation of plasmid DNA

Individual bacterial colonies were picked from agar plates using sterile plastic disposable pipette tips, placed (one colony/tube) into 30ml sterile glass test-tubes containing 5ml of LB/ampicillin (100µg/ml) and grown overnight with constant agitation to saturation at 37°C. Bacteria in 1.5ml aliquots of these cultures were pelleted by centrifugation at 5000 X g for 2 minutes. Supernatant was removed and the bacterial pellet was resuspended in 100µl of an ice-cold solution containing 50mM glucose, 25mM Tris-Cl (pH 8.0) and 10mM EDTA (pH 8.0). Bacteria were lysed by addition of 200µl of 0.2M NaOH, 1% SDS; protein was precipitated by addition of 150µl 3M potassium acetate, 5M acetic acid. Following gentle mixing by inversion and subsequent flicking with fingertips, tubes were centrifuged for 5 minutes at 14000 X g at 4°C, and the supernatant poured into fresh microcentrifuge tubes. DNA was extracted by the addition of an equal volume of phenol:chloroform to eliminate protein, vortexing thoroughly, and centrifuging at 14000 X g for 2 minutes at room temperature. The upper aqueous layer was removed, placed in a fresh microcentrifuge tube and DNA was precipitated by addition of one volume of isopropanol and centrifugation for 10 minutes at 4°C. The resulting pellet was rinsed with 70% ethanol, air dried and resuspended in 50-100µl TE containing 10µg/ml RNase A which had been preboiled to inactivate DNase.

2.3.1.6 Quantification of DNA

DNA was quantitated by spectrophotometry (DU-640B or DU-62, Beckman) at 260nm. DNA quantity was calculated as one optical density unit at 260nm represented 50µg/ml of double stranded DNA (Maniatis, 1982).

2.3.1.7 Restriction Enzyme digests

Restriction digest analysis was carried out on the isolated DNA in order to confirm the generation of recombinant plasmid DNA. DNA was digested with restriction endonuclease enzymes using conditions and buffers suggested and supplied by the manufacturer (Promega Corp or Boerhinger Mannheim). Typically 500ng-1µg of DNA was digested in a volume of 10-20µl using the appropriate buffer supplied by the manufacturer. Typically an excess of enzyme was used (5-10 units per digest) and digests were incubated at 37°C for up to 3 hours.

2.3.1.8 DNA agarose gel electrophoresis

One to two percent agarose (SeaKem LE, Seaplaque or MetaPhor high resolution agarose, FMC Bioproducts) was prepared in 1 x TAE and dissolved by heating in a microwave oven. Ethidium bromide 0.5µg/ml was added to the gel to allow visualisation of the DNA under ultraviolet light at 254nm. Gels were electrophoresed at 70-100V in 1 xTAE buffer and molecular weight markers (*Hind*III/*Eco*RI digested DNA or 1Kb DNA ladder, Life Technologies) were run alongside the samples. Gels were then photographed under ultraviolet illumination at 254nm using Polaroid 667 Land film.

2.3.1.9 Sequence analysis

An ABI automated sequencer was used for double stranded sequencing of recombinant plasmid containing insert DNA of the correct molecular weight, using the dideoxy method of Sanger 1977 (Sanger, 1977). Sequencing data was obtained courtesy of Pfizer Central Research, Sandwich, Kent.

2.3.2 Human and rat Y5 PCR

2.3.2.1 Oligonucleotide design

Oligonucleotide primers were designed from regions of the human and rat Y5 receptor open reading frame, as illustrated in figure 2.4. Typically each primer encompassed 20-25 bases and was between 50-60% GC rich. These oligonucleotides were synthesised commercially by Oswel DNA Service (University of Southampton) and were dissolved in sterile water. (See Appendix II for sequences of Y5 specific designated primers). All oligonucleotides were checked for complementary hybridisation with the DNA data bank using the *blast* or *fasta* programme (Wisconsin Package Version 9.1, Genetics Computer Group (GCG) software, Madison, Wisc).

2.3.2.2 Human and Rat Y5 receptor PCR

2 nanograms of Marathon-Ready cDNA (Clontech) or 1µg human genomic DNA (Promega Corp) were used in a PCR reaction with 1µM oligonucleotide primers. 10 X reaction buffer containing 25mM $MgCl_2$ (Boerhinger Mannheim), 200µM dNTP mix (Boerhinger Mannheim), 50% glycerol and 2.5 units *Taq* polymerase (Boerhinger Mannheim) were added and the reaction volume was increased to 100µl with nuclease free water (Promega Corp). The reaction was carried out in a thin walled 0.5ml microcentrifuge tube, and after gentle mixing the tube was then subjected to cycled temperature conditions. After an initial denaturation step at 94°C for 3 minutes, the reaction was allowed to cycle 30 times through a sequence of temperatures: 1) denaturation of template: 94°C for 1 1/2 minutes, 2) primer annealing at 58°C for 1 1/2 minutes, 3) DNA polymerisation at 72°C for 2 minutes. A final elongation step at 72°C for 15 minutes was also performed to ensure generation of full length products. *Taq* DNA polymerase was used to amplify cDNA fragments homologous to the NPY Y5 receptor. Unless otherwise stated the annealing temperature used was as stated above. Reaction mixtures containing no DNA were used as negative controls for the PCR reaction, and positive control reactions were carried out using primers homologous to a region of the glyceraldehye 3-phosphate dehydrogenase (G3PDH) gene, at a reaction concentration of 0.2µM (Clontech).

2.3.2.3 Recombinant plasmid generation

The PCR products generated using the thermostable *Taq* DNA polymerase were cloned into the pCR2.1 cloning vector using the TA cloning kit (Invitrogen) following manufacturer instructions.

2.3.2.4 Ligation

T4 DNA ligase (R&D Systems) was used to catalyse the formation of a phosphodiester bond between adjacent 3'-hydroxyl groups and 5'-phosphate termini in DNA (Weiss, 1986). The cloning vector pCR2.1 (Invitrogen) was used as a recipient vector for the PCR fragments generated in the above reactions. A 10µl reaction volume typically
contained 10X ligase buffer, 50ng pCR2.1 vector, 50-100ng PCR product, 5 units T4 DNA ligase. The ligation reaction was allowed to proceed overnight at 14°C.

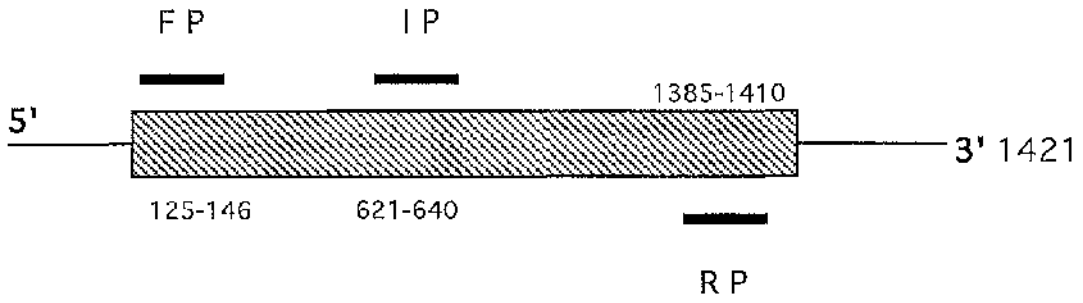
Figure 2.4. Oligonucleotide primers schematic.

Schematic of cDNA sequences and the relevant oligonucleotide primers designed for PCR purposes. Hatched regions on the cDNA correspond to translated sequence, with wide bars representing oligonucleotide primers used in the PCR reaction.
FP = Forward primer
IP = Internal primer
RP = Reverse primer

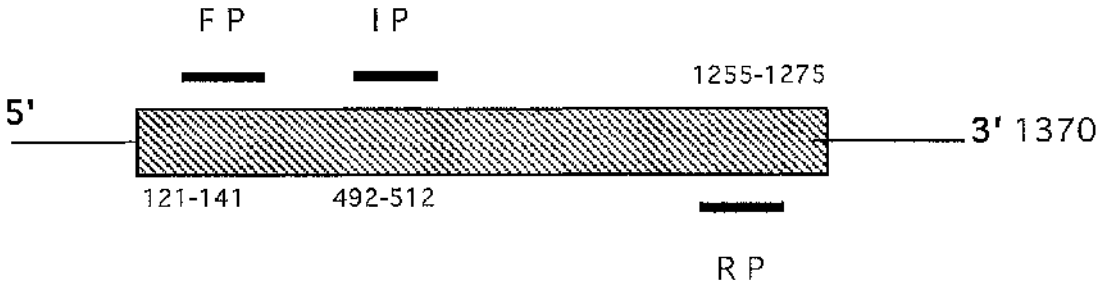
A: Rat Y5 receptor : PCR fragments; FP & RP = 1285bp
IP & PR = 789bp

B: Human Y5 receptor: PCR fragments; FP & RP = 1154bp
IP & RP = 783bp

A) Rat Y5 receptor



B) Human Y5 receptor



2.3.2.5 Transformation of bacteria with DNA

A 50µl aliquot of One Shot (Invitrogen) competent cells was thawed on ice for each sample of DNA to be transformed. 2µl 0.5M β-mercaptoethanol were added to the thawed cells and gently mixed with a sterile pipette tip. 2µl of DNA from the ligation reaction described above containing between 10-100ng DNA was added to the suspension of competent bacteria, and after gentle mixing, each sample was incubated on ice for 30 minutes. The bacteria/DNA mixture was then subjected to "heat shock" by incubation at 42°C for 30 seconds, followed by incubation on ice for 2 minutes. Two hundred and fifty microlitres of SOC media was then added and the mixture incubated at 37°C for 1 hour. Aliquots of 50µl and 200µl were then spread onto LB agar plates containing 100µg/ml ampicillin, IPTG and X-Gal. Control transformations were carried out as described in section 2.3. 1.3.

2.3.2.6 Isolation of plasmid DNA

Plasmid minipreps were prepared using a Qiaprep Spin plasmid kit (Qiagen). Individual bacterial colonies were picked from agar plates using sterile plastic disposable pipette tips, placed (one colony/tube) into 30ml sterile glass test-tubes containing 5ml of LB/ampicillin (100µg/ml) and grown overnight with constant agitation to saturation at 37°C. Bacteria in 1.5ml aliquots of these cultures were pelleted by centrifugation at 5000 X g for 10 minutes. The pellet was completely resuspended in 250µl of cell resuspension solution buffer P1 (supplied) by pipetting up and down. Cell lysis solution buffer P2 (supplied), 250µl, was added to the suspension and the contents gently mixed by inversion; lysis was complete in 5 minutes when the solution became clear and viscous. Neutralisation solution buffer N3 (supplied), 350µl, was added and the preparation immediately mixed by gentle inversion of the microcentrifuge tube several times. Insoluble protein was removed by centrifugation at 14000 X g for 10 minutes at room temperature. The DNA-containing supernatant was applied to a Qiaprep column (supplied) and centrifuged at 14000 X g for 1 minute at room temperature, to bind DNA on the column resin . Flow through containing no DNA was discarded and the spin columns washed in 500µl wash buffer PB (supplied) before centrifuging again at 14000 X g for 1 minute and discarding any flow through. The spin columns were then washed in 750µl buffer PE (supplied) containing ethanol, and centrifuged at 14000 X g for 1 minute. To remove all traces of liquid, the column was then centrifuged again for 1 minute at 14000 X g. The columns were then placed in fresh 1.5ml microcentrifuge tubes and plasmid DNA eluted from the column by applying 50µl nuclease free water (Promega) to the centre of each column and centrifuging for 1 minute at 14000 X g after allowing the column to stand for 1 minute. The columns were discarded and DNA stored at -20°C.

Restriction digest analysis was performed as described in section 2.3.1.6 in order to establish the success of recombinant plasmid generation. Typically the restriction endonuclease *Eco*RI was used, as the vector pCR2.1 contains 2 *Eco*RI sites on either side of the multiple cloning site, and digestion with this enzyme allows insert to be excised effectively, the results of which can be easily observed by DNA gel electrophoresis (section 2.3.1.7).

2.3.3 Northern dot blot analysis of human Y5 distribution

A DNA probe encompassing part of the human Y5 open reading frame (obtained by PCR as described in section 2.3.2.2) was radioactively labelled as described in section 2.3.3.2 and used to analyse a human master RNA blot (Clontech) in order to determine the distribution of the human Y5 receptor.

A human RNA master blot containing 50 high quality human Poly A^+ RNA samples (Clontech) from various brain regions and peripheral tissues was prehybridised for at least 3 hours at 42°C in 50mls ExpressHyb hybridisation solution (Clontech). After addition of labelled probe, hybridisation was allowed to proceed overnight at 42°C with constant agitation. Following overnight hybridisation the master blot was washed to remove any non specific binding of radiolabelled probe. The blot was washed twice in a solution of 2XSSC/0.1% SDS at room temperature for 20 minutes and then washed a further two times for 20 minutes in 0.2X SSC/01%SDS at 50°C. Once satisfied that sufficient removal of any non specific radioactivity had been achieved, the blot was wrapped in cling film to prevent dehydration. The poly A^+ samples on the master blot had been normalised (prior to purchase) to the mRNA expression levels of eight housekeeping genes in order to ensure accurate assessment of mRNA abundance in different tissues. A human ubiquitin control cDNA probe (one of the housekeeping genes used in the normalisation process) was supplied as a positive control cDNA probe and used to confirm consistent hybridisation for all samples on the master blot (data not shown).

2.3.3.1 Signal detection

The blot was exposed to phosphorimager screens with cassettes and the resulting images captured from the screen to a database using a bio-imaging analyser (Fujix BAS100 MacBAS, Fuji Photo Film Co Ltd).

The blot was then exposed to film (Kodak X-OMAT-AR) with an intensifying screen, and allowed to expose at -70°C, before developing film using conventional photographic technology.

2.3.3.2 Rat Y5 probe preparation

The partial human Y5 PCR encompassing a 800 base pair fragment of the human Y5 open reading frame obtained by PCR on human genomic DNA as described in section 2.3.2.2 was radiolabelled as described:

^{32}-P labelled fragments used for blot hybridisation were prepared by random-primed DNA synthesis by the method of Feinberg (Feinberg and Vogelstein, 1983) using a kit (High Prime, Boehringer Mannheim). In a typical reaction, approximately 50-100ng DNA was strand separated by incubating at 94°C for 10 minutes in a final volume of 11µl. The DNA was then snap-cooled on ice and 4µl of 5 x High Prime solution (supplied), containing 4 units Klenow polymerase, 0.125mM dATP, dTTP, dGTP in a stabilisation buffer and 50% glycerol were added to the tube. 50µCi [α^{32}P] dCTP (3000Ci/mmol, Amersham) were added and the reaction incubated for 45 minutes at 37°C. The reaction was terminated by incubating at 65°C for 10 minutes and unincorporated nucleotide removed using Chromaspin TE 10 columns (Clontech). The reaction contents were applied to Chromaspin spin columns, and centrifuged at room temperature for 5 minutes at 3,000 X g. The unincorporated radionucleotide remained attached to the column matrix after elution of the incorporated labelled DNA from the column upon centrifugation. Labelled DNA was eluted from the Chromaspin columns in 50µl TE and liquid scintillation spectrophotometry used to determine label incorporation and DNA specific activity. DNA was routinely labelled to a specific activity of approximately 1 x 10^9 dpm/µg. The labelled DNA was strand separated by incubating at 94°C for 10 minutes immediately prior to use in hybridisation of Northern, Southern or dot blots.

2.4 Results and Discussion

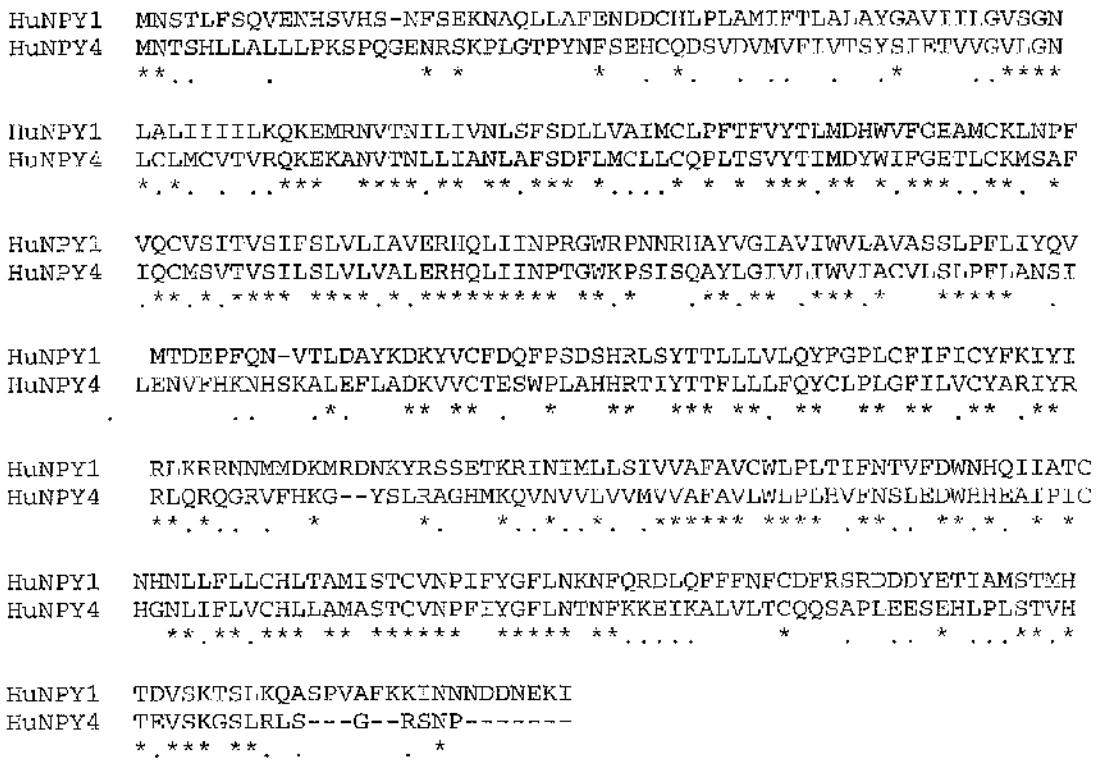
2.4.1 Sequence alignments of the Y1, Y2 and Y4 receptors

When this project started, the sequence of three human NPY receptors had been defined using molecular cloning. To assist the design of degenerate primers to use in the identification of novel NPY receptors, the sequences of the human receptors were aligned in order to identify regions of homology. Alignments of the human Y1 and Y4 receptors is shown in figure 2.5, and alignment of all three receptors is shown in figure 2.6.

In figure 2.5 showing the sequence alignments of the human Y1 and Y4 receptors, the amino acids shared between the two receptors are denoted by an asterisk.

Figure 2.5. Alignment of Y1 and Y4 sequences.

HuNPY1 = Human Y1 receptor
HuNPY4 = Human Y4 receptor
Asterisks (*) represent amino acids conserved between receptor sequences and, dashes mark gaps introduced to optimise alignments.


HuNPY1 MNSTLFSQVENHSVHS-NFSEKNAQLLAFENDDCHLPLAMIFTLALAYGAVIILGVSGN
HuNPY4 MNTSHLLALLLPKSPQGENRSKPLGTPYNFSEHCQDSVDVMVFIVTSYSIETVVGVLGN
HuNPY1 LALIIIILKQKEMRNVTNILIVNLSFSDLLVAIMCLPFTFVYTLMDHWVFGEAMCKLNPF
HuNPY4 LCLMCVTVRQKEKANVTNLLIANLAFSDFLMCLLCQPLTSVYTIMDYWIFGETLCKMSAF
HuNPY1 VQCVSITVSIFSLVLIAVERHQLIINPRGWRPNNRHAYVGIAVIWVLAVASSLPFLIYQV
HuNPY4 IQCMSVTVSILSLVLVALERHQLIINPTGWKPSISQAYLGIVLIWVIACVLSLPFLANSI
HuNPY1 MTDEPFQN-VTLDAYKDKYVCFDQFPSDSHRLSYTTLLLVLQYFGPLCFIFICYFKIYI
HuNPY4 LENVFHKNHSKALEFLADKVVCTESWPLAHHRTIYTTFLLLFQYCLPLGFILVCYARIYR
HuNPY1 RLKRRNNMMDKMRDNKYRSSETKRINIMLLSIVVAFAVCWLPLTIFNTVFDWNHQIIATC
HuNPY4 RLQRQGRVFHKG--YSLRAGHMKQVNVVLVVMVVAFAVLWLPLHVFNSLEDWHHEAIPIC
HuNPY1 NHNLLFLLCHLTAMISTCVNPIFYGFLNKNFQRDLQFFFNFCDFRSRDDDYETIAMSTMH
HuNPY4 HGNLIFLVCHLLAMASTCVNPFIYGFLNTNFKKEIKALVLTCQQSAPLEESEHLPLSTVH
HuNPY1 TDVSKTSLKQASPVAFKKINNNDDNEKI
HuNPY4 TEVSKGSLRLS---G--RSNP-------

Alignments of the Y1 and Y4 receptors revealed that 42% sequence homology was exhibited between these receptors at the amino acid level, with transmembrane regions displaying 57% . In figure 2.6 showing the sequence alignments of the human Y1, Y4 and Y2 receptors, the amino acids shared between all three receptors are denoted by an asterisk. The Y1 and Y2 receptors, surprisingly share only 31% overall sequence identity, with 40% in transmembrane regions. This level of homology for receptors that recognise the same ligand is surprisingly low, indeed it is the lowest percentage reported for receptors that bind the same ligand (Larhammar, 1996b). Of the three receptors, the Y2 receptor, as outlined in figure 2.6 exhibits low sequence identity to both Y1 and Y4 receptors. The lack of apparent sequence homology between the Y2 and Y1 or Y4 receptors has been attributed, in certain reports, to evolutionary divergence, which has resulted in structural diversity of the NPY receptor gene family. However, both the Y1 and Y2 subtypes display conservation across species, having 94% identity between human and rat, thus the evolutionary rate for Y1 and Y2 receptors appears to be in the slower range for G-protein coupled receptors as each displays 94% homology between man and rat (Larhammar, 1996b).

A slow rate of evolution in combination with great sequence divergence between the Y1 and Y2 receptors would suggest that the gene duplication that generated Y1 and Y2 from a common ancestral gene took place long ago, presumably before the origin of vertebrates (Larhammar, 1996b). Another possibility to explain the structural difference between Y1 and Y2 would be functional convergence of structurally very different receptors i.e., Y1 and Y2 have evolved NPY/PYY binding separately. This possibility chelates support from the seemingly very different modes of interaction of Y1 and Y2 with the ligand. As discussed in Chapter 1 section 1.6, the Y1 receptor binds to both the N-and C-terminus of NPY and related PP family members, whereas the Y2 receptor exhibits almost exclusive binding to the C-terminus (Wahlestedt, 1986). Sequence comparisons have revealed that the positions identified to interact with ligands differ between the Y1 and Y2 receptors, and subsequent mutagenesis studies confirmed these findings (Walker, 1994). The greater percentage identity observed between Y1 and Y4 receptors also supports this theory as both these receptors bind to, and require both termini of their ligands for receptor activation.

Varying degrees of conservation across species are also observed within this receptor family. The Y4 receptor exhibits only modest sequence identity between human and rat; one of the lowest percentages reported for orthologous G-protein coupled receptors between different orders of mammals. The low Y4 conservation contrasts with the reported high identities for Y1 and Y2 receptors, suggesting a rapid evolutionary rate for the Y4 receptor, which like its ligand, PP is also rather divergent between man and

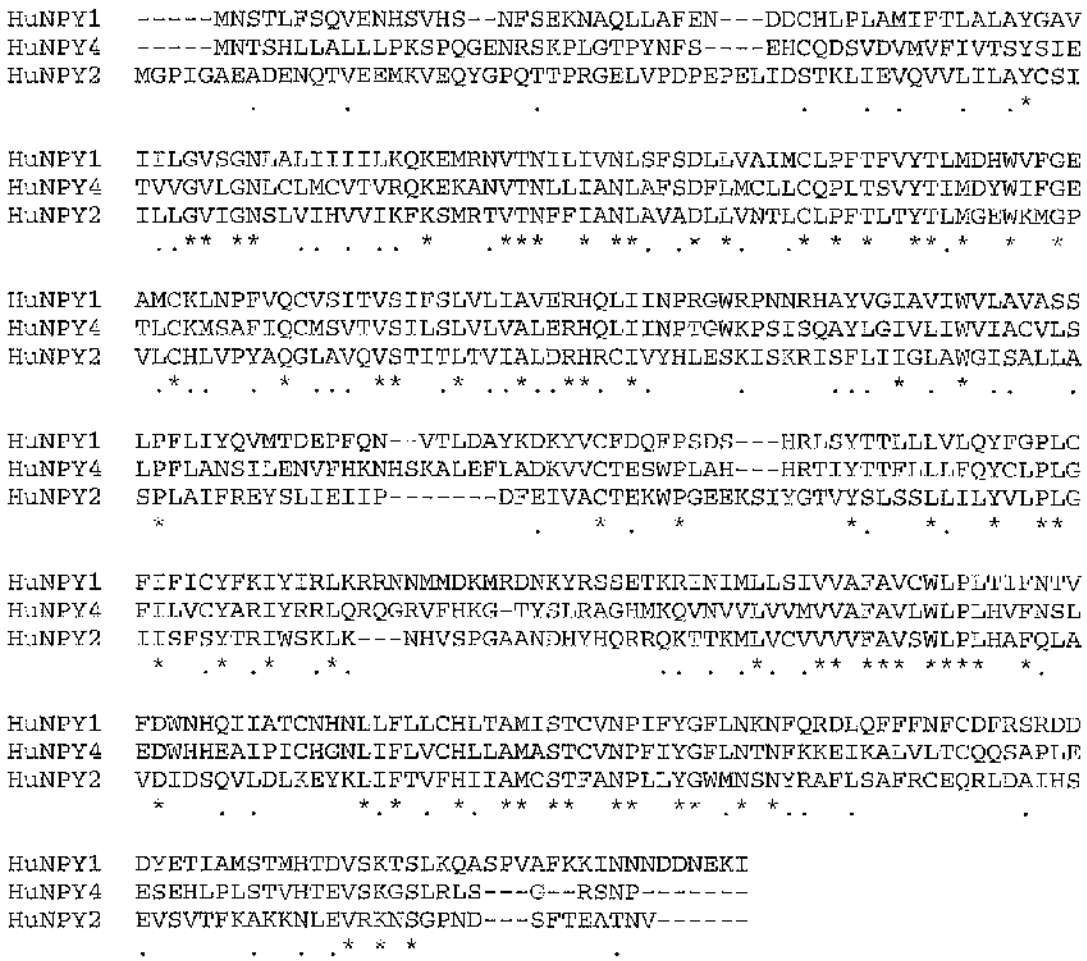
Figure 2.6. Alignment of Y1, Y2 and Y4 sequences.

HuNPY1 = Human Y1 receptor

HuNPY2 = Human Y2 receptor

HuNPY4 = Human Y 4 receptor

Asterisks (*) denote amino acids conserved between receptor sequences and, dahses mark gaps introduced to optimise alignments.


HuNPY1 -----MNSTLFSQVENHSVHS--NFSEKNAQLLAFEN---DDCHLPLAMIFTLALAYGAV
HuNPY4 -----MNTSHLLALLLPKSPQGENRSKPLGTPYNFS----EHCQDSVDVMVFIVTSYSIE
HuNPY2 MGPIGAEADENQTVEEMKVEQYGPQTTPRGELVPDPEPELIDSTKLIEVQVVLILAYCSI
HuNPY1 IILGVSGNLALIIIILKQKEMRNVTNILIVNLSFSDLLVAIMCLPFTFVYTLMDHWVFGE
HuNPY4 TVVGVLGNLCLMCVTVRQKEKANVTNLLIANLAFSDFLMCLLCQPLTSVYTIMDYWIFGE
HuNPY2 ILLGVIGNSLVIHVVIKFKSMRTVTNFFIANLAVADLLVNTLCLPFTLTYTLMGEWKMGP
HuNPY1 AMCKLNPFVQCVSITVSIFSLVLIAVERHQLIINPRGWRPNNRHAYVGIAVIWVLAVASS
HuNPY4 TLCKMSAFIQCMSVTVSILSLVLVALERHQLIINPTGWKPSISQAYLGIVLIWVIACVLS
HuNPY2 VLCHLVPYAQGLAVQVSTITLTVIALDRHRCIVYHLESKISKRISFLIIGLAWGISALLA
HuNPY1 LPFLIYQVMTDEPFQN--VTLDAYKDKYVCFDQFPSDS---HRLSYTTLLLVLQYFGPLC
HuNPY4 LPFLANSILENVFHKNHSKALEFLADKVVCTESWPLAH---HRTIYTTFLLLFQYCLPLG
HuNPY2 SPLAIFREYSLIEIIP-------DFEIVACTEKWPGEEKSIYGTVYSLSSLLILYVLPLG
HuNPY1 FIFICYFKIYIRLKRRNNMMDKMRDNKYRSSETKRINIMLLSIVVAFAVCWLPLTIFNTV
HuNPY4 FILVCYARIYRRLQRQGRVFHKG-TYSLRAGHMKQVNVVLVVMVVAFAVLWLPLHVFNSL
HuNPY2 IISFSYTRIWSKLK---NHVSPGAANDHYHQRRQKTTKMLVCVVVVFAVSWLPLHAFQLA
HuNPY1 FDWNHQIIATCNHNLLFLLCHLTAMISTCVNPIFYGFLNKNFQRDLQFFFNFCDFRSRDD
HuNPY4 EDWHHEAIPICHGNLIFLVCHLLAMASTCVNPFIYGFLNTNFKKEIKALVLTCQQSAPLE
HuNPY2 VDIDSQVLDLKEYKLIFTVFHIIAMCSTFANPLLYGWMNSNYRAFLSAFRCEQRLDAIHS
HuNPY1 DYETIAMSTMHTDVSKTSLKQASPVAFKKINNNDDNEKI
HuNPY4 ESEHLPLSTVHTEVSKGSLRLS---G--RSNP-------
HuNPY2 EVSVTFKAKKNLEVRKNSGPND---SFTEATNV------

rat, with 8 replacements in 36 positions and suggests that PP and its receptor have co-evolved at a rapid pace. The great sequence variability between species for the Y4 receptor is mirrored in pharmacology and tissue distribution as discussed in Chapter 1 section 1.6. In man the Y4 receptor appears to bind human PP with a similar affinity to PYY and NPY, implying that all three peptides may serve as ligands at physiological concentrations, whereas the rat and mouse Y4 receptors seem to be more selective for PP (Larhammar, 1996b). Thus within the same family there are highly conserved receptors and peptide ligands as well as one rapidly evolving receptor and ligand.

2.4.2 Human foetal brain PCR

Figure 2.3 illustrates the regions of Y1, Y4 and Y2 receptor sequence which exhibited adequate amino acid conservation for oligonucleotide primer design.
After performing the PCR reaction described in section 2.3.1.2, using the primers described in 2.3.1.1, following the cycling parameters outlined and running an aliquot on a 1% agarose gel, a product of the predicted molecular weight (800 base pairs) was obtained as illustrated in figure 2.7a. This PCR product was subcloned into the p-TAG vector as described in sections 2.3.1.2, 2.3.1.3 and 2.3.1.4. DNA was isolated as described in section 2.3.1.5 and restriction digest with the enzymes *Eco* R I and *Xho I* revealed that the PCR product had been successfully cloned into the p-TAG vector (see figure 2.7b). DNA was prepared for sequencing as detailed in section 2.3.1.8, however subsequent sequence analysis failed to find any homology between the DNA obtained by PCR and any of the cloned NPY receptor subtypes. Upon further investigation, the sequence appeared to correspond to vector containing the Lac Z gene, and also shared homology with regions of sequence from the large T antigen. From these results it would appear that the degenerate oligonucleotide primers annealed to the expression vector which the foetal brain library was constructed in (CDM 8), as opposed to regions of the cDNA corresponding to Y1, Y2 or Y4 receptors.

2.4.3 Sequence alignments of the Y1, Y2, Y4 and Y5 receptors

During the course of this thesis, an additional NPY receptor Y5 was reported. This new sequence was then compared to the previous alignments. Figure 2.8 shows alignments of the protein sequences of the Y1, Y2, Y4 and Y5 receptors, with identical amino acids denoted by an asterisk.
From these sequence alignments, the Y5 receptor appeared to be another divergent form being as different to both the Y1 and Y2 receptors as the latter are to one another, even though the Y5 receptor gene is located on the same chromosome as the Y1 receptor. Despite the low sequence

Figure 2.7 Results of the degenerate PCR carried out on human foetal brain cDNA.

A: Paired degenerate oligonucleotide primers (see figure 2.3 and Appendix II) designed to conserved regions of the human NPY Y1, Y2 and Y4 receptors were used to amplify cDNA with sequence homology from a human foetal brain cDNA library. An aliquot of 10µl of 100µl total volume was electrophoresed on an agarose gel as outlined in section 2.3.1.7. DNA was visualised under ultraviolet irradiation; photograph exposure time 0.5-1.0 seconds.

Lane 1: PCR product obtained from PCR reaction carried out with human foetal brain cDNA and degenerate oligonucleotide primers. Product size = 800bp (***).

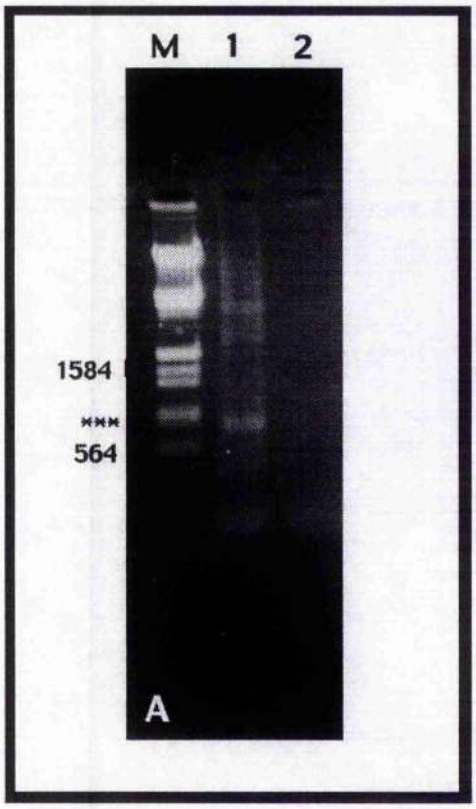
Lane 2: Negative control PCR reaction. No product obtained as expected.

Restriction digested DNA markers (M) were electrophoresed alongside reaction products. *Hind* III/*EcoR* I-digested λ DNA.

B: The 800bp PCR product obtained as shown in figure 2.7A lane I was subcloned into the p-TAG vector.
Following isolation of miniprep DNA, the recombinant plasmid was digested with restriction enzymes *Eco*RI and *Xho* I in order to confirm the generation of recombinant plasmid DNA.

Lanes 2 and 9 contain PCR insert of 800bp (***).

Restriction digested DNA markers (M) were electrophoresed alongside reaction products. *Hind* III/*EcoR* I-digested λ DNA.


M 1 2
1584
***
564
A

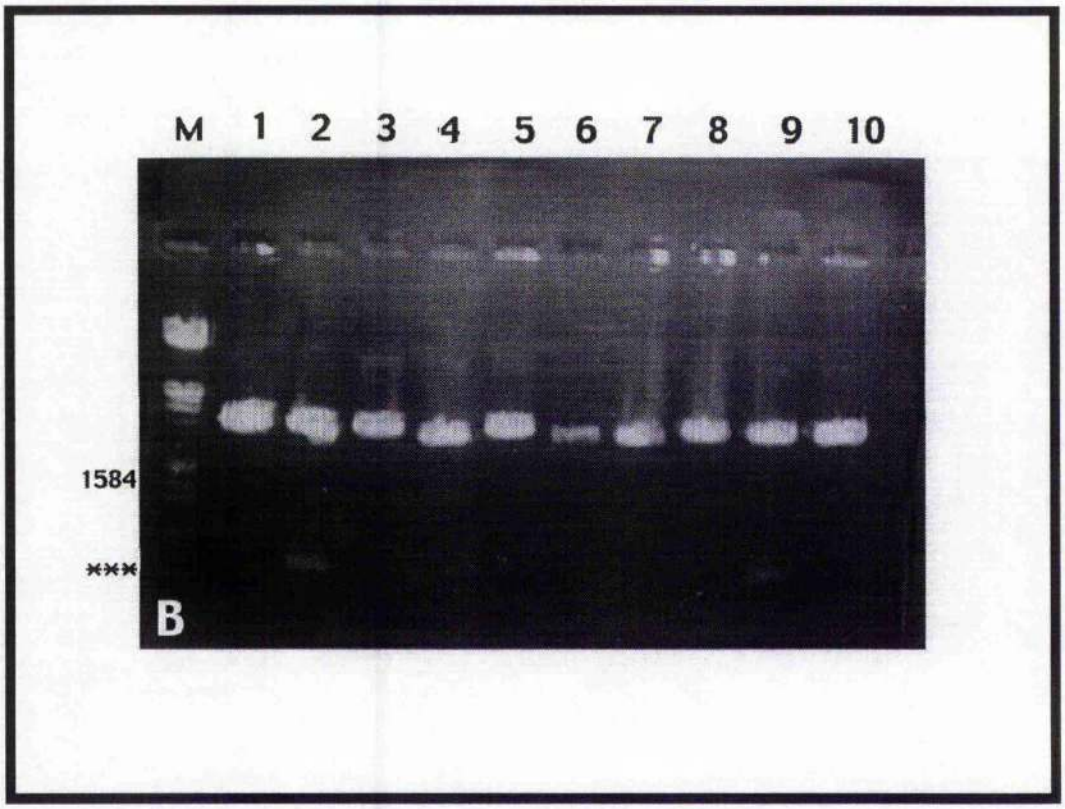

M 1 2 3 4 5 6 7 8 9 10
1584
***
B

Figure 2.8. Alignment of NPY-family receptor sequences.

HuNPY1 = Human Y1 receptor
HuNPY2 = Human Y2 receptor
HuNPY4 = Human Y4 receptor
HuNPY5 = Human Y5 receptor
Asterisks (*) denote amino acids conserved between receptor sequences, and dashes mark gaps introduced to optimise alignments.


HuNPY1 ----------MNSTLFSQVENHSVHSNFSEKNAQLLAFENDDCHLPLAMIFTLALAYGAV
HuNPY4 ---------MNTSHLLALLLPKSPQGENRSKPLGTPYNFSEHCQDSVDVMVFIVTSYSIE
HuNPY2 MGPIGAEADENQTVEEMKVEQYGPQTTPRGELVPDPEPELIDSTKLIEVQVVLILAYCSI
HuNPY5 ---------MDLELDEYYNKTLATENNTAATRNSDFPVWDDYKSSVDDLQYFLIGLYTFV
HuNPY1 IILGVSGNLALIIIILKQKEMRNVTNILIVNLSFSDLLVAIMCLPFTFVYTLMDHWVFGE
HuNPY4 TVVGVLGNLCLMCVTVRQKEKANVTNLLIANLAFSDFLMCLLCQPLTSVYTIMDYWIFGE
HuNPY2 ILLGVIGNSLVIHVVIKFKSMRTVTNFFIANLAVADLLVNTLCLPFTLTYTLMGEWKMGP
HuNPY5 SLLGFMGNLLILMALMKKRNQKTTVNFLIGNLAFSDILVVLFCSPFTLTSVLLDQWMFGK
HuNPY1 AMCKLNPFVQCVSITVSIFSLVLIAVERHQLIINPRGWRPNNRHAYVGIAVIWVLAVASS
HuNPY4 TLCKMSAFIQCMSVTVSILSLVLVALERHQLIINPTGWKPSISQAYLGIVLIWVIACVLS
HuNPY2 VLCHLVPYAQGLAVQVSTITLTVIALDRHRCIVYHLESKISKRISFLIIGLAWGISALLA
HuNPY5 VMCHIMPFLQCVSVLVSTLILISIAIVRYHMIKHPISNNLTANHGYFLIQYILPLVCLTV
HuNPY1 LPFLIYQVMTDEPFQN--VTLDA----------YK
HuNPY4 LPFLANSILENVFHKNHSKALEF----------LA
HuNPY2 SPLAIFREYSLIEIIP----------DF
HuNPY5 SHTSVCRSISCGLSNKENRLEENEMINLTLHPSKKSGPQVKLSGSHWSYSFIKKHRRRYS
HuNPY1 DKYVCFDQFPSDS----HRLSYTTLLLVLQYFGPLCFIFICYFKIYIRLKRR-NNMMDKM
HuNPY4 DKVVCTESWPLAH----HRTIYTTFLLLFQYCLPLGFILVCYARIYRRLQRQ GRVFHKG
HuNPY2 EIVACTEKWPGEEK-SIYGTVYSLSSLLILYVLPLGIISFSYTRIWSKLK----NHVSPG
HuNPY5 KKTACVLPAPERPSQENHSRILPENFGSVRSQLSSSSKFIPGVPTCFEIKPEENSDVHEL
HuNPY1 RDNKYRSSETKR---INIMLLSIVVAFAVCWLPLTIFNTVFDWNHQIIATCNHNLLFLLC
HuNPY4 TYS-LRAGHMKQ---VNVVLVVMVVAFAVLWLPLHVFNSLEDWHHEAIPICHGNLIFLVC
HuNPY2 AANDHYHQRRQK---TTKMLVCVVVVFAVSWLPLHAFQLAVDIDSQVLDLKEYKLIFTVF
HuNPY5 RVKRSVTRIKKRSRSVFYRLTILILVFAVSWMPLHLFHVVTDFNDNLISNRHFKLVYCIC
HuNPY1 HLTAMISTCVNPIFYGFLNKNFQRDLQFFFNFCDFRSRDDDYETIAMSTMHTDVSKTSLK
HuNPY4 HLLAMASTCVNPFIYGFLNTNFKKEIKALVLTCQQSAPLEESEHLPLSTVHTEVSKGSLR
HuNPY2 HIIAMCSTFANPLLYGWMNSNYRAFLSAFRCEQRLDAIHSEVSVTFKAKKNLEVRKNSGP
HuNPY5 HLLGMMSCCLNPILYGFLNNGIKADLVSLIHCLHM----------
HuNPY1 QASPVAFKKINNNDDNEKI
HuNPY4 LSGRSNP----------
HuNPY2 NDSFTEATNV----------
HuNPY5 ----------

homology, there appears to be a clustering of amino acid sequence similarity between the Y1 and Y4 receptors, while the Y2 and Y5 receptors clearly define another family. The receptors Y2 and Y5 are equally distantly related to one another as to the Y1/Y4 group, and it is interesting to note that the Y1/Y4 group and the Y2/Y5 group are more distantly related to one another than any other G-protein coupled receptors that bind to the same endogenous ligand (Larhammar, 1996b). Possible explanations for this phenomenon are that the Y1, Y2 and Y5 receptors have evolved rapidly and hence accumulated differences in a short period of time. However, the similarity between species for any one receptor suggests that this is unlikely. For instance, the Y1 receptor is highly conserved between *Xenopus laevis* and mammals. The Y5 receptor, like the Y1 and Y2 receptors exhibits great homology across species, displaying 87% overall amino acid identity between man and rat, with 99% identity observed in transmembrane regions. Therefore the evolutionary rate for the Y5 receptor also appears to be in the slower range for G-protein coupled receptors. Another possibility is that the four genes arose long ago and have diverged from one another over a substantial period of time.

In 1996, a fifth member of the NPY receptor family was described, initially in mouse, and subsequently in primates and humans. This receptor was designated y6, after considerable debate regarding the nomenclature of this NPY receptor subtype (Weinberg, 1996; Gregor, 1996 and Matsumoto, 1996). The y6 receptor is a functional receptor in mice, but represents a pseudogene in man and primates due to a premature stop codon which results in the formation of a truncated receptor protein lacking the characteristic seven transmembrane domains, and fails to bind PP-fold peptides due to an inability to express the receptor protein. Mutagenesis studies of the pseudogene to restore the frame results in a protein with the predicted seven transmembrane domain topology but still fails to bind members of the PP-fold family of peptides. Therefore in humans this receptor does not represent a functional NPY receptor subtype, and as a result protein alignments with this subtype were not carried out. When the amino acid sequence of the y6 receptor has been aligned with the other cloned NPY receptor subtypes in other species, the y6 receptor appears to exhibit sequence similarity with the Y1 and Y4 receptors, representing a clustering of amino acid sequence between these three receptors while the Y2 and Y5 receptors clearly define another family (Gehlert, 1998).
The dendrogram illustrated in figure 2.9 demonstrates the apparent divergence of the receptors that bind the NPY-family of peptides.

Figure 2.9. Distance tree for NPY family peptide receptors.

Branch lengths correspond to sequence divergence calculated from the alignment in figure 2.8 using the CLUSTAL programme for GCG (Wisconsin Package Version 9.1, Genetics Computer Group (GCG) software, Madison, Wisc).

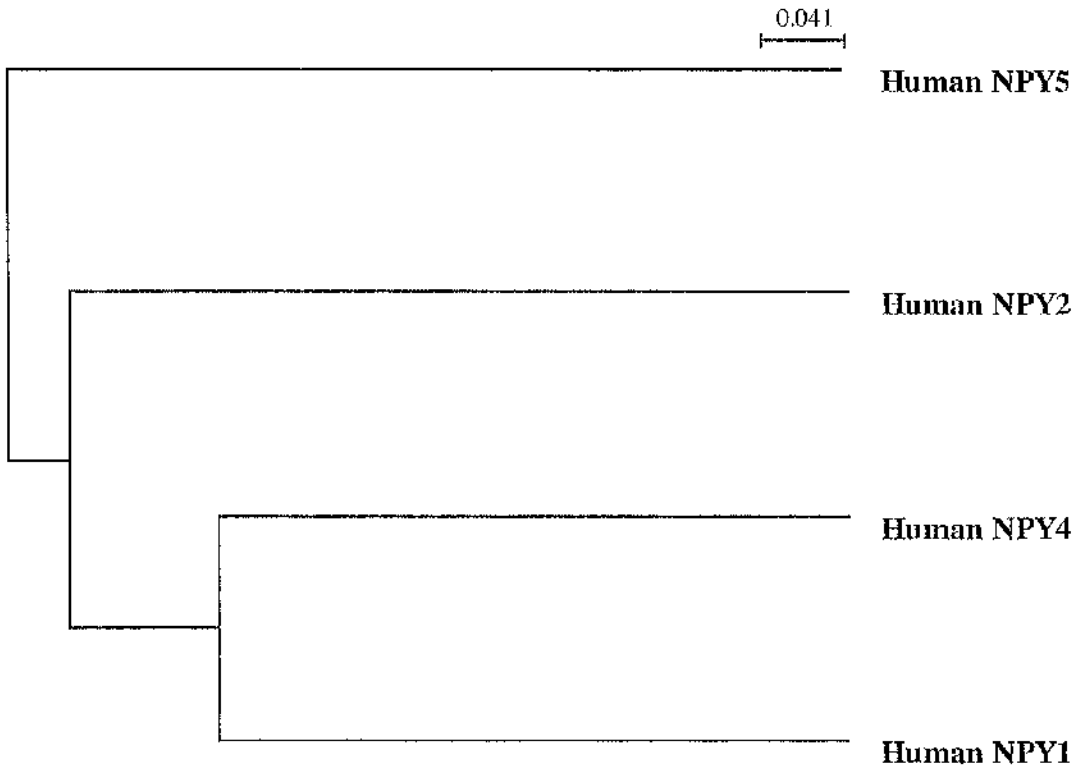

0.041
Human NPY5
Human NPY2
Human NPY4
Human NPY1

Figure 2.10 Identification of the rat NPY Y5 receptor in rat brain cDNA.

A: Paired oligonucleotide primers (see figure 2.4A and Appendix II) designed to the rat Y5 open reading frame were used to amplify corresponding cDNA fragments from rat brain cDNA.
Aliquots of 10μl of 100μl total volume were electrophoresed on an agarose gel as outlined in section 2.3.1.7. DNA was visualised under ultraviolet irradiation; photograph exposure time 0.5-1.0 seconds.

Lanes 1 and 3: PCR product obtained from rat brain cDNA using rat Y5 homologous oligonucleotide primers. Product size = ~800bp (***).

Lane 2: Negative control PCR reaction containing no DNA template. No product obtained as expected.

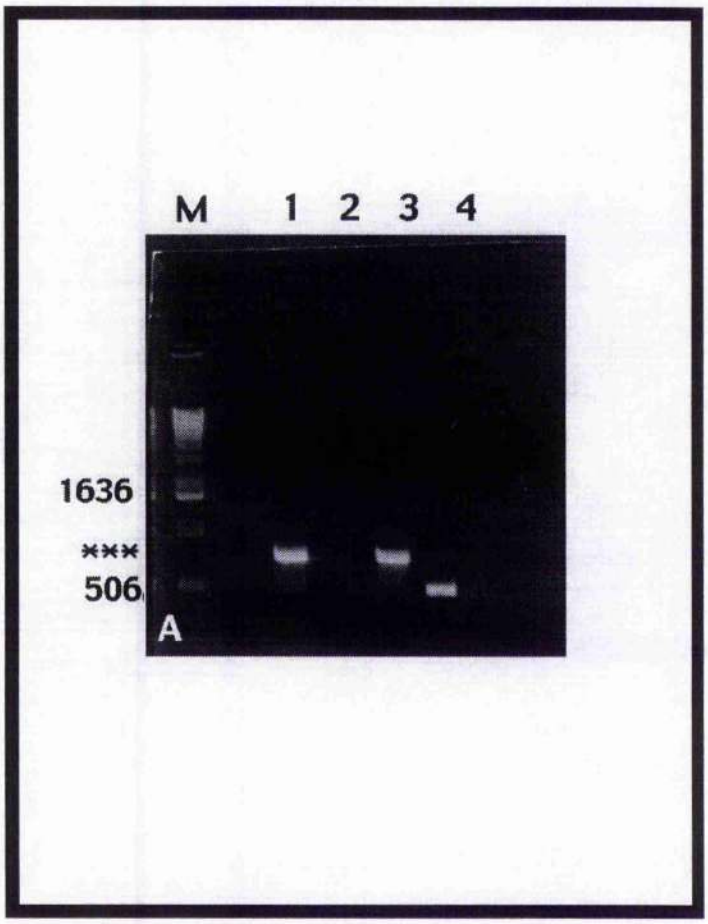
Lane 4: Positive control PCR product obtained from rat brain cDNA using G3PDH primers. Product size =500bp.

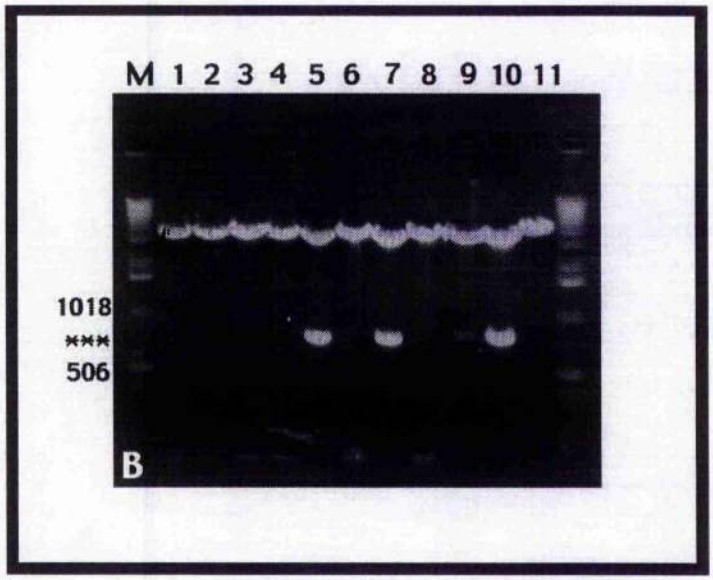
Restriction digested DNA markers were electrophoresed alongside reaction products. Ikb DNA ladder (Life Technologies) (M).

B: The 800bp PCR products amplified from the rat brain cDNA using rat Y5 homologous oligonucleotide primers were subloned into the pCR 2.1 vector. Following isolation of miniprep DNA, the recombinant plasmid was digested with the restriction enzyme *Eco*RI in order to confirm the generation of recombinant plasmid DNA.

Lanes 5, 7, 9 and 10 contain the PCR insert of 800bp(***).

Restriction digested DNA markers (M) were electrophoresed alongside reaction products. IKb DNA ladder (Life Technologies).


M 1 2 3 4
1636
***
506
A


M 1 2 3 4 5 6 7 8 9 10 11
1018
***
506
B

2.4.4 Human and rat Y5 receptor PCR

Figure 2.4 illustrates the regions of the human and rat Y5 receptor sequence where oligonucleotide primers were designed.

PCR reactions were carried out as described in section 2.3.2.2, using combinations of primers designed to yield either full length or partial Y5 cDNA products. A partial rat Y5 product was obtained using rat brain cDNA (Clontech) as a template (Figure 2.10a). Subsequent subcloning into the vector pCR2.1 was confirmed by restriction digest analysis using *Eco*R I to excise the 800 base pair PCR product from mini-prep DNA prepared from the transformed plasmid (Figure 2.10b). Sequence analysis of those minipreps containing the target sequence revealed that the PCR product obtained was homologous to a region of the rat Y5 open reading frame. However, despite the use of multiple alternative 5' oligonucleotides, a full length rat Y5 product was unobtainable (see figure 2.4 for schematic of rat Y5 primer design and Appendix II for sequence of primers).

A longer length human Y5 PCR product of 1200 base pairs was obtained using human hypothalamic cDNA (Clontech) as template (Figure 2.11a). However despite repeated efforts and employing different strategies, this PCR product proved impossible to subclone. The experience encountered here with both the rat and human PCR has been observed in other laboratories working in the field (Dan Larhammar personal communication) and although the reasons for this are unclear, it may reflect secondary structure at the 5' end of this receptor. This secondary structure does not appear to be the result of a highly GC rich region of sequence, as the sequence fails to exhibit 5' GC abundance.

A partial human Y5 PCR product was obtained using human genomic DNA (Promega Corp) as template (Figure 2.11b). Subsequent subcloning of this 800 base pair product into the vector pCR2.1 was confirmed upon analysis of restriction digests with *Eco*R I (Figure 2.11c). Sequence analysis of those minipreps containing the target sequence revealed that the PCR product obtained was homologous to a region of the human Y5 open reading frame.

Both the rat and human Y5 sequences appear to be correct and no mutants have been introduced during the PCR procedure. This is significant, as the use of *Taq* polymerase in the PCR reactions may have resulted in the introduction of mutants into the amplified DNA. As *Taq* DNA polymerase does not have 3' to 5' exonuclease activity, this polymerase does not possess proofreading properties like other thermostable polymerases such as Vent or *Pfu* polymerase which have the ability to excise incorrectly incorporated nucleotides during the synthesis of DNA strands complementary to the template. Therefore, the fidelity of this

Figure 2.11 Identification of the human Y5 receptor in human hypothalamic cDNA and human genomic DNA.

A: Paired oligonucleotide primers (see figure 2.4B and Appendix II) designed to the human Y5 open reading frame were used to amplify corresponding cDNA fragments from human hypothalamic cDNA. Primer annealing temperature for amplification of human Y5 PCR products was varied, as follows:

Lane 1: Annealing temperature 50°C
Lane 3: Annealing temperature 55°C
Lane 5: Annealing temperature 58°C
Lane 7: Annealing temperature 60°C
Lane 9: Annealing temperature 62°C

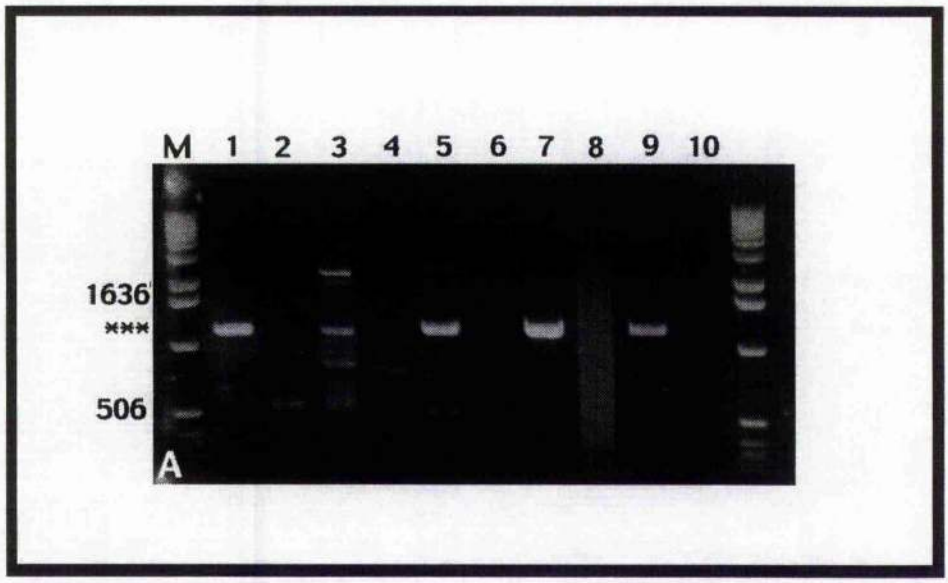
Aliquots of 10µl of 100µl total volume were electrophoresed on an agarose gel as outlined in section 2.3.1.7. DNA was visualised under ultraviolet irradiation; photograph exposure time 0.5-1.0 seconds.
PCR products of predicted size 1200bp (***) were obtained with each of the annealing temperatures described above using the human Y5 homologous primers. Lane 10 represents the negative control reaction containing no product as expected. Positive control reactions were carried out as described (data not shown).

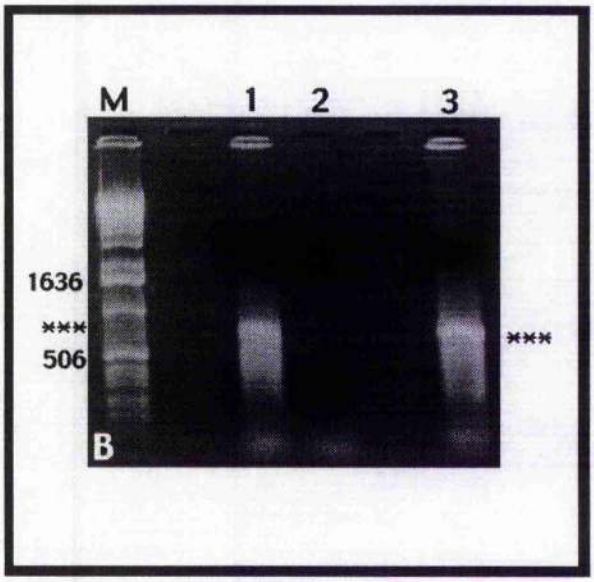
Restriction digested DNA markers (M) were electrophoresed alongside reaction products. 1Kb DNA ladder (Life Technologies).

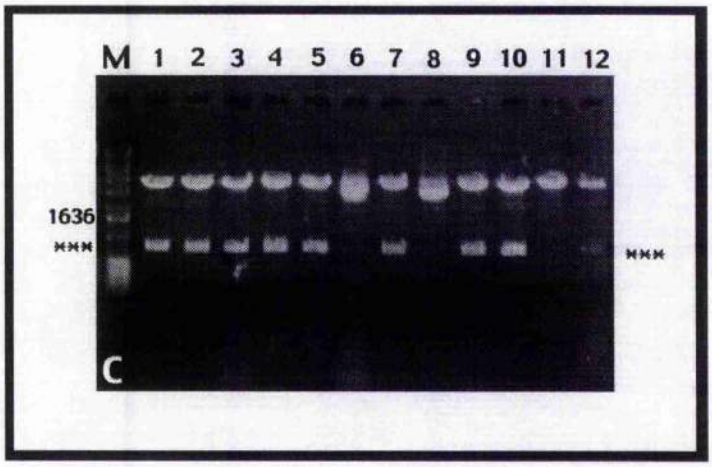
B: Paired oligonucleotide primers (see figure 2.4B and Appendix II) designed to the human Y5 open reading frame were used to amplify corresponding cDNA fragments from human genomic DNA.
Lanes 1 and 3 : PCR product obtained from human genomic DNA using human Y5 primers. Product size 800bp (***).
Lane 2: Negative control
All other lanes empty.

C: The 800bp PCR products amplified from the human genomic DNA using human Y5 homologous primers were subcloned into the vector pCR2.1. Miniprep DNA was digested with the restriction enzyme *Eco*RI in order to confirm the generation of recombinant plasmid DNA.

All lanes with the exception of lanes 6, 8 and 11 contain the PCR insert of 800bp (***)


M 1 2 3 4 5 6 7 8 9 10
1636
***
506
A


M 1 2 3
1636
***
506
***
B


M 1 2 3 4 5 6 7 8 9 10 11 12
1636
***
***
C

polymerase is not as high as that exhibited by other DNA polymerases such as Vent or *Pfu* polymerase, which are between 5- to 15-fold greater than *Taq* polymerase. Although both Vent and *Pfu* polymerases have higher thermostabilty than *Taq* polymerase, making them more robust enzymes, the inability of these enzymes to generate 3' dA overhangs on their reaction products, leaving blunt-ended fragments, made the use of *Taq* polymerase more favourable in the experiments carried out, as the subcloning procedure employed relied upon the presence of 3'dA overhangs.

2.4.5 Human RNA master blot

Analysis of the signals detected after screening the human RNA master blot (Clontech) containing 50 different poly A^+ selected RNA samples from human tissues, detected Y5 receptor mRNA readily in kidney, and to a lesser extent in small intestine, liver and lung. No signal was detected in other tissues including the spleen (Figure 2.12). No signal was detected in the Poly A^+ RNA from various brain regions. However, as hypothalamic Poly A^+ RNA was not present on the master blot, this result is not entirely surprising as Y5 mRNA is believed to be concentrated predominantly in this brain region, with levels of expression extremely low or absent in other areas of the brain (Gerald, 1996). The finding of abundant Y5 receptor mRNA in the kidney was surprising as the initial report described a lack of the mRNA in peripheral tissues. To confirm this finding, PCR was carried out as described in section 2.3.2.2 using a human kidney cDNA library (Clontech) as a source of template and human Y5 primers. A product of correct molecular weight was obtained confirming the presence of Y5 mRNA signal detected by Northern blot analysis (Figure 2.13). The Y5 receptor has been postulated to play a role in the renal effects of NPY where it is involved in mediating the enhancement of diuresis and natriuresis (Bischoff, 1997).

Figure 2.12 Analysis of the distribution of the human NPY Y5 receptor by Northern blot analysis.

A human RNA master blot (Clontech) containing 50 different poly A^+ selected RNA samples was screened with a ^{32}P-labelled cDNA probe encompassing a region of the human Y5 open reading frame cDNA.

Human Y5 mRNA was readily detected in kidney, and to a lesser extent in small intestine, liver and lung. No human Y5 mRNA was detected in other tissues including the spleen.

1=kidney
2=liver
3=small intestine
4=lung

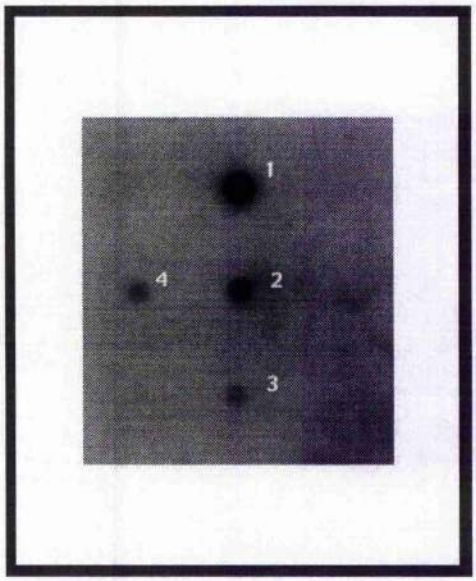

1
4
2
3

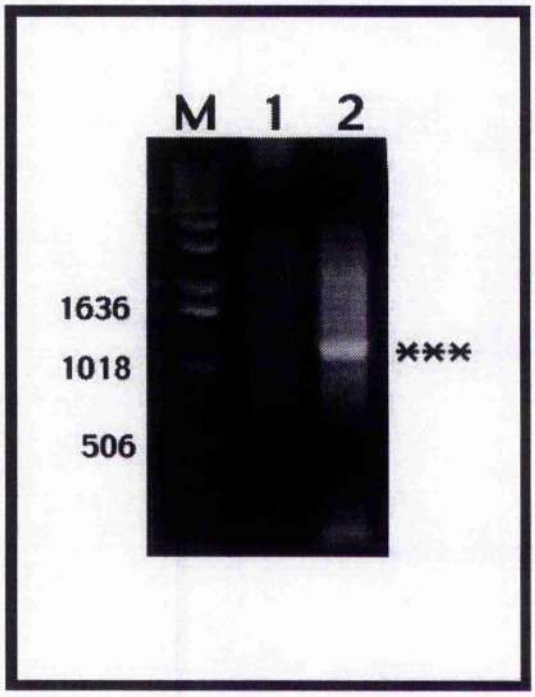
Figure 2.13 Confirmation of the presence of the human Y5 receptor in the kidney by PCR analysis.

Paired oligonucleotide primers (see figure 2.4B and Appendix II) designed to the human Y5 open reading frame were used to amplify corresponding cDNA fragments from a human kidney cDNA library.
An aliquot of 10µl of 100µl total volume were electrophoresed on an agarose gel as outlined in section 2.3.1.7. DNA was visualised under ultraviolet irradiation; photograph exposure time 0.5-1.0 seconds.

Lane 1: Negative control PCR reaction containing no DNA template. No product was obtained as expected.

Lane 2: PCR product obtained from human kidney cDNA using human Y5 homologous primers. Product size 1200bp.

Restriction digested DNA markers (M) were electrophoresed alongside reaction products. 1kb DNA ladder (Life Technologies).


M
1
2
1636
1018
506
***

2.5 Summary

1)Structural alignments of the sequences of the Y1, Y2, Y4 and Y5 receptors demonstrate that they are members of the large superfamily of G-protein coupled receptors, which are membrane spanning receptors and exhibit a characteristic seven transmembrane domain structure.

2)The NPY family of receptors are extremely diverse. Despite recognising the same ligand(s), namely members of the PP-fold family of peptides including NPY, PYY and PP, the cloned receptors for NPY display a structural diversity exhibiting low percentage identities between family members even in transmembrane regions. The diversity observed may reflect different structural requirements of NPY, which are essential in order to elicit activation of receptor subtypes.

3) There appear to be two families of NPY receptors, as even with the low sequence homology displayed between subtypes, there is a clustering of amino acid sequence between the Y1, Y4 and y6 receptors, while the Y2 and Y5 receptors clearly define another family.

4) Members of the NPY receptor family appear to display significant homology between species, with interspecies variation minimal. This is demonstrated by the high percentage identities in receptor amino acid sequences observed between man and rat for the Y1, Y2 and Y5 receptors. The Y4 receptor does not exhibit species conservation of receptor sequence, a phenomenon which may reflect the variability of the PP ligand across species, as Pancreatic polypeptide is believed to be a rapidly evolving ligand which displays considerable divergence between man and rat.

Chapter 3

Isolation and characterisation of a rat Y5 genomic clone.

Chapter 3

3.1 Introduction

The role of neuropeptides, in particular NPY, in the physiology of feeding behaviour has gathered tremendous recognition as a result of findings which document alterations in endogenous levels of these neuropeptides following starvation and satiety. The potent stimulatory effects NPY elicits on ingestive behaviour are well characterised and of widespread interest. The pharmcological profile known to mimic NPY stimulated feeding could not be attributed to the previously described NPY receptors and the G-protein coupled receptor believed to mediate NPY induced eating was cloned from rat hypothalamus in 1996 (Gerald, 1996). This new receptor (known as Y5) appeared to demonstrate an identical pharmacology to that known to promote NPY feeding in satiated rats (Gerald, 1996 and Kalra, 1990).

The physiology of feeding is extremely complex, involving numerous interactions between neurotransmitters, neuropeptides and hormones known to play a role in the regulation of this fundamental behaviour. This complex circuitry undoubtedly includes NPY, which together with other established mediators of feeding behaviour (see section 1.9) serves to maintain energy homeostasis and nutrient balance. Following the cloning of the Y5 receptor, the published cDNA sequence encoding the mRNA for this receptor provided a valuable tool for studying the regulation of this crucial gene. By gaining knowledge on the control of this gene, a major constituent in the network of feeding regulation may be further understood, in particular, as regards interactions with the many other entities in the complex circuitry.

3.1.1 The role of the Y5 gene in the regulation of feeding

In order to establish further the role of NPY and the Y5 receptor in the regulation of feeding behaviour, an experimental strategy was employed which would enable the identification and characterisation of the Y5 receptor gene. To determine the molecular organisation and regulation of the rat Y5 receptor gene, isolation and characterisation of a Y5 genomic clone was necessary in order to identify exon and intron organisation. By determining the genomic organisation of the Y5 gene, the 5'UT region of this gene could be identified and putative regulatory sequence elements of the promoter region could be identified. These can then be exploited to carry out promoter studies and determine the pattern of regulation for the Y5 gene. The cloning of the Y5 receptor cDNA (Gerald, 1996) enabled the design of oligonucleotide primers homologous to the Y5 receptor which could be radiolabelled and used in the screening of a rat genomic library to identify a Y5 genomic clone.

However, the NPY Y1 receptor has also been implicated in the control of feeding (Kanatani, 1996, 1998). The structure of the gene encoding the Y1 receptor has been previously determined, and is characterised by the presence of a single 97-bp intron in the coding region following transmembrane V (Herzog, 1993), and the existence of several alternative 5' exons that allow for the regulation of tissue-specific expression of the receptor (Ball, 1995).

3.1.2 The use of lambda (λ) bateriophage in genomic library screening.

The large cloning capacity of lambda (λ) bacteriophages allows the construction of highly redundant genomic libraries, which are an important tool in the identification and characterisation of receptor genes. The subsequent characterisation of phage recombinants identified upon library screening, can prove to be a complex procedure: a) Purification of large amounts of phage DNA is time consuming when compared with the ease of working with high copy vectors, b) Restriction mapping can prove tedious because the insert of a λ recombinant is smaller than the vector backbone, and c) Subcloning of insert fragments can present difficulties due to the lack of unique restriction sites flanking the insert and the low cloning efficiencies for large fragments. The novel λPS vector (Nehls, 1994) used to construct the rat genomic library screened in the experiments described in this chapter, circumvents these problems as it features an automatic plasmid subcloning facility of insert fragments, and therefore combines the advantage of large insert genomic libraries with the convenience of working with high copy plasmids. The λPS vector is a replacement vector of lambda phage origin which can accommodate inserts of up to 20Kb, thereby allowing the complete representation of complex genomes in a reasonable number of recombinants. The linear vector contains two loxP sites in direct orientation flanking a high copy plasmid backbone and the insert. Recombination between these two sites is mediated by Cre recombinase. The Cre-lox site specific recombination system is particularly simple and well characterised, and is accomplished with the use of Cre, a 48 KDa protein which is capable of catalysing the recombination between directly repeated sites on the same molecule to excise the intervening segment (figure 3.1). This leads to the excision of the multi-copy plasmid from the phage genome, and is termed automatic subcloning as it obviates the need for tedious subcloning of the insert (Sauer, 1987 and 1988). The use of Bluescript facilitates further manipulation of excised plasmid DNA, as demonstrated by the convenience of working with high copy plasmids .

3.2 Experimental strategy

The experiments described in this chapter were carried out with the aim of identifying and characterising the rat Y5 receptor gene, the rationale behind this strategy ultimately aimed at determining the pattern of regulation of expression for this gene which is strongly implicated in the control of food intake.

3.2.1 Identification of a positive clone

The published cDNA sequence encoding the rat Y5 open reading frame was exploited in order to design oligonucleotide primers bearing homology to the 5' region of the rat Y5 receptor. These were subsequently used to screen a rat genomic library. Hybridisation screening of the rat genomic library with radiolabelled oligonucleotide primers homologous to the Y5 receptor cDNA was carried out in order to identify a rat Y5 genomic clone (Benton, 1977). Identification of a positive clone following the initial primary screening of the library, permitted a secondary screening process and the subsequent identification of a positive clone. This was then used to isolate plasmid DNA bearing the NPY Y5 gene.

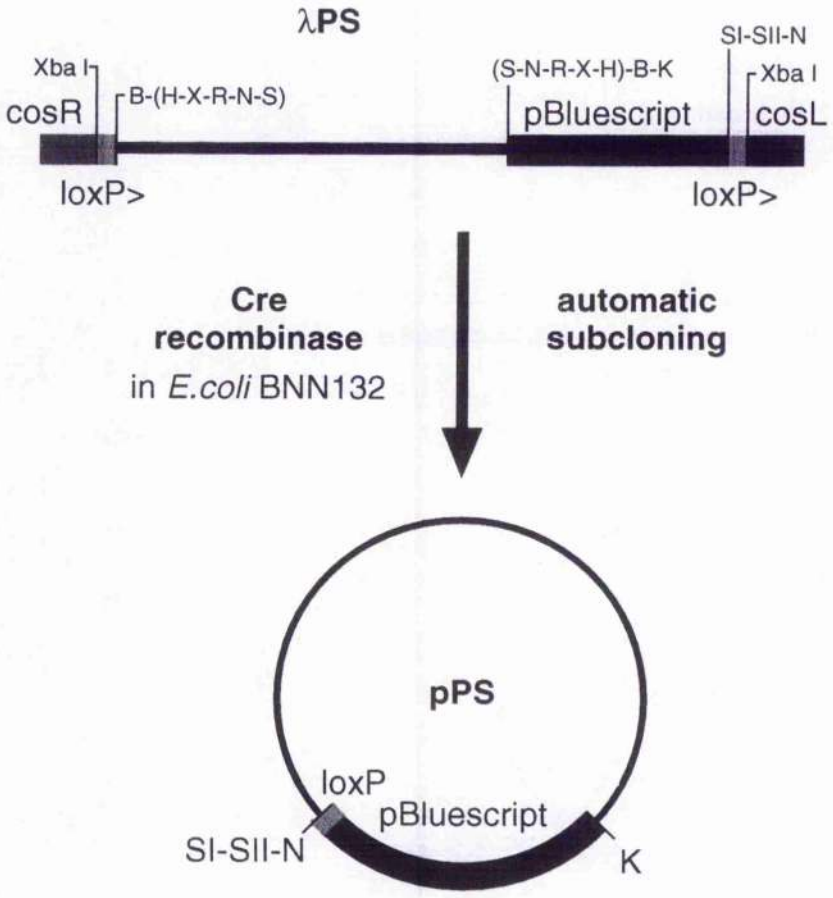
3.2.2 Isolation and analysis strategy

The Cre lox excision system was used to subclone the isolated DNA clone into Bluescript and restriction digest analysis was carried out on the plasmid DNA in order to assess insert size and to determine by diagnostic restriction digest whether the isolated genomic DNA contains rat Y5 receptor sequence.

3.2.3 Southern blot analysis strategy

Southern blot analysis was carried out on the isolated DNA in order to identify any regions of homology between the isolated DNA and the rat Y5 receptor. A DNA probe encompassing a region of the rat Y5 open reading frame was used to detect regions of rat Y5 sequence within the isolated genomic DNA, and to identify the 5' region of the Y5 gene. Identification of sequence bearing homology to the rat Y5 receptor was confirmed using PCR technology and subsequent sequence analysis.
The experimental strategy employed in the experiments described in this chapter is summarised in figure 3.2

Figure 3.1. Schematic of the λPS vector structure, detailing the Cre recombinase automatic subcloning facility.


λPS
Xba I
cosR
B-(H-X-R-N-S)
(S-N-R-X-H)-B-K
pBluescript
SI-SII-N
Xba I
cosL
loxP>
loxP>
Cre
recombinase
in E.coli BNN132
automatic
subcloning
pPS
loxP
pBluescript
SI-SII-N
K

3.3 Chapter specific methods.

3.3.1 Oligonucleotide design.

Oligonucleotide primers specific for regions of messenger RNA (mRNA) coding for the rat Y5 receptor (Gerald, 1996) were used as radiolabelled probes in the rat genomic library screening in order to isolate the rat Y5 genomic clone by hybridisation screening. A pictorial representation of the primers alongside the Y5 mRNA is given in figure 3.3 and the oligonucleotide sequences can be found in Appendix II. Care was taken in oligonucleotide design to avoid sequences which might contribute to secondary structure and to incorporate a relatively high proportion of G and/or C residues to achieve higher dissociation temperature (T_D) values. Oligonucleotides were synthesised commercially by Oswel DNA Service (University of Southampton) and reconstituted in water. All oligonucleotides were checked for complementary hybridisation with the DNA data bank using the *blast* or *fasta* programmes for GCG (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisc).

3.3.2 End labelling (5') of oligonucleotides.

T4 polynucleotide kinase (T4PNK) was used to phosphorylate the 5'-end of oligonucleotides with ^{32}P to a specific activity of 2.5 X 10^9 dpm/µg, as outlined (Maxam and Gilbert, 1980). In a typical reaction, 1µg of oligonucleotide was labelled using a 5'-DNA terminus labelling system (Life Technologies) in a 25µl reaction volume, with 10µCi [$\gamma^{32}P$] ATP (5000Ci/mmol, Amersham). One microlitre of labelled product was spotted onto DEAE ion exchange paper (DE-81, Whatman) and analysed using liquid scintillation spectrophotometry to determine

Figure 3.2. Schematic depicting experimental strategy for rat Y5 genomic clone identification.

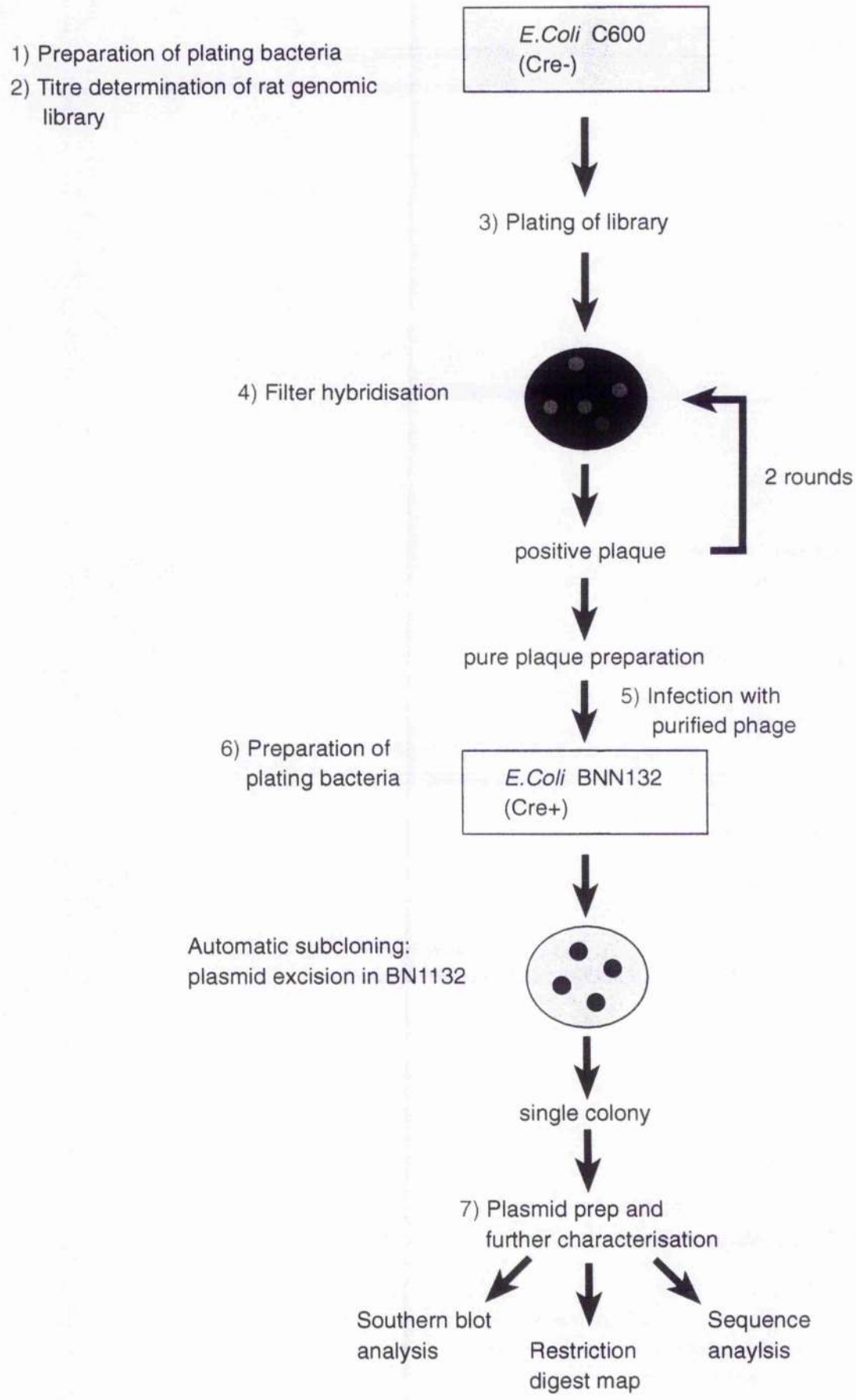
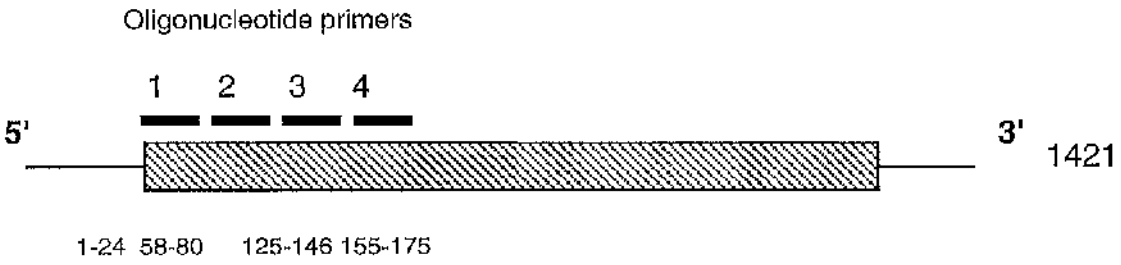

1) Preparation of plating bacteria
2) Titre determination of rat genomic library
E.Coli C600
(Cre-)
3) Plating of library
4) Filter hybridisation
2 rounds
positive plaque
pure plaque preparation
5) Infection with purified phage
6) Preparation of plating bacteria
E.Coli BNN132
(Cre+)
Automatic subcloning:
plasmid excision in BN1132
single colony
7) Plasmid prep and further characterisation
Southern blot analysis
Restriction digest map
Sequence anaylsis

Figure 3.3. Oligonucleotide primers schematic.

Schematic of rat Y5 cDNA sequence and the oligonucleotide primers used for genomic library screening purposes. Hatched regions on the cDNA correspond to translated sequences.
Oligonucleotide primers 1, 2 and 3 represent those used in genomic library screening procedure.
Oligonucleotide primers 1, 2 ,3 and 4 represent those used in Southern blot analysis.

Rat Y5 receptor



label incorporation and specific activity. Samples containing no enzyme were also analysed as negative controls to compare incorporation of radioactivity. All samples were washed in 0.5M sodium phosphate to remove free nucleotides before counting. CHROMASPIN TE 10 columns (Clontech) were used to purify the oligonucleotides before use.

3.3.3 Genomic library screening.

3.3.3.1 Preparation of plating bacteria (C600).

The bacterial strain C600 (Cre-) was prepared in order to prepare serial dilutions of the genomic library for titre determination. A single colony of the bacterial strain was grown in 50ml LB++ in a 250ml flask, with vigorous agitation at 37°C to saturation (usually overnight). The culture was then centrifuged at 2500 X g for 10 minutes to pellet the bacteria, which were subsequently resuspended in 25ml 20mM $MgSO_4$.

3.3.3.2 Titre determination of rat genomic library.

The titre of the rat genomic library was assessed in order to ensure that all clones within the library were represented in the library plating and subsequent screening. Serial dilutions of the library were prepared in SM solution (Appendix I). Three hundred microlitres of the C600 plating bacteria were infected with 1µl of a 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} library dilution and allowed to incubate for 30 minutes at 37°C. LB/$MgSO_4$ plates were prepared, as was LB/top agar, which was cooled to 55°C before addition of the infected bacteria. The infected bacteria and LB /top agar mixture was poured onto prewarmed (37°C) LB/$MgSO_4$ plates, allowed to set, and incubated in an inverted position at 37°C overnight to allow plaque growth. Plaques were counted following overnight incubation and the exact titre of the plaque forming units (pfus) of the library stock was calculated, as the number of plaques obtained is equivalent to the number of phage present in 1µl of the corresponding serial dilution. The titre of the library was calculated as 5.4 X 10^7 λ phage/µl. As the suggested number of pfus to guarantee complete representation of the rat genome in one plating is 2 X 10^6 pfus, the estimated titre value of 5.4 X 10^7 pfus was therefore sufficient to ensure representation of the rat genome. The recommended number of pfus per plate was calculated at 20-50 X 10^3 pfu's per 12cm X 12cm plate, therefore a 1:1000 dilution of the library was required. Three millilitres of C600 plating bacteria were infected with 10µl of the library dilution calculated to yield 50 X 10^3 pfus, and incubated at 37°C for 30 minutes. Following incubation, 9mls of LB/top agar were added to the infected bacteria and the sample poured onto prewarmed (37°C) LB/$MgSO_4$ plates. After cooling at room temperature, the plates were incubated at 37°C overnight in an inverted position. Plates were checked at regular intervals in order to assess plaque formation and growth, as plaque growth was optimal when individual plaques were readily observed,

and no plaque diffusion was present. When optimal plaque growth had been obtained, plates were stored at 4°C until transfer to nylon membrane.

3.3.3.3 Transfer of plaques to nylon membrane.

In order to screen the rat genomic library with the Y5 specific radiolabelled oligonucleotide primers, transfer of the plaque DNA in the pfus to nylon membrane was necessary. Twenty plates containing 50 X 10^3 pfus, and representing the entire rat genome were subjected to the following procedure in order to ensure efficient transfer of the plaque DNA to the nylon membrane. Nylon membrane (Hybond, Amersham) was carefully placed on top of the pfu containing agar, ensuring the entire surface of the plate was covered by the membrane. A syringe was used to form a series of holes at the edge of the membrane to facilitate orientation following the screening process. The membrane was positioned correctly on the plates and carefully removed after 2 minutes. Duplicate lifts were carried out as described, leaving the membrane on the plates for 4 minutes to ensure efficient transfer. After drying at room temperature for 10 minutes, the membranes were denatured by submerging in a solution containing 1.5M NaCl and 0.5M NaOH for 2 minutes, neutralised in a solution containing 1.5M NaCl and 0.5M Tris-HCl (pH8.0) for 5 minutes. The membranes were then rinsed in 2 X SSC solution for 2 minutes, air-dried between 2 sheets of filter paper and UV crosslinked at 700,000μJ for approximately 30 seconds with an ultraviolet crosslinker (Amersham) to cross-link nucleic acids to the membrane.

3.3.3.4 Filter hybridisation.

Filters were equilibrated in prehybridisation solution containing 5 X SSC, 5 X Denhardts, 0.1% SDS, 20mM NaH_2PO_4 and 100mg/ml sheared and denatured salmon sperm DNA at 60°C with rotation for at least 3 hours (in a Techne hybridisation vessel). After addition of ^{32}P labelled oligonucleotide probe, hybridisation was allowed to proceed overnight at 60°C with constant agitation. Following overnight hybridisation, the hybridisation solution was removed and the filters were washed twice for 15 minutes at 60°C in a solution of 5 X SSC/0.1%SDS. Filters were then subjected to more stringent washing conditions, by washing twice for 15 minutes at 60°C in a solution of 3 X SSC/0.1%SDS. Once satisfied that sufficient removal of any non specific radioactivity had been achieved the filters were wrapped in cling film to prevent dehydration. Membranes containing no DNA, but treated identically to the filters containing bacteriophage DNA, were used as negative controls in order to ensure hybridisation between the probe and DNA on the membrane was specific.
Detection of a positive signal was carried out as described in section 2.3.3.1, and positive plaques were picked from the corresponding agar plates following careful alignment of filter and plate, to ensure isolation of the correct plaque was achieved. Single plaques were picked

using an inverted 1ml pipette tip, transferred to an eppendorf tube containing 500µl SM/20µl chloroform, vortexed and stored at 4°C until further processing was carried out.

3.3.3.5 Isolation of plasmid DNA from bacteriophage.

The bacterial strain BNN132 (Cre+), which expresses Cre recombinase essential for the excision of the DNA from the λ phage vector, was prepared as described in the manufacturers instructions (Mo Bi Tec). The BNN132 strain was streaked on to LB agar plates containing 25µg/ml kanamycin and grown overnight at 37°C. A single colony of the Cre+ recombinase containing strain was grown in 50ml LB++ in a 250ml flask, with vigorous agitation at 37°C to saturation (usually overnight). The culture was then centrifuged at 2500 X g for 10 minutes to pellet the bacteria, which were subsequently resuspended in 25ml 20mM $MgSO_4$.

The bacteria were infected with the purified phage by adding 50µl purified phage to 200µl of Cre+ plating cells, and incubating at 37°C for 30 minutes, following gentle mixing. The bacteria and phage mixture were then plated on to LB agar plates containing 50 µg/ml ampicillin, inverted and incubated at 37°C overnight. Following overnight incubation, a single colony was picked and grown to saturation with vigorous agitation at 37°C in 5mls of LB/ampicillin(100µg/ml). Plasmid "miniprep" DNA was isolated as described in section 2.3.2.4.

3.3.4 Plasmid "Maxipreparation"

Large-scale plasmid preparations were performed using a kit (Qiafilter maxi kit, Qiagen). The method used was as instructed in the manufacturers protocol. The plasmid containing strain of *E.Coli* was grown overnight in 100ml LB medium containing appropriate selective antibiotic, at 37°C with vigorous shaking. The bacterial cells were harvested by centrifugation at 6000 X g for 15 minutes at 4°C, and the pellet resuspended in 10ml of buffer P1 (supplied). To this, 10ml of lysis buffer P2 (supplied) was added, mixed carefully by inversion and incubated at room temperature for 5 minutes. Following incubation at room temperature, 10ml of chilled neutralisation buffer P3 (supplied) was added to the lysate, mixed immediately but gently by inversion and poured directly into the barrel of a QIA filter cartridge (supplied). The lysate was then incubated for 10 minutes at room temperature before pushing the liquid through the filter, removing insoluble complexes containing chromosomal DNA, salt, detergent and proteins which form during the neutralisation step. The cleared lysate was then loaded onto a previously equilibrated QIAGEN-tip 500 by gravity flow. The selectivity of the resin in the QIAGEN-tip ensures that only plasmid DNA binds, while degraded RNA, cellular proteins, and metabolites are not retained and appear in the flow-through fraction. The QIAGEN-tip was then washed with 2 X 30ml buffer QC (supplied) to ensure removal of contaminants. The plasmid DNA was then eluted from the QIAGEN-tip with 15ml of high salt

buffer QF (supplied) and precipitated by the addition of 10.5ml of isopropanol at room temperature, followed by centrifugation at 15,000 X g for 30 minutes at 4°C. The DNA pellet obtained following centrifugation was washed in 5ml room temperature 70% ethanol to remove residual salt and centrifuged at 15,000 X g for 10 minutes at room temperature. The pellet obtained was air-dried for 5-10 minutes and the DNA resuspended in 1ml of TE buffer pH 8.0.

3.3.5 Southern blot analysis

Following restriction digest of DNA as described in section 2.3.1.6, DNA was transferred from agarose gels to membrane by capillary action for subsequent hybridisation analysis as described (Southern, 1975); nylon charge-modified membrane (Hybond, Amersham) was substituted for nitrocellulose and transfer was allowed to proceed overnight. After electrophoresis and photography, the gel was soaked for 45 minutes in 1.5M NaCl/0.5M NaOH at room temperature to denature the DNA. The gel was then neutralised by soaking in a solution containing 2M Tris pH 7.4/2M NaOH for 40 minutes at room temperature. The gel was then laid on several layers of filter paper (Whatmann 3MM) soaked in 10 X SSC; these layers were overlaid on a filter paper wick extending from a tray filled with 10 X SSC. A piece of nylon membrane, cut to the size of the gel and nicked in the corner for orientation purposes, was prewet in 5 X SSC and laid on top of the gel such that all bubbles were excluded between the gel and membrane. Three layers of solution saturated filter paper were placed on top of the membrane avoiding trapping of bubbles and then a 4-6 cm pile of dry absorbant paper was placed on top. A glass plate and a 1Kg weight added weight needed to ensure contact of dry paper with wet filter paper. The following day, the damp blot was exposed, 30 seconds each side to ultraviolet light at 254nm to cross-link nucleic acid to the membrane and to assess transfer efficiency. Blots were then air-dried and stored between sheets of filter paper.

3.3.6 Recombinant plasmid generation-1.4Kb Pst fragment

The 1.4kb DNA fragment generated by digestion of genomic DNA with the restriction endonuclease *Pst*I was cloned into Bluescript (SK-) for plasmid DNA preparation and sequencing. One microgram of vector DNA was digested to completion with *Pst*I. Simultaneously, genomic DNA was digested to completion using *Pst*I and electrophoresed through low melting point agarose gel. The 1.4Kb fragment was excised using a scalpel blade and heated to 65°C to melt the agarose. The vector fragment was then subjected to dephosphorylation using Shrimp Alkaline Phosphatase (SAP) (Amersham). This enzyme removes the 5' phosphate from DNA ends thereby preventing religation of the vector. This enzyme was inactivated by incubating at 65°C for 15 minutes following dephosphorylation. The DNA fragment was ligated using T4 DNA ligase (Boehringer Mannheim) in 1X low salt buffer (Boehringer Mannheim: Appendix I) and 1mM ATP incubated at 15°C overnight. DNA was transformed as described in section 2.3.1.4 and plasmid DNA was isolated as described in section 2.3.2.4.

3.4 Results.

3.4.1 Genomic library screening.

A rat genomic DNA library constructed in λPS phage vector (Mo Bi Tec) was screened with a series of ^{32}P end-labelled oligonucleotides homologous to 5' regions of the rat Y5 open reading frame (figure 3.3). Six positive clones were obtained from 2 X 10^6 bacteriophage plaques following the primary screening procedure described in section 3.3.3. Hybridisation screening of duplicate filters confirmed the presence and location of these clones which were subsequently isolated, and prepared for a further cycle of screening. Three positive clones were obtained from 2.6 X 10^3 bacteriophage, again following the procedure described in section 3.3.3. The positive clones were isolated for preparation of plasmid DNA from the bacteriophage.

3.4.2 Diagnostic restriction analysis.

Three colonies were obtained following excision of the plasmid DNA from the targeted bacteriophage plaques using the Cre+ recombinase system to facilitate subcloning of the DNA insert into Bluescript. Plasmid DNA was prepared as described in sections 2.3.2.4.and 3.3.3.6.

Following isolation of the excised DNA from targeted bacteriophage preparations, restriction digest analysis was carried out on the DNA in order initially to determine whether the isolated genomic DNA contained any sequence with homology to the rat Y5 receptor. DNA was subjected to digestion with the restriction endonucleases *Bgl II* and *NcoI*, as the Bluescript vector lacks restriction sites for these enzymes, making them ineffective at digestion of the vector. These enzymes were used to digest the isolated genomic DNA as these enzymes would not generate vector fragments. The use of other restriction enzymes may generate vector fragments. The MAPSORT programme for GCG (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisc.) permitted a detailed analysis of the restriction enzyme sites present within the rat Y5 open reading frame (Gerald, 1996), and revealed that the rat Y5 cDNA contains restriction sites for both *Bgl* II and *Nco*I enzymes. Following digestion to completion of 10μg DNA with both restriction enzymes and gel electrophoresis analysis, the DNA fragments generated enabled diagnostic analysis to be carried out. The generation of DNA fragments corresponding to the molecular weight predicted by the rat Y5 map would suggest that the isolated DNA may contain regions of rat Y5 sequence.

The inserts generated from restriction digestion using *Bgl* II and *Nco*I yielded a fragment of the predicted molecular weight, as determined from the restriction map of the rat Y5 cDNA. The presence of the 600bp predicted fragment suggested that the isolated genomic DNA contains rat Y5 sequence. Fragments of different molecular weights were also obtained, as the large genomic DNA insert is likely to contain numerous restriction sites for the enzymes used. The other fragments generated upon restriction digestion with *Bgl* II and *Nco*I were as follows; 200bp, 400bp, 700bp, 800bp, 1000bp, 1200bp, 1800bp, 2500bp and 4000bp.

Figure 3.4a illustrates the restriction digest analysis of the isolated genomic DNA following digestion and gel analysis as described in sections 2.3.1.6 and 2.3.1.7

3.4.3 Restriction digest determination of insert size

In order to determine the size of the genomic insert, restriction analysis was carried out using endonucleases which are known to possess restriction sites in the cloning region

Figure 3.4. Restriction digest analysis of isolated genomic DNA.

A: Diagnostic isolated genomic DNA digest.

10µg genomic DNA was digested to completion with *Bgl* II and *Nco*I and electrophoresed on an agarose gel as oultined in section 2.3.1.7. DNA was visualised under ultraviolet irradiation; photograph exposure time 0.5-1.0 seconds.

Lanes 1and 2: The migration position of the 600bp genomic DNA fragment predicted by the rat Y5 cDNA restriction map is denoted by ***.

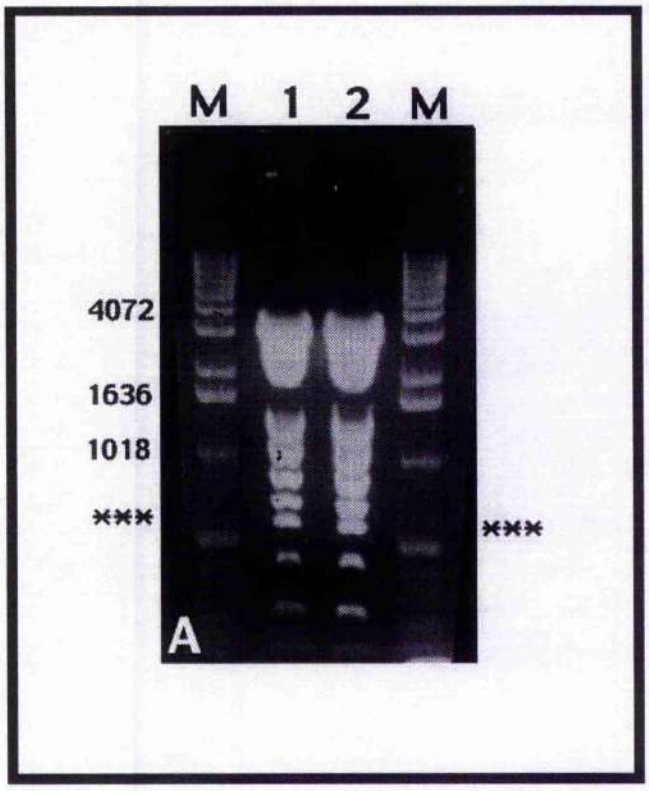
Restriction digested DNA markers (M) were electrophoresed alongside digested genomic DNA. 1Kb ladder (Life Technologies).

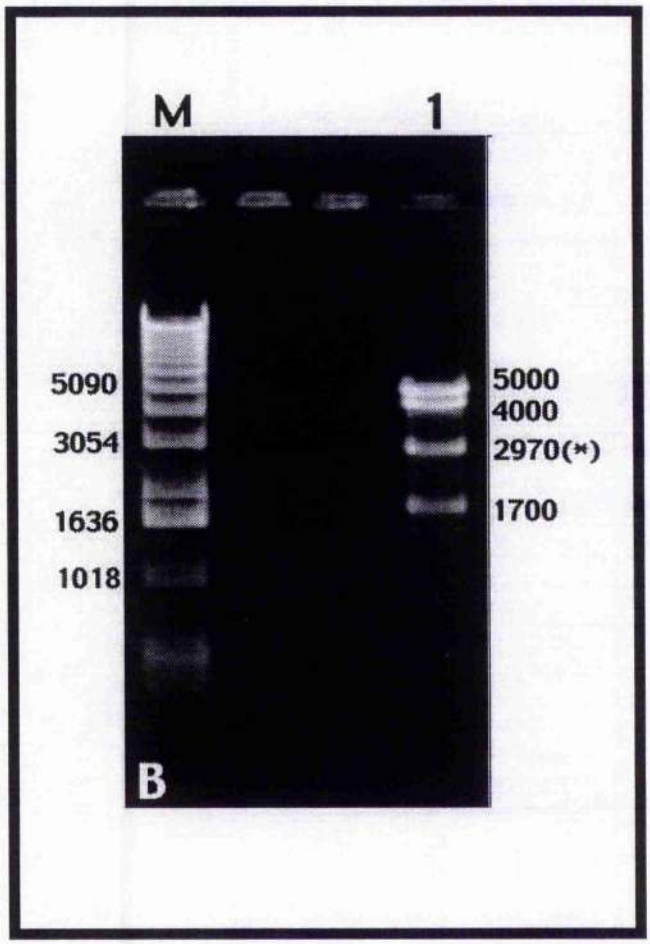
B: Restriction digest analysis of insert size.

10µg genomic DNA was digested to completion with *Kpn*I and *Not*I and treated as described above.

Lane 1: The migration position of the genomic DNA fragments generated and the Bluescript vector (*) are denoted in base pair values.

Restriction digested DNA markers (M) were electrophoresed alongside digested genomic DNA. 1Kb ladder (Life Technologies).


M 1 2 M
4072
1636
1018
***
***
A


M 1
5090
3054
1636
1018
5000
4000
2970(*)
1700
B

of the vector. By performing restriction analysis with *Kpn*I and *Not*I, which have restriction sites 5' and 3' of the insert, the insert size was readily determined from the DNA fragments generated. The size of the genomic DNA calculated as 10.5kb as shown in figure 3.4b.

3.4.4 Restriction analysis and cloning of a partial Y5 receptor gene.

The restriction enzymes *Bam*HI, *Hind*III, *Nco*I , *Not*I and *Pst*I were used to digest 10µg genomic DNA, in order to establish the number of restriction sites present within the insert and to obtain smaller DNA fragments for Southern blot analysis. The results of restriction digest analysis of the genomic DNA following gel electrophoresis are shown in figure 3.5a. As illustrated in figure 3.5a, the restriction endonucleases *Hind*III and *Pst*I were shown to be effective at digestion of the isolated genomic DNA, as observed by the generation of multiple DNA fragments. The restriction endonucleases *Bam* HI, and *Not*I were ineffective and failed to fragment the DNA. Consistent with one internal site, *Nco* I linearised the isolated genomic DNA. The size of the fragments obtained upon digestion with *Hind*III and *Pst*I were as follows: *Hind*III 3500bp and 7000bp, *Pst*I 1400bp, 2700bp and 6000bp.

Southern blot analysis of the restriction digests shown in figure 3.5a was carried out (as described in section 3.3.3.7) using a region of the rat Y5 receptor cDNA as a probe (see figure 2.4a) in order to identify restriction fragments which may harbour regions of the rat Y5 gene. This probe comprises base pairs 621-1410 of the rat NPY Y5 cDNA, which contain the open reading frame of this receptor from transmembrane domain 5 (TM5) to the carboxy-terminal region of the receptor protein. As illustrated in figure 3.5b, Southern analysis revealed regions of homology between the rat Y5 cDNA probe and the 2.7 Kb fragment generated by *Pst*I.

3.4.5 The open reading frame of the rat NPY Y5 receptor between TM5 and the C-terminal region of the protein contains no introns

In order to confirm the presence of a region of the rat Y5 receptor gene within the isolated genomic DNA, the restriction digest was repeated, using 10µg DNA and the restriction endonuclease *Pst*I. The DNA from the 2.7Kb *Pst*I generated fragment was excised from the gel and purified using a kit (Qiaquick gel extraction, Qiagen). Following purification, the DNA was used as a template in a PCR reaction with oligonucleotide primers homologous to the rat Y5 receptor. Figure 2.4a highlights the combination of primers used in the PCR reaction, which was carried out as described in section 2.3.2.2, subjected to 40 cycled temperature conditions and an annealing temperature of 60°C. Negative control reactions were carried out as described previously in section 2.3.2.2.

Figure 3.5. Southern blot analysis of isolated genomic DNA.

A: Restriction analysis of isolated genomic DNA
10μg per lane of isolated genomic DNA was digested to completion with the following restriction enzymes:
Lane a :*Hind* III, lane b: *Bam*HI, lane c: *Nco* I, lane d: *Not* I, lane e: *Pst*I, lane f: *Hind* III, lane g: *Bam*HI, lane h: *Pst*I, lane i: *Not* I and lane j: *Nco* I.
DNA was electrophoresed on an agarose gel as outlined in section 2.3.1.7.and visualised under ultraviolet irradiation; photograph exposure time 0.5-1.0 seconds.

Restriction digested DNA markers (M) were electrophoresed alongside digested genomic DNA. 1Kb ladder (Life Technologies).

B: Autoradiogram of a Southern blot of the gel in figure 3.5A hybridised to a ^{32}P-labelled rat NPY Y5 cDNA probe encompassing the rat Y5 open reading frame from TM5 to the C-terminus of the receptor protein.
Lanes e and h contain *Pst* I digested genomic DNA. A single band corresponding to the 2.7Kb PstI fragment (***) hybridised to the radiolabelled probe.


M a b c d e f g h i j M
3054
1636
1018
A

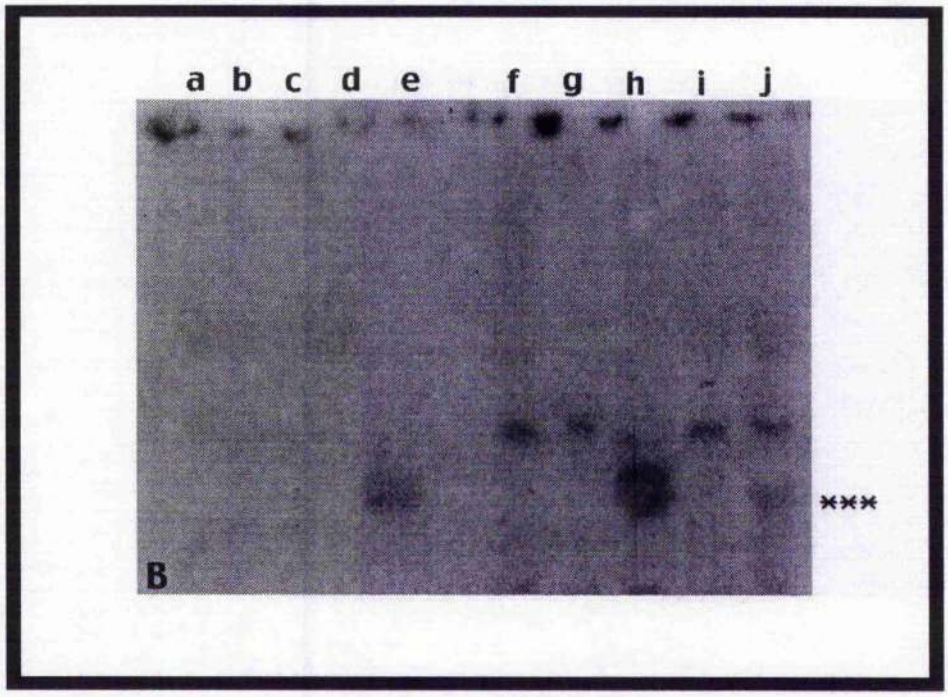

a b c d e f g h i j
***
B

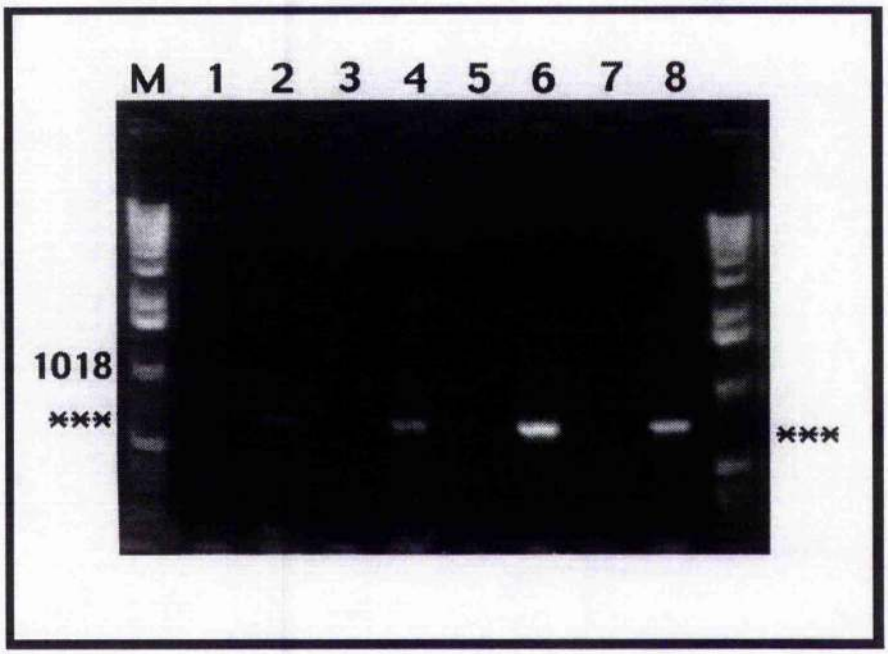
Figure 3.6. Identification of a partial rat NPY Y5 receptor gene within the isolated genomic DNA .

Paired oligonucleotide primers (see figure 2.4A and Appendix II) designed to the rat Y5 open reading frame were used to amplify corresponding DNA fragments from the isolated genomic DNA. The 2.7Kb fragment generated by *Pst*I digestion which hybridised to a radiolabelled probe encompassing a region of the rat Y5 open reading frame (figure 3.5B) was used in the PCR reaction described (section 3.4.5).

Lanes 1, 3, 5 and 7: No PCR products obtained using a rat Y5 cDNA 5' forward primer and reverse primer (figure 2.4A). Expected PCR product molecular weight =1200bp.

Lanes 2, 4, 6, and 8 : PCR products of expected molecular weight were obtained using a rat Y5 cDNA internal 5' primer and reverse primer (figure 2.4A). Expected PCR product molecular weight = 800bp (***).

Restriction digested DNA markers (M) were electrophoresed alongside digested genomic DNA. 1Kb ladder (Life Technologies).


M 1 2 3 4 5 6 7 8
1018
***
***

As demonstrated in figure 3.6, gel electrophoretic analysis of the PCR products revealed that products of the predicted molecular weight were obtained using only one of the primer combinations. The internal forward primer (IP) and reverse primer (RP) (figure 2.4 A) generated a PCR product of the molecular weight of 800bp. This molecular weight product was identical to that predicted from the cDNA sequence. Subsequent sequence and bioinformatic analysis of the PCR products obtained confirmed that the DNA shared 100% sequence identity with rat Y5 cDNA over this region (Gerald, 1996). These results suggest that the region of the rat Y5 gene situated between the internal primer (TM5) and reverse primer (C-terminus) (figure 2.4A) does not contain an intron in the sequence, as identical PCR products were obtained from cDNA (figure 2.10a) and genomic DNA.

3.4.6 Identification of the 5' untranslated region of the rat Y5 gene by restriction digest analysis and Southern blotting.

In order to examine regulation of the Y5 gene, cloning and characterisation of the 5' flanking region of the gene is necessary to enable promoter studies to be carried out. In order to construct a map of the 5' region of the rat Y5 receptor gene, Southern analysis was carried out on isolated genomic DNA digested to completion with restriction endonucleases *Hind*III and *Pst*I (figure 3.7A).

Following digestion of 10µg DNA and gel electrophoretic analysis, DNA was transferred to nylon membrane and Southern blot analysis was carried out using ^{32}P end-labelled oligonucleotide primers homologous to the 5' end of the rat Y5 open reading frame (figure 3.3). Four individual identical Southern blots were carried out and each probed with a different radiolabelled oligonucleotide primer corresponding to different regions of the 5' region of the cDNA.

The results of the Southern hybridisations described above are shown in figure 3.7B. The regions of homology observed between the radiolabelled oligonucleotide probes and the digested genomic DNA are tabulated below in table 3.1.

HindIII fragments X = 7000bp
Y = 3500bp

*Pst*I fragments X = 6000bp
Y = 2700bp
Z = 1400bp

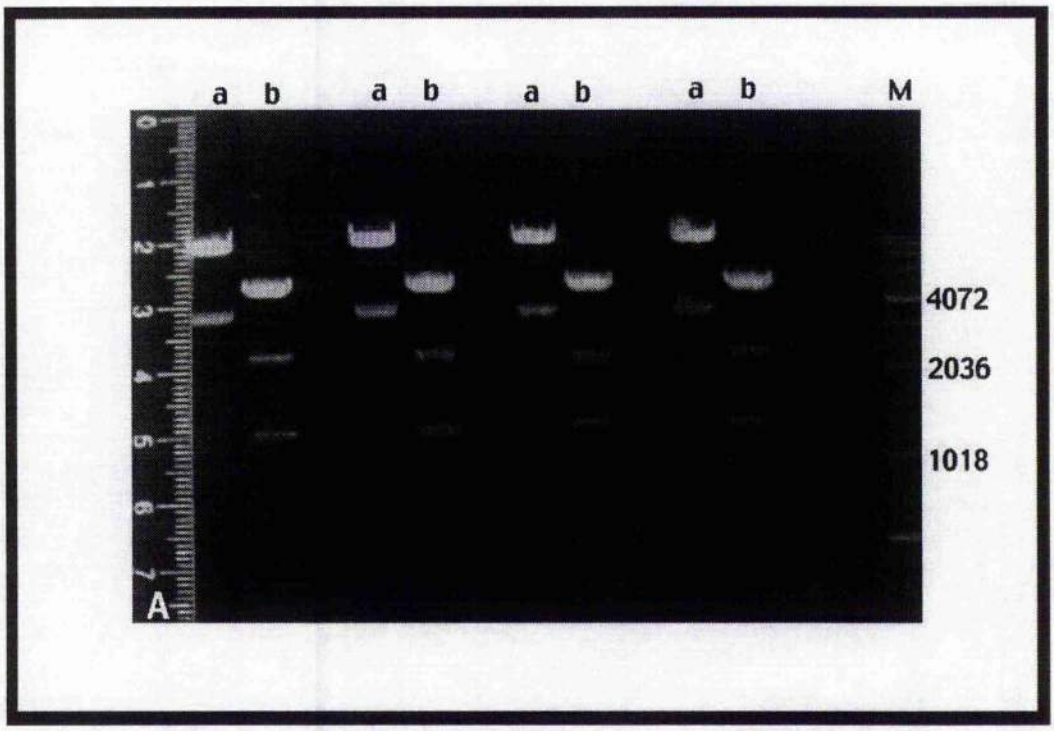
Figure 3.7 Identification of the 5' untranslated region of the rat Y5 gene by restriction digest analysis and Southern blotting.

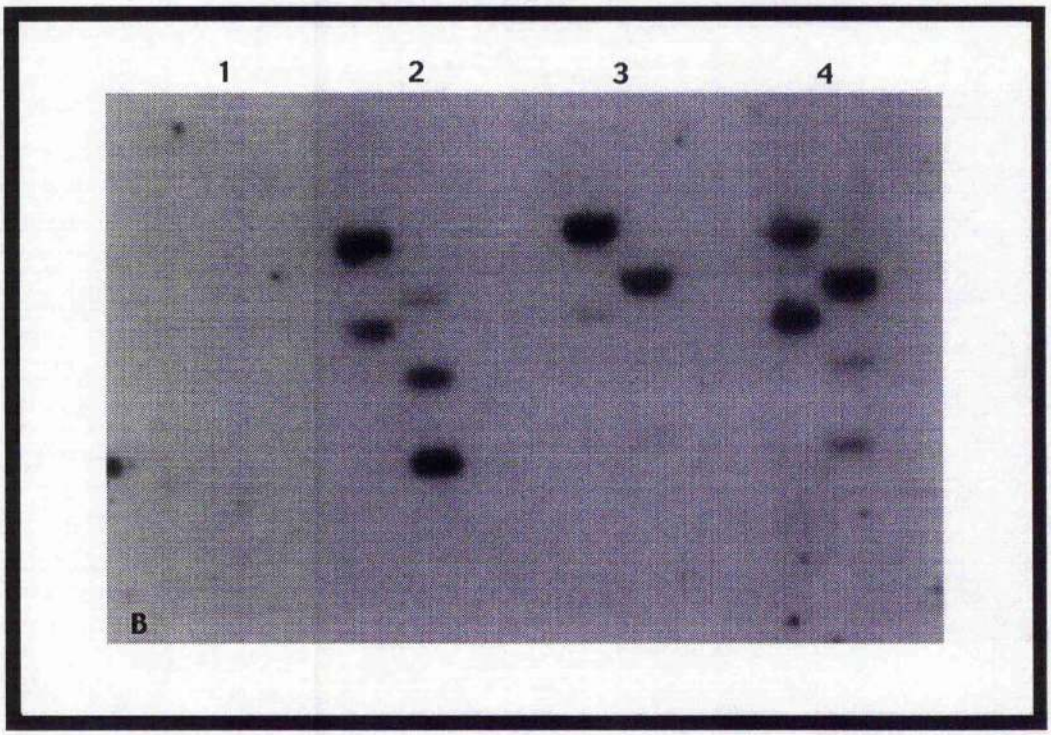
A: Restriction analysis of isolated genomic DNA
10μg per lane of isolated genomic DNA was digested to completion with the following restriction enzymes:
Lanes a: *Hind* III
Lanes b: *Pst*I
DNA was electrophoresed on an agarose gel as outlined in section 2.3.1.7. and visualised under ultraviolet irradiation; photograph exposure time 0.5-1.0 seconds.

Restriction digested DNA markers (M) were electrophoresed alongside digested genomic DNA. 1Kb ladder (Life Technologies).

B: Autoradiogram of the four individual identical Southern blots of the gel in figure 3.8A hybridised to a ^{32} P-end labelled oligonucleotide primers homologous to different regions of the 5' end of the rat Y5 open reading frame see figure 3.3 and Appendix II).

The radiolabelled oligonucleotide primer used to probe each blot is denoted numerically as illustrated (numbers 1-4), and the pattern of hybridisation obtained is described in detail in table 3.1


a b a b a b a b M
4072
2036
1018
A


1 2 3 4
B

	Oligo 1	Oligo 2	Oligo 3	Oligo 4
*Hind*III X	—	++++	+++	+++
*Hind*III Y	—	+++	—	+++
*Pst*I X	—	—	+++	+++
*Pst*I Y	—	+++	—	—
*Pst*I Z	—	++++	—	—

Table 3.1 (+++ and ++++denote hybridisation of radiolabelled oligonucleotide to genomic DNA fragment.).

As the table above and figure 3.7B demonstrate, the radiolabelled oligonucleotide primer 2 hybridises strongly to the smallest *Pst*I generated fragment of 1.4Kb, demonstrating that regions of homology exist between this oligonucleotide primer and the 1.4Kb fragment of genomic DNA. As this oligonucleotide is homologous to the region of the Y5 cDNA encompassing the functional translation codon, this *Pst*I generated fragment of DNA may contain sequence from the 5'-untranslated region of the Y5 gene. The 1.4Kb fragment of DNA was subcloned into the vector Bluescript (SK) as described in section 3.3.6, and DNA was prepared for sequencing (section 2.3.1.9) and further analysis.

3.4.7 Sequence analysis of the 1.4Kb *Pst*I fragment

Sequence analysis of this DNA has revealed difficulty in identifying regions of homology with the cDNA which may suggest that this fragment only contains a very small exon. The 2 oligonucleotides (3 & 4) downstream of the second primer hybridised to the largest fragment of *Pst* I suggesting that an intron separates the sequences between oligonucleotide 2 and oligonucleotides 3 and 4.

3.5 Discussion.

3.5.1 Isolation of a genomic rat Y5 receptor gene clone from a rat genomic library

Screening of an amplified rat genomic library yielded 3 positive clones when hybridised to oligonucleotide primers homologous to the rat Y5 open reading frame. The number of bacteriophage plaques screened in the amplified rat genomic library was 2 $X10^6$. As the amplified library was calculated to possess a redundancy of 10, the number of bacteriophage plaques screened represents 10 rat genomes. The rat genome is likely to contain a single Y5 gene, as the other members of the NPY receptor family are encoded by single copy genes (Larhammer, 1992., Gerald, 1995., Lundell, 1995., and Gregor, 1996).

3.5.2 Diagnostic restriction digest analysis

The restriction digest carried out with the endonucleases *Bgl* II and *Nco*1, which are ineffective at digestion of the vector DNA, and are known to possess restriction sites within the rat Y5 cDNA, enabled a diagnostic strategy to be employed. The rat Y5 cDNA restriction map predicted a fragment size of molecular weight 600bp upon digestion with the aforementioned endonucleases. As the isolated genomic DNA yielded a fragment of this size when digested to completion with *Bgl* II and *Nco*I, this suggested that the genomic DNA obtained from the library screening may contain DNA sequence corresponding to the rat Y5 receptor gene. The results of this diagnostic digest enabled the investigation and characterisation of the isolated genomic DNA to progress further.

3.5.3 Identification of a partial rat Y5 receptor gene.

Southern blot analysis was carried out in order to obtain DNA fragments of a manipulative size, and to confirm the presence of rat Y5 receptor DNA within the isolated genomic DNA. Restriction enzymes known to be 6 base pair recognition site restriction enzymes were selected at random and used to fragment the genomic DNA. As illustrated in figure 3.6a, the endonucleases *Hind*III and *Pst*I generated multiple DNA fragments, confirming the presence of restriction sites for these enzymes within the genomic DNA. Restriction sites for the endonucleases *Bam*HI and *Not*I do not appear to be present in the isolated genomic DNA, as these failed to fragment the DNA upon digestion to completion.

Southern analysis with a ^{32}P-labelled cDNA probe encompassing a region of the open reading frame of the rat Y5 cDNA revealed that the 2700bp *Pst*I fragment was positive by hybridisation suggesting homology exists between the genomic DNA and this region of the rat Y5 cDNA. Following isolation and purification of this genomic DNA fragment, a PCR strategy was employed using oligonucleotide primers homologous to the rat Y5 cDNA (figure 2.4a). Sequence analysis of the resultant PCR product obtained confirmed that this fragment of genomic DNA contains a region of the rat Y5 open reading frame. In addition, as the sequence obtained for the PCR of the genomic fragment was identical to the cDNA, this indicates that the region of the NPY Y5 gene between TM5 and the C-terminal region contains no introns.

3.5.4 Identification of the rat Y5 5' untranslated region.

As the regulation of feeding behaviour undoubtedly involves interactions between numerous neurotransmitters, neuropeptides and hormones known to play a role in mediating this fundamental function, further knowledge regarding the regulation of a major component in this complex circuitry would provide invaluable insight into one of the critical pathways responsible for energy homeostasis. In order to investigate further the regulation of the Y5 gene, identification of the promoter region of the gene is essential. By identifying the promoter region, sequence motifs characteristic of binding sites for transcription factors may be located and reporter gene constructs designed in order to examine regulation of this crucial gene.

Recent published work implies that the organisation of the 5' end of this gene is very complex (Herzog, 1997). Indeed, this genomic structure has been extremely difficult to subclone and evaluate (H.Herzog, personal communication). This may explain the difficulty encountered in this project in defining the 5' end of this genomic fragment. Thus, oligonucleotide 1 failed to hybridise to any of the genomic fragments, suggesting that this region of the DNA is absent in this clone. Primer 2 which encompasses the proposed translation initiation codon (ATG) hybridised strongly to the smallest *Pst* I fragment suggesting that at least one exon encoding the 5' region of the gene is located in this fragment. However, following sequence analysis, it has proved difficult to identify regions of homology implying either that the hybridisation observed is false or that the exon is extremely small. Oligonucleotides 3 and 4 hybridise to the largest *Pst* I fragment whereas the region encoding the open reading frame (from transmembrane domain 5 to the C-terminal region of receptor protein) is present on the 2.7 Kb *Pst* I fragment. This implies that there is at least one intron between oligonucleotide 4 and the open reading frame oligonucleotide encompassing part of transmembrane domain 5.

As a role for the NPY Y1 receptor subtype in the control of NPY induced feeding behaviour has been suggested (Kanatani, 1996, 1998), it is noteworthy that the NPY Y1 receptor gene and the Y5 receptor gene are transcribed in opposite directions from a common promoter region on chromosome 4q31-q32. (Herzog, 1997). One of the alternatively spliced 5'exons of the Y1 receptor (1C) is also an integral part of the coding region of the Y5 receptor, as exon 1C of the Y1 receptor gene, if translated from the opposite strand, encodes sequences corresponding to the third large intracellular loop of the Y5 receptor (Herzog, 1997). The close proximity of these two NPY receptor genes suggests that they have evolved from a gene duplication event, the Y5 receptor gene probably evolving by a gene duplication event from the Y1 receptor gene, which is now encoded in the opposite orientation 23Kb upstream of exon 2 of the Y1 gene. Of particular interest is the fact that the small intron interrupting the coding sequence of the Y1 gene is converted into a functional sequence within the Y5 gene. The transcription of both

genes from opposite strands of the same DNA sequence suggests that transcriptional activation of one will have an effect on the regulation of gene expression of the other. As both Y1 and Y5 genes are implicated in the regulation of food intake, coordinate expression of their specific genes may be important in the modulation of NPY activity.

3.6 Summary

1)Both the NPY Y1 and Y5 receptor genes which are implicated in the regulation of NPY induced feeding, are situated in close proximity to each other on chromosome 4q31-q32. Indeed, the Y1 and Y5 receptor genes lie on alternate overlapping strands of the same region of the chromosome. They are likely to have evolved from a gene duplication event, with the Y5 receptor gene probably evolving as a result of a gene duplication from the Y1 receptor gene.

2)Exon 1c of the Y1 receptor gene is an integral part of the coding region of the Y5 receptor, corresponding to the third large intracellular loop of the Y5 receptor (Herzog, 1997).

3) As both Y1 and Y5 receptor genes are believed to be involved in the modulation of NPY elicited eating, coordinate regulation of the expression of these genes may play an important role in mediating this characteristic NPY activity.

4)Three positive clones were isolated from a rat genomic library following hybridisation screening with radiolabelled oligonucleotide primers homologous to the rat Y5 receptor cDNA.

5)Southern blot analysis revealed that a *Pst*I generated genomic DNA fragment contained regions of homology with the open reading frame of the rat Y5 cDNA. Subsequent PCR and sequence analysis confirmed that this genomic DNA fragment encoded a region of the rat Y5 open reading frame and that this part of the rat Y5 gene is intronless.

6)Southern blot analysis with oligonucleotide primers homologous to the 5' region of the rat Y5 open reading frame revealed that an oligonucleotide primer encoding the functional translation initiation codon hybridised to a *Pst*I generated genomic DNA fragment, suggesting that this DNA fragment may contain 5'UT region.

Chapter 4

Novel receptor cloning : Genetrapper™.

Chapter 4

4.1 Introduction

The potent orexigenic effects of NPY were first identified in 1983 (Clark, 1983). Since the initial findings of NPY induced stimulation of feeding were described, the role of NPY as a physiological mediator of ingestive behaviour has become well established. The NPY receptor subtype involved in mediating the potent augmentation in eating behaviour elicited by this neuropeptide and has also attracted widespread interest, and the field of study surrounding the feeding receptor for NPY has emerged as a rapidly evolving and highly competitive area of study. As the pharmacology of the cloned NPY receptor subtypes failed to mimic the pharmacology known to elicit NPY induced feeding, the tremendous interest associated with this peptides role in feeding was fuelled further. In 1996 an expression cloning strategy was reported to have identified the feeding receptor for NPY (Gerald, 1996). The receptor was designated Y5, and the pharmacology of the receptor expressed from this cDNA was shown to mirror that required to promote NPY-elicited eating. However, the role of the Y5 receptor as the sole mediator involved in the stimulation of feeding mediated by NPY was to prove contentious, as reports describing NPY Y1 receptor involvement in the feeding response to NPY (Kanatani, 1996, 1998) resulted in considerable controversy surrounding the receptor subtype responsible for NPY mediated stimulation of feeding. In addition, studies were reported implicating a novel, previously unidentified NPY receptor subtype as the receptor involved in mediating feeding behaviour. Thus, the precise nature of the receptor involved in mediating feeding behaviour remains unclear.

The wealth of interest intrinsic to this area of research is generated by the potential therapeutic benefits associated with the development of an antagonist to the NPY feeding receptor, as the increasing prevalence of obesity creates an enormous market for developing an effective appetite suppressant.

The hypothalamus is the brain region believed to co-ordinate and regulate signals relating to the control of nutrient and energy homeostasis. This brain region is densely innervated with NPY containing nerve fibres (Allen, 1983), and local injection of NPY elicits the most potent effects on food intake (Stanley, 1985). Thus, the receptor subtype responsible for mediating NPY induced stimulation of ingestive behaviour is likely to be located within the hypothalamus.

In an attempt to isolate novel NPY receptor family members, a novel cloning technology was employed. The Genetrapper™ cDNA positive selection system (Life Technologies) facilitates the rapid isolation of cDNA clones from DNA prepared from a cDNA library. As the desired

NPY receptor subtype is believed to be distributed within the hypothalamus, a human hypothalamic cDNA library was constructed to use in conjunction with this technology.

4.2 Experimental Strategy

The experimental rationale behind the Genetrapper™ cDNA Positive Selection system (Life Technologies Inc) is summarised in figure 4.1. A series of oligonucleotides homologous to individual NPY receptor gene family members, and one degenerate oligonucleotide selected from a conserved region within two NPY receptor genes were designed in order to isolate novel NPY receptor family members using the Genetrapper™ methodology described.

4.3 Chapter specific methods

Unless otherwise stated, the materials used in the experiments described below were supplied by the manufacturer (Life Technologies Inc).

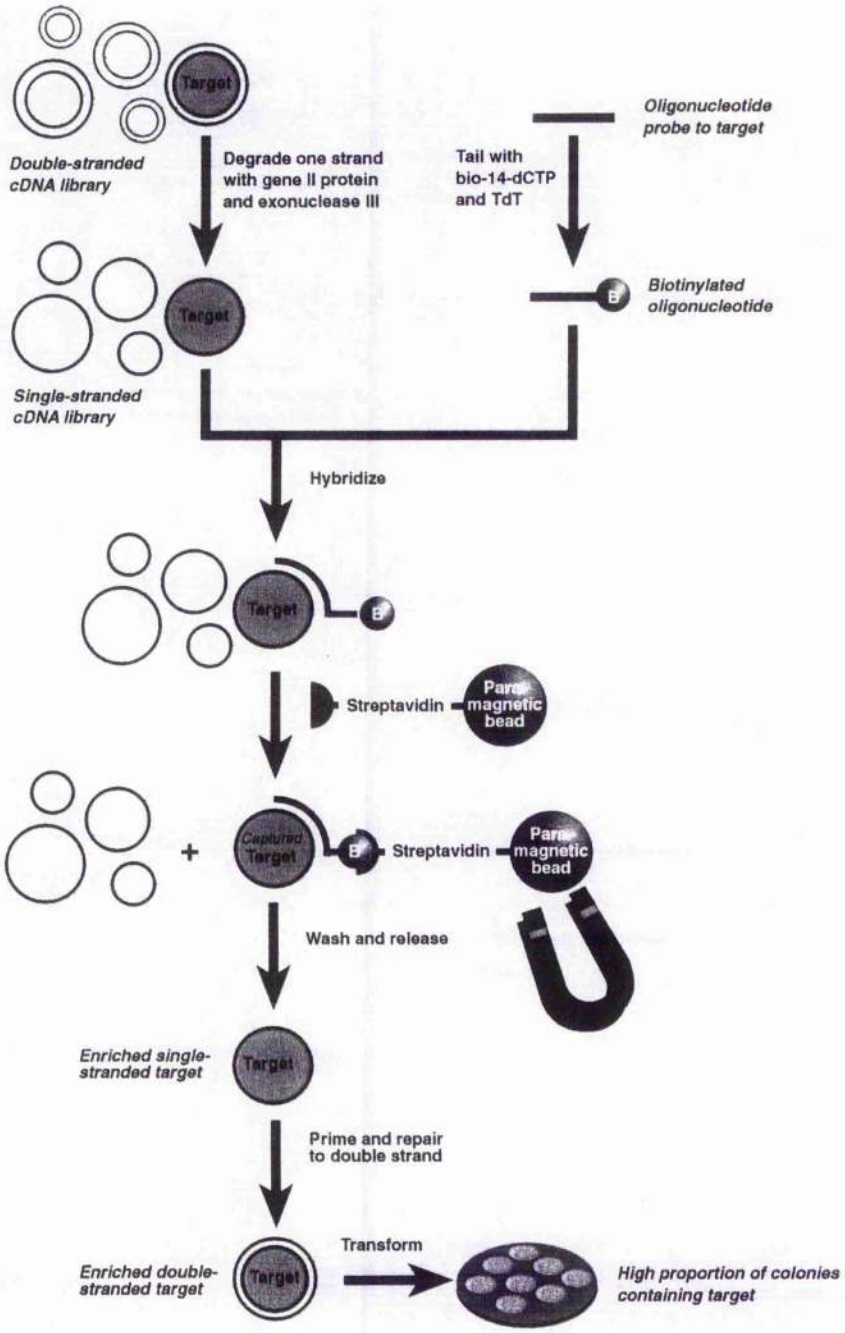
4.3.1 Human hypothalamic cDNA library construction.

The cDNA library used in the experiments described in this chapter was constructed from human hypothalamus in the plasmid based mammalian expression vector pSPORT 1 (Life Technologies Inc). Briefly, five micrograms of human hypothalamic Poly A^+ RNA (Clontech) were used as template for cDNA synthesis using a kit (SUPERSCRIPT Plasmid System, Life Technologies Inc). Fragments were size selected over 1Kb and directionally cloned into *SalI*/*NotI* sites of pSPORT 1. A detailed description of the cDNA library construction is described in the following sections.

4.3.1.1 First strand synthesis.

5 micrograms of human hypothalamic PolyA^+ RNA were added to *Not*1 primer adaptor and denatured for 60 seconds at 94 °C before chilling rapidly on ice. To the mRNA and adaptor primer mix, 5 X first strand buffer (supplied), 0.1M DTT (supplied) and 10mM dNTP mix (supplied) were added before incubating at 45°C for 2 minutes. Following incubation, 5µl (250 units) Superscript II Reverse Transcriptase were added to the mixture and the reverse transcriptase reaction allowed to proceed at 45°C for 1 hour.

Figure 4.1. Schematic of experimental strategy depicting the Genetrapper™ technology.


Target
Double-stranded cDNA library
Degrade one strand with gene II protein and exonuclease III
Oligonucleotide probe to target
Tail with bio-14-dCTP and TdT
B
Biotinylated oligonucleotide
Target
Single-stranded cDNA library
Hybridize
Target
B
Streptavidin
Para magnetic bead
+
Captured Target
B
Streptavidin
Para magnetic bead
Wash and release
Enriched single-stranded target
Target
Prime and repair to double strand
Transform
Enriched double-stranded target
Target
High proportion of colonies containing target

4.3.1.2 Second strand synthesis.

The following components were added on ice in the order shown to the first strand reaction; 93µl DEPC treated water, 30µl 5 X second strand buffer (supplied), 3µl 10mM dNTP mix, 10 units *E.coli* DNA ligase, 40 units *E.coli* DNA polymerase I and 2 units *E.coli* RNAse H. The total reaction volume of 150µl was vortexed to mix and incubated at 16°C for 2 hours before adding 10 units T4 DNA polymerase and incubating at 16°C for a further 5 minutes. The cDNA was precipitated as described below in section 4.3.1.3.

4.3.1.3 cDNA precipitation.

To the reaction mixture prepared above in section 4.3.1.2, 10µl 0.5M EDTA and 150µl phenol:chloroform:isoamyl alcohol (25:24:1) were added prior to vortexing and centrifuging at room temperature for 5 minutes at 14000 X g to separate the phases.
The upper, aqueous layer was removed, transferred to a fresh eppendorf tube and the cDNA precipitated by adding 70µl of 7.5M NH_4OAc, 500µl absolute ethanol (-20°C), and centrifuging at room temperature for 20 minutes at 14,000 X g.
Following careful removal of the supernatant the pellet was overlaid with 70% ethanol (-20°C) and subjected to centrifugation for 2 minutes at 14,000 X g. The cDNA was then dried at 37 °C for 10 minutes to evaporate residual ethanol.

4.3.1.4 *Sal I* adaptor ligation.

The following reagents were added on ice, in the order shown to the cDNA prepared as described in sections 4.3.1.1-4.3.1.4 ; 25µl DEPC treated water, 5 X T4 DNA ligase buffer, 20µg *Sal* I adaptors and 5 units T4 DNA ligase. The reagents were mixed gently and incubated at 16°C for a minimum of 16 hours (usually overnight). The cDNA was then extracted and precipitated as described in section 4.3.1.3.

4.3.1.5 *Not I* digestion.

The following reagents were added on ice, in the order shown to the cDNA from the *Sal* I adaptor addition described in section 4.3.1.4; 41µl DEPC treated water, 5µl REact 3 buffer and 4.8 units *Not I* . The reaction was allowed to proceed at 37°C for 2 hours before adding 50µl of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuging at room temperature for 5 minutes at 14,000 X g to separate the phases. The upper aqueous layer was removed and run through a CHROMOSPIN TE 1000 column (Clontech). The cDNA was then precipitated with

7M NH_4OAc, absolute ethanol and glycogen (20µg/µl). The resulting cDNA pellet was washed with 70% ethanol and dried at 37°C for 5 minutes before resuspending in 10µl water.

4.3.1.6 Size selection of the cDNA inserts.

In order to effectively construct a cDNA library containing an average insert size above 1kb, the human hypothalamic cDNA prepared as described in sections 4.3.1.1-4.3.1.5 was electrophoresed in an agarose gel containing ethidium bromide (10mg/ml) at 100 Volts for 1 hour. Human hypothalamic cDNA was visualised using ultraviolet illumination at 300-360nm to minimise damage to the DNA (figure 4.2). The cDNA was then excised from the gel in a size selection procedure whereby only cDNA above 1Kb was selected. This procedure facilitates the cloning of large inserts. After weighing the agarose sample, 100µl buffer saturated phenol was added per µg of agarose, the sample vortexed for 1 minute and then dropped into liquid nitrogen for approximately 1 minute before centrifuging at room temperature for 15 minutes at 14,000 X g. To the lower phenol phase, 100µl distilled water was added, and the sample vortexed, dropped into liquid nitrogen and centrifuged as described above. Following centrifugation, the upper aqueous layer was removed, and pooled together with the upper aqueous layer obtained initially. An equal volume of phenol/chloroform was added to the sample, which was vortexed and centrifuged as described. Following removal of the upper aqueous layer, an equal volume of chloroform was added and the sample vortexed and centrifuged as detailed above. The upper aqueous layer containing the size selected cDNA was precipitated with absolute ethanol and 7.5M NH_4OAc. The resulting pellet was washed in 75% ethanol and resuspened in 10µl distilled water after drying.

4.3.1.7. Ligation of cDNA to the vector.

The following ligation reaction was carried out in order to clone the size selected cDNA fragments directionally into a *SalI/Not*I site in the vector pSPORT 1 (Life Technologies Inc).
5 X DNA ligase buffer, 2.5µg/ml pSPORT 1, *Not I-Sal I* cut, 10ng cDNA and DEPC treated water were mixed in a final reaction volume of 19µl. One microlitre of T4 DNA (50U/ml) ligase was added and the reaction incubated overnight at 16°C followed by a further incubation for 3 hours at room temperature .

4.3.1.8 Introduction of Ligated cDNA into *E.coli* by transformation

The ligated cDNA was purified by adding 5µl of yeast tRNA and 12.5µl 7.5M NH_4OAc to the ligation reaction described above in section 4.3.1.7. The ligated cDNA was then precipitated by adding absolute ethanol (-20°C), vortexing and centrifuging at room temperature for 20 minutes at 14,000 X g. The resulting supernate was removed and the DNA pellet overlaid with 70% ethanol before centrifuging for 2 minutes at room temperature and 14,000 X g. The ligated cDNA was then dried at 37°C for 10 minutes before resuspending in 10µl sterile water. DNA was introduced into electrotransformable cells by adding 1µl of the purified ligated cDNA to 40µl of electrotransformable ELECTROMAX DH10B cells (Life Technologies). The ligated cDNA was introduced into the cells with a Gene Pulser electroporator (Biorad) using a field strength of 1.8 k, 25µF and a pulse length of approximately 4ms. 960µl of SOC medium was added to the electroporated cells which were then incubated at 37°C for 1 hour with vigorous agitation.

Serial dilutions of 100µl of the transformed bacteria were prepared in LB medium, and then plated on to 100mm LB agar plates containing 100µg/ml ampicillin. The plates were then inverted and incubated overnight at 37°C. The resulting colonies from the serial dilutions were counted in order to assess the initial titre of the human hypothalamic cDNA library- which was calculated as containing 2 X 10^6 clones. The titre of the library was assessed by multiplying the average number of colonies by the volume of the library, and then dividing by the plating volume.

The remaining transformed cells in 1ml LB were preserved by storing in 1ml 80% (w/v) glycerol at -70°C. In order to assess the percentage of clones containing inserts, a selection of individual bacterial colonies were picked and grown overnight for isolation of plasmid DNA as described in section 2.3.2.4. Following isolation of miniprep DNA, restriction digest analysis was carried out as described in section 2.3.1.6 using the restriction endonucleases *Not*I and *Sal*I, as these restriction sites are present in the multiple cloning site of the pSPORT 1 vector and will therefore enable any insert to be generated upon digestion. Gel electrophoretic analysis was performed as described in section 2.3.1.7 and revealed that 85% of the clones contained cDNA inserts.

4.3.1.9 Semi-solid amplification of human hypothalamic cDNA library.

The human hypothalamic cDNA library was amplified using a semi-solid amplification method which prevents biasing of insert size upon amplification, and ensures that large cDNA inserts are amplified.

A solution of 2 X LB containing 0.3% Seaprep agarose (FMC Bioproducts) was prepared and ampicillin added at a final concentration of 200µg/ml. This media was prewarmed to 37°C and at least 1.5-3 X 10^6 cfu (colony forming units) of cells from the library were added. Aliquots of 25ml were pipetted into 50ml tubes and placed in ice water for between 20-60 minutes or at 4°C until the agarose set. The aliquoted media containing agarose and cDNA library was placed at room temperature for 60 minutes followed by incubation at 30°C for 48 hours. By incubating at 30°C large inserts were successfully amplified. After 48 hours, the mixture containing amplified colonies/agarose was centrifuged at 5,800 X g for 20 minutes at room temperature. Following centrifugation, the supernatant was discarded and the bacterial pellet resuspended in 100ml of 2 X LB containing 12.5% glycerol. A 100µl aliquot was removed for analysis, the remaining amplified library was aliquoted, quickly frozen on dry ice and stored at -70°C. Following semi-solid amplification the titre of the library was assessed as 7.8 X 10^{10}.

4.3.2 Oligonucleotide design.

The Genetrapper™ cDNA Positive Selection system requires oligonucleotides complementary to a segment of the target cDNA to isolate effectively homologous cDNA clones from the appropriate cDNA library. As the experimental rationale behind the experiments described in this chapter was aimed at the cloning of novel NPY receptor family members, the cDNA sequences of the known NPY receptor family members were used to design oligonucleotides for Genetrapper™. For efficient cDNA capture and repair, care was taken when designing oligonucleotides to ensure that the G + C content was between 50% to 60% and that the oligonucleotides were between 16 to 25 nucleotides in length, in order to obtain an optimal yield of desired cDNA clones, while minimising the number of background colonies. As the isolation of a full length cDNA clone is desired, oligonucleotides were designed from the 5' region of the cDNA sequences of the other NPY receptor family members. Oligonucleotides were synthesised commercially (Life Technologies Inc), reconstituted in 1 X TE buffer and checked for complementary hybridisation with the DNA databank using the *blast* or *fasta* programmes for GCG (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisc).

4.3.3 Genetrapper™ .

Figure 4.1 is a schematic which summarises the Genetrapper™ methodology described in detail in the following section.

4.3.3.1 Biotinylation of oligonucleotides

Following reconstitution of oligonucleotides in TE buffer at a final concentration of 1µg/µl, oligonucleotides were biotinylated at the 3' end using the enzyme Terminal Deoxynucleotidyl Transferase and Biotin-14-dCTP to enable efficient capture of oligonucleotide/cDNA hybrids using streptavidin coated paramagnetic beads.

The oligonucleotides were biotinylated in a reaction in which the following components were added in the order described; 5µl 5 X Terminal Deoxynucleotidyl Transferase (TdT) buffer, 3 µg oligonucleotide, 5µl Biotin -14-dCTP, autoclaved distilled water sufficient to bring the volume to 23µl and 2µl Terminal Deoxynucleotide Transferase(TdT). The reaction components were mixed by pipetting, centrifuged at room temperature for 2 seconds at 14,000 x g and incubated at 30°C for 1 hour. The biotinylated oligonucleotides were precipitated by adding 1µl of (20µg /µl) glycogen, 26µl 1M Tris-HCl (pH 7.5) and 120µl ethanol, vortexing and storing on dry ice for 10 minutes before centrifuging at 4°C for 30 minutes at 14,000 X g.
The supernatant was carefully removed and 200µl of 70% ethanol layered over the pellets, before centrifuging at 4°C for 2 minutes at 14,000 x g. The ethanol wash was removed and repeated once before drying the pellets at room temperature for 10 minutes and dissolving in 15µl TE buffer. 3µl of biotinylated oligonucleotide was removed for analysis of biotinylation reaction products.

4.3.3.2 Preparation of double stranded DNA from a human hypothalamic cDNA library.

One hundred millilitres of LB containing 100µg/ml ampicillin was inoculated with 1ml of human hypothalamic cDNA library containing 1 X 10^9 cells. The bacteria were grown to saturation overnight at 30°C with vigorous agitation. The bacteria were pelleted by centrifuging at 4,800 X g for 15 minutes at 4°C, the supernatant was discarded and the pellet resuspended in 10mls of buffer I (Appendix I) containing RNAse. 10mls of buffer II (Appendix I) were added to the resuspended bacteria, mixed by inversion and incubated at room temperature for 5 minutes. To this mixture, 10mls of cold 7.5M NH_4OAc were added, the solution mixed by inversion and the cell mixture placed on ice for 10 minutes. Following incubation on ice, the sample was centrifuged at 3,000 X g for 15 minutes at 4°C. The resulting supernatant was poured through cheesecloth into a fresh centrifuge tube, an equal

volume of ice-cold isopropanol was added, the contents of the tube were mixed well and centrifuged at 3,000 X g for 15 minutes at 4°C. Following centrifugation, the supernatant was discarded and the pellet resuspended in 500µl of buffer I and transferred to a fresh microcentrifuge tube. The solution was clarified by centrifuging at 4°C for 1 minute at 14,000 X g and transferring the supernatant to a fresh tube. The DNA containing supernatant was incubated at 37°C for 30 minutes, followed by incubation at 65°C for 5 minutes. The supernatant sample was divided into 2 equal parts and an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) added to each before vortexing to mix and centrifuging at room temperature for 5 minutes at 14,000 X g. The upper aqueous layer was removed from each sample and the phenol extraction procedure was repeated at least 3 times or until the interface between the 2 phases appeared clear. An equal volume of cold isopropanol was added to each sample, followed by centrifugation at 4°C for 15 minutes at 14,000 X g. The supernatant was discarded and 70% ethanol added to each sample, before centrifuging as above. The supernatant was carefully removed and the DNA pellets dried at room temperature and resuspended in 50µl TE buffer. The double stranded human hypothalamic cDNA was quantified (section 2.3.1.6) before subsequent procedures were carried out.

4.3.3.3 Generation of single stranded DNA with Gene II and Exo III.

As the Genetrapper™ rationale relies upon the formation of hybrids between biotinylated oligonucleotides and an appropriate cDNA library, the generation of single stranded cDNA inserts is essential.

The following reaction was prepared in order to convert the double stranded (ds) phagemid DNA to single stranded (ss) DNA. The replication initiator protein GeneII is a site specific endonuclease that binds to the f1 ori in phagemid vectors and nicks the viral strand of the supercoiled DNA. For each oligonucleotide hybridisation, the following were added to a microcentrifuge tube at room temperature: 10 X GeneII buffer, 5µg ds human hypothalamic cDNA, 12µl distilled water and 1µl GeneII. The reaction mixture was vortexed and centrifuged at room temperature for 2 seconds at 14,000 X g to collect contents before incubating at 30°C for 25 minutes. The reaction was then incubated at 65°C for 5 minutes and immediately chilled on ice for 1 minute. 1µl of the mixture was transferred to a tube containing 9µl of TE buffer and 2µl of gel loading dye for gel analysis. After nicking by Gene II, Exo III was used to digest the nicked strand from the 3' end, leaving a ss covalently-closed DNA.
To the remaining 19µl, 2µl of Exo III were added before vortexing, centrifuging at room temperature for 2 seconds at 14,000 X g and incubating at 37°C for 60 minutes. Following incubation, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and the mixture vortexed thoroughly and centrifuged at room temperature for 5 minutes at 14,000 X g to separate the phases. The upper aqueous layer (18µl) was transferred to a fresh tube and

stored at 4 °C, 1µl being transferred to a fresh tube containing 9µl TE buffer and 2µl gel loading dye for gel analysis. Before proceeding to the cDNA capture hybridisation, it was ensured that the nicked form of double stranded DNA generated by Gene II treatment was completely converted to single stranded DNA following Exo III digestion by examining the Gene II/ Exo III digested cDNA on an agarose gel (see results figure 4.5).

4.3.3.4 cDNA Capture hybridisation.

Hybrid formation between the biotinylated oligonucleotides and the ss DNA was performed as follows:
The biotinylated oligonucleotides were diluted to 10ng/µl in TE buffer before using in the capture reaction. Six microlitres of 4 X hybridisation buffer was incubated at 37°C for 2 minutes before adding the GeneII/ExoIII treated DNA and mixing by pipetting. The DNA was denatured in a 95°C water bath for 1 minute and chilled immediately on ice for 1 minute before adding 1µl of diluted biotinylated oligonucleotide (10ng), mixing and incubating at 37°C for 1 hour.

4.3.3.5 Streptavidin bead preparation.

The streptavidin-coated paramagnetic beads used to capture the hybrids were gently mixed by pipetting to ensure complete resuspension. 45µl of the bead suspension was required for each reaction and these were washed prior to use. Tubes containing the 45µl aliquots of beads were inserted into a microcentrifuge magnet and left to stand for 2 minutes, after which the supernatant was removed whilst retaining the tubes in the magnet. 100µl of TE buffer was added to the beads which were resuspended in the solution by tapping. The beads were again placed against the magnet for 2 minutes, the supernatant removed and the beads resuspended in 30µl of TE buffer.

4.3.3.6 cDNA Capture.

The hybridisation mixture was removed from 37°C, centrifuged at room temperature for 2 seconds at 14,000 X g, and the prepared paramagnetic beads added. The suspension was incubated for 30 minutes at room temperature and mixed frequently (using a Vortex Genie [Fisher] on setting 3). The tubes were then inserted into the magnet for 2 minutes to capture the magnetic beads, before discarding the supernatant. 100µl of wash buffer was added to resuspend the beads, which were placed again in the magnet for 2 minutes before removing and discarding the supernatant. This washing step was repeated again and 100µl of wash buffer was added to the beads to resuspend before transferring to a clean tube. The tubes were

then inserted into the magnet for 5 minutes, after which the supernatant was removed and discarded, and this wash step repeated again.
Elution buffer (1X) was prepared in TE buffer at pH 8.0, 20µl was added to the washed magnetic beads and mixed by pipetting. The beads were then incubated at room temperature for 5 minutes, vortexing gently at 10 second intervals, before inserting into magnet for a 5 minute period. The supernatant containing the captured cDNA clones was removed and transferred to a fresh tube. Elution from the magnetic beads was repeated by resuspending them in 15µl of TE buffer and insertion into the magnet for a further 5 minutes, following which the supernatant was pooled with the first elution supernatant.
The tube containing the combined supernatants was then inserted into the magnet for 10 minutes to remove any remaining paramagnetic beads. The supernatant (35µl) was transferred to a fresh tube and 1µl of glycogen, 18µl of 7.5M NH_4OAc and 135µl of ice-cold ethanol were added, before mixing well and storing at -20°C overnight to precipitate the captured cDNA.

The captured cDNA was precipitated by centrifuging at 4°C for 30 min at 14,000 X g, carefully removing the supernatant from the pellet and washing in 100µl of 70% ethanol before centrifuging at room temperature for 2 minutes at 14,000 X g . The ethanol was carefully removed and the pellets dried at room temperature for 10 minutes, before dissolving in 10µl of TE buffer .

4.3.3.7 Repair of captured cDNA

Before beginning the repair reaction to enrich further the targeted cDNA clone, a thermocycler was programmed for one cycle with the following parameters: 90°C denaturing step for 1 minute, 55°C annealing step for 30 seconds and 70°C extension step for 15 minutes.
DNA primer/repair mixture was prepared for each capture reaction by adding the following to a fresh microcentrifuge tube: 11µl autoclaved distilled water, 0.5µl 10mM dNTP mix, 0.5µl repair enzyme (supplied), 2µl 10 X repair buffer, 5µl captured DNA and 50ng oligonucleotide (not biotinylated). The mixture was mixed by pipetting and centrifuged at room temperature for 2 seconds at 14,000 X g. The repair mix was then placed in the pre-programmed thermocycler and the repair reaction allowed to proceed. Following the repair cycle, the mixture was centrifuged at room temperature for 2 seconds at 14,000 X g. Repaired captured DNA was precipitated by adding 1µl glycogen, 18µl 7.0M NH_4OAc, 135µl ethanol and 15µl distilled water, mixing and incubating on dry ice for 30 minutes. The repair mix was then centrifuged at 4°C for 30 minutes at 14,000 X g. Following centrifugation, the supernatant was removed and 100µl 70% ethanol added to wash the pellet, before centrifuging for a further 15 minutes at 4°C and 14,000 X g. The pellet was air dried and dissolved in 10µl TE buffer.

4.3.3.8 Electroporation of the captured DNA.

2µl of the captured DNA was added to 40µl of electrotransformable ELECTROMAX DH10B cells (Life Technologies Inc). The captured DNA was introduced into the cells using a Gene Pulser (Biorad) using a field strength of 2.5 kV and a pulse length of approximately 4ms. Following electroporation, 960µl of SOC medium was added to the electroporated cells which were subsequently incubated at 37°C for 1 hour with vigorous agitation.
The transformed electroporated captured DNA was plated onto 22cm x 22cm LB agar bioassay plates containing 100µg/ml ampicillin. The plates were incubated, in an inverted position, overnight at 37°C.

4.3.3.9 Transfer of selected library to filters.

The colonies produced following the electroporation of the DH10B cells with the selected library were picked using a robot (Biopicker, Biorobotics Ltd) and grown up in 96-well microtitre plates in medium suitable for storage at -80°C (freezing medium). A gridding robot (Biogridder, Biorobotics Ltd) was used to grid samples of the clones onto Hybond-N nylon membrane (Amersham) in a known array. After growing up overnight at 30°C on the membrane, the clones were alkaline denatured in a solution containing 0.5M NaOH/1.5M NaCl for 7 minutes, neutralised in a solution containing 1.5M NaCl/1M Tris pH 8.0 for 3 minutes and washed in 2 X SSC for 2 minutes. The DNA was then UV cross-linked to the filter (UV Stratalinker 2400).

4.3.4 Screening for novel NPY receptor family members.

Replica filters of the selected library were screened for various members of the NPY receptor family in an attempt to isolate novel family members. Oligonucleotide probes were radiolabelled with γ-^{32}P ATP as described in section 3.3.2.5 and DNA probes labelled with α-^{32}P-dCTP as described in section 2.3.3.2.
Hybridisation was carried out as described in section 3.3.3.4 at 50°C in ExpressHyb prehybridisation/hybridisation solution (Clontech). Filters were then washed at low stringency (6 X SSC/0.1%SDS, 2 X 20 minutes, room temperature) followed by a high stringency wash (6 X SSC/0.1%SDS, 2 X 20 minutes, 50°C). Following autoradiography as described in section 2.3.3.1, positive clones were picked from the original 96 well microtitre plates, DNA isolated as described in section 2.3.2.4 and the sequence determined.

4.4 Results

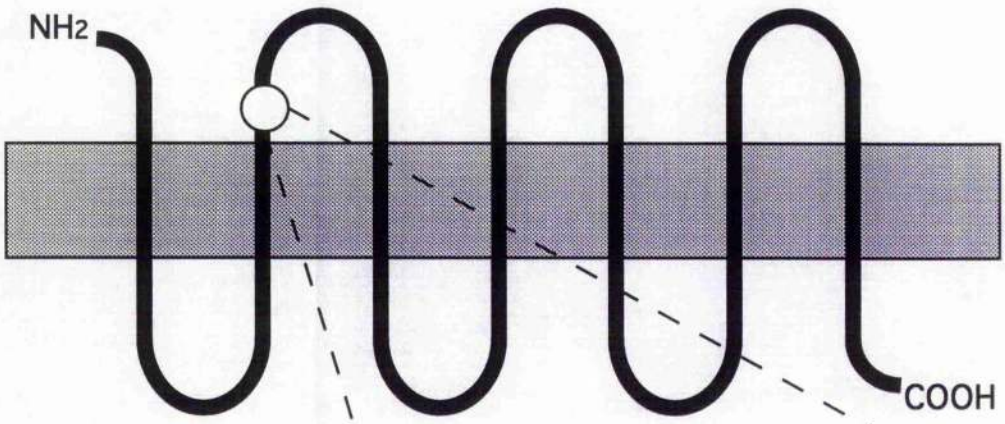
4.4.1 Oligonucleotide design.

In an attempt to isolate novel NPY receptor family members, in particular novel receptor subtypes involved in the feeding response to NPY, the nucleotide sequences encoding the cDNA's of the cloned NPY receptor genes were aligned to facilitate design of a degenerate oligonucleotide based upon the known sequence of these receptors. However, as discussed in Chapter 2, the members of the NPY receptor gene family are particularly divergent (Larhammar, 1996) and low percentage sequence identity is exhibited between receptor subtypes. As a result a degenerate oligonucleotide could not be designed based upon the sequences of the cloned human NPY receptors (Y1, Y2, Y4, Y5 and y6), as no readily identifiable region with reasonable degeneracy (<1000) could be identified despite lengthy sequence analysis. The degeneracy value is of tremendous importance, as an oligonucleotide degeneracy greater than 1000 will result in a high background signal upon applying the Genetrapper™ technology. As the receptor subtype involved in mediating the feeding response to NPY is believed to be encoded by a rarely expressed mRNA (section 1.7), the presence of numerous clones with sequence identity unrelated to NPY receptor subtypes, will significantly impinge upon the ability to detect any novel NPY receptor sequences.

Due to the diversity of the NPY receptor gene family, as detailed above and in chapter 2, only one degenerate oligonucleotide was designed and this was based on the nucleotide sequences of the human Y1 and y6 receptors. Figure 4.2 is a diagrammatic representation of the degenerate oligonucleotide design.

The remaining oligonucleotides used in the Genetrapper™ procedure were a series of individual oligonucleotides homologous to the Y2, Y4 and Y5 NPY receptor subtypes

Figure 4.2 Schematic of rational design of human Y1/y6 receptor degenerate oligonucleotide.



Human NPY Y1 Receptor AATGTTACCAACATCCTGATTGTGAAC
Human NPY y6 Receptor AATTTCACCAGCATACTGATTGCCAAT

Degenerate Y1/y6 Receptor Oligonucleotide

TTACCAACATCCTGATTGTG
C G A CC

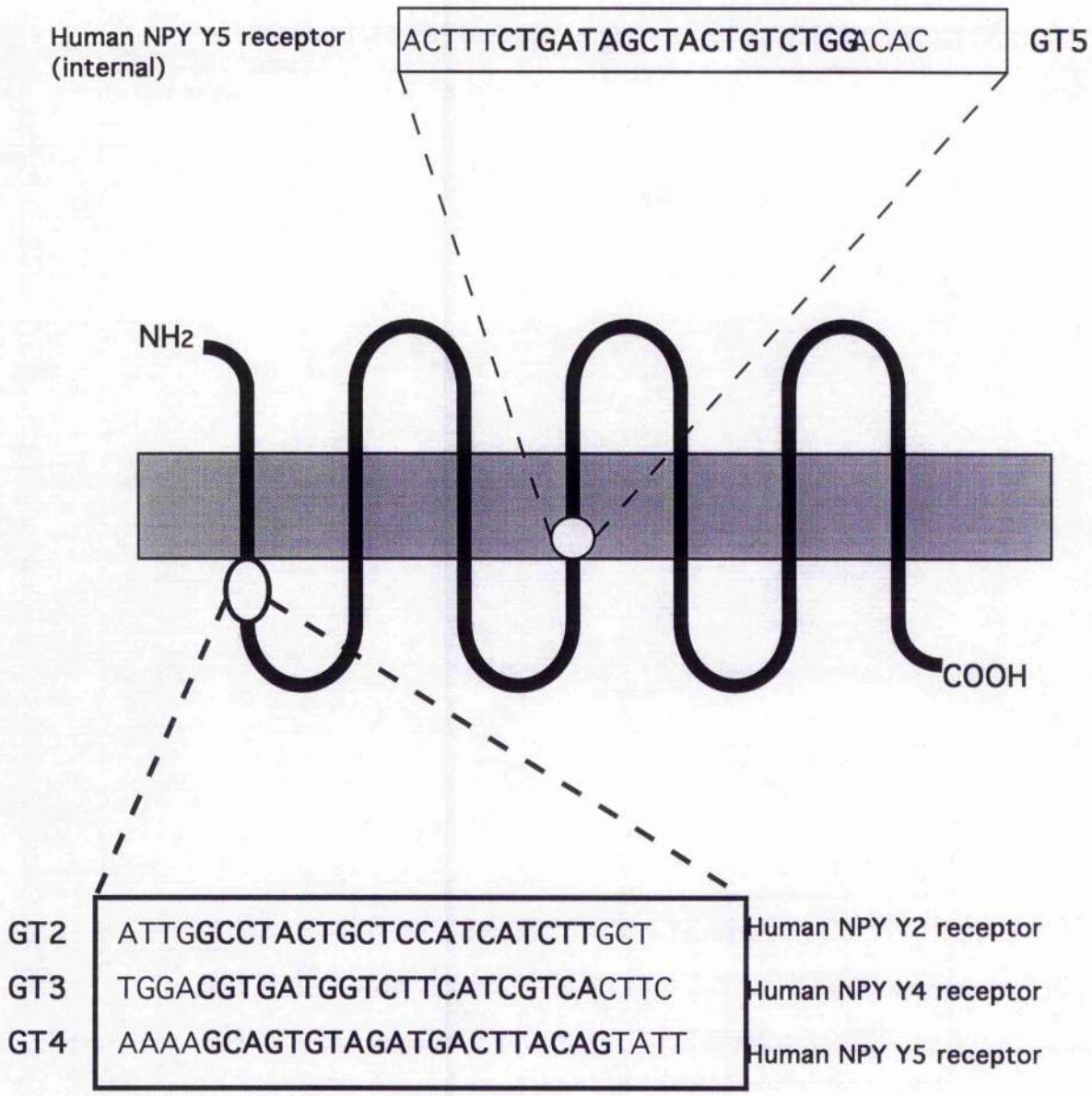
GT1

Degeneracy	2	x2	x2	x2x2

Total degeneracy = 32

Figure 4.3 Schematic of Genetrapper™ oligonucleotide design.

Characters in bold denote the nucleotide sequence of each oligonucleotide primer.
GT2= human Y2 receptor homologous oligonucleotide
GT3= human Y4 receptor homologous oligonucleotide
GT4= human Y5 receptor homologous oligonucleotide
GT5= human Y5 receptor homologous oligonucleotide (internal)


Human NPY Y5 receptor
(internal)
ACTTTCTGATAGCTACTGTCTGGACAC
GT5
NH2
COOH
GT2
ATTGGCCTACTGCTCCATCATCTTGCT
Human NPY Y2 receptor
GT3
TGGACGTGATGGTCTTCATCGTCACTTC
Human NPY Y4 receptor
GT4
AAAAGCAGTGTAGATGACTTACAGTATT
Human NPY Y5 receptor

as outlined in figure 4.3. The sequences of the oligonucleotides used in the Gentrapper™ experiments can be found in Appendix II. A number of laboratories have reported difficulty cloning the 5' end of the cDNA for the various NPY receptors. Therefore, a further primer GT5 was designed that lies internal to the open reading frame of the NPY Y5 receptor. This primer was intended to be used as a control for the Genetrapper™ approach.

4.4.2 Analysis of the oligonucleotide biotinylation reaction products.

3µl of formamide stop buffer was added to the 3µl of biotinylated oligonucleotide prior to vortexing and centrifuging at room temperature for 2 seconds at 14,000 x g. Dilutions of unbiotinylated oligonucleotides were prepared to run as standards in order to determine the concentration of biotinylated oligonucleotide. As each oligonucleotide was reconstituted at a concentration of 1µg/µl, a series of standard dilutions of non-biotinylated oligonucleotides were prepared at concentrations of 500ng, 200ng and 50ng. 5µl of each oligonucleotide dilution was then mixed with 5µl of formamide stop buffer, vortexed and centrifuged at room temperature for 2 seconds at 14,000 x g.

The biotinylated oligonucleotides and standard dilutions were loaded on to a 15% TBE/Urea gel (Novex) and gel electrophoresis was carried out for 1.5 hours at 180 volts in 1xTBE buffer, before staining with ethidium bromide (10mg/ml) to visualise the oligonucleotide DNA and photographing with ultraviolet illumination at 254nm.
Figure 4 .4 illustrates analysis of the oligonucleotide biotinylation reaction following gel electrophoresis.
Following gel analysis, the concentration of the biotinylated oligonucleotides was determined as 16.5ng/µl for oligonucleotides GT3, GT4 and GT5, and 7ng/µl for GT1 and GT2.

4.4.3 Analysis of GeneII and ExoIII digestion products

To determine whether the dsDNA had been successfully converted to ss DNA gel electrophoretic analysis was carried out. The DNA digested with Gene II or GeneII-Exo III was electrophoresed for 1.5 hours at 100 Volts in a 0.8% agarose gel prepared in 1x TBE. A sample of the original ds human hypothalamic DNA was electrophoresed alongside the GeneII and ExoIII treated samples.

The Gene II and GeneII-Exo III treated DNA samples were compared to the undigested double stranded phagemid DNA (figure 4.5).
From the gel analysis of the GeneII and ExoIII digested DNA illustrated in figure 4.5, the double stranded human hypothalamic cDNA appears to have been converted to

Figure 4.4 Gel analysis of Genetrapper™ oligonucleotide biotinylation reaction products.

Biotinylated oligonucleotides were electrophoresed on an acrylamide gel as described (section 4.4.2). DNA was visualised under ultraviolet irradiation and photograph exposure time was 0.5-1.0 seconds.

Lane 1: Biotinylated oligonucleotide GT3
Lane 2: Biotinylated oligonucleotide GT4
Lane 3: 500ng non-biotinylated oligonucleotide GT4
Lane 4: 200ng non-biotinylated oligonucleotide GT4
Lane 5: Biotinylated oligonucleotide GT5
Lane 6: 50ng non-biotinylated oligonucleotide GT5

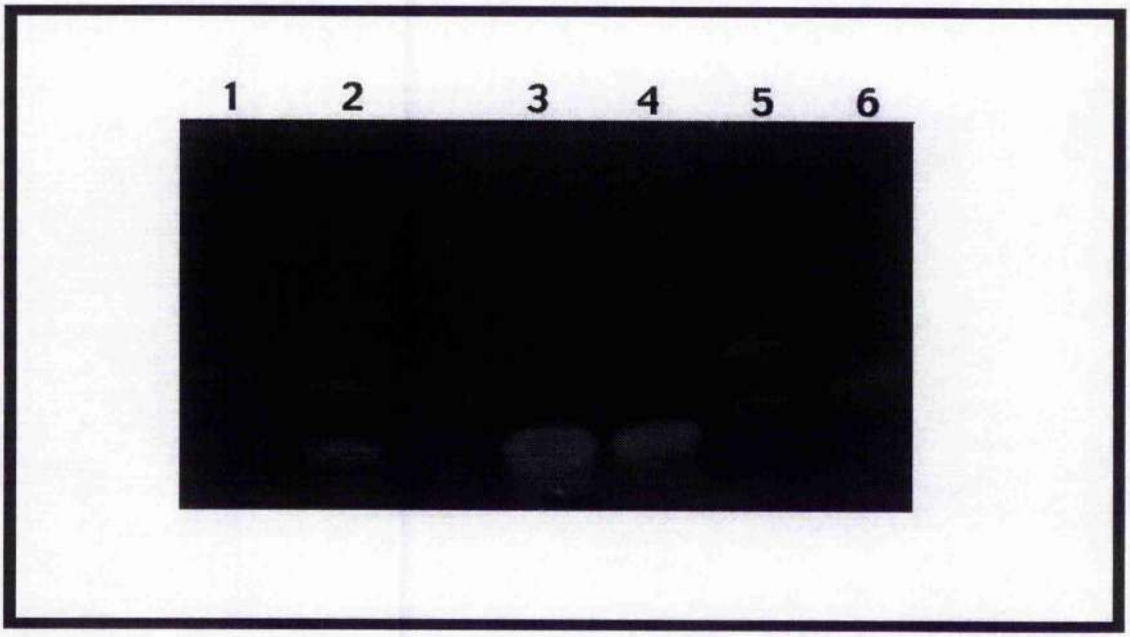
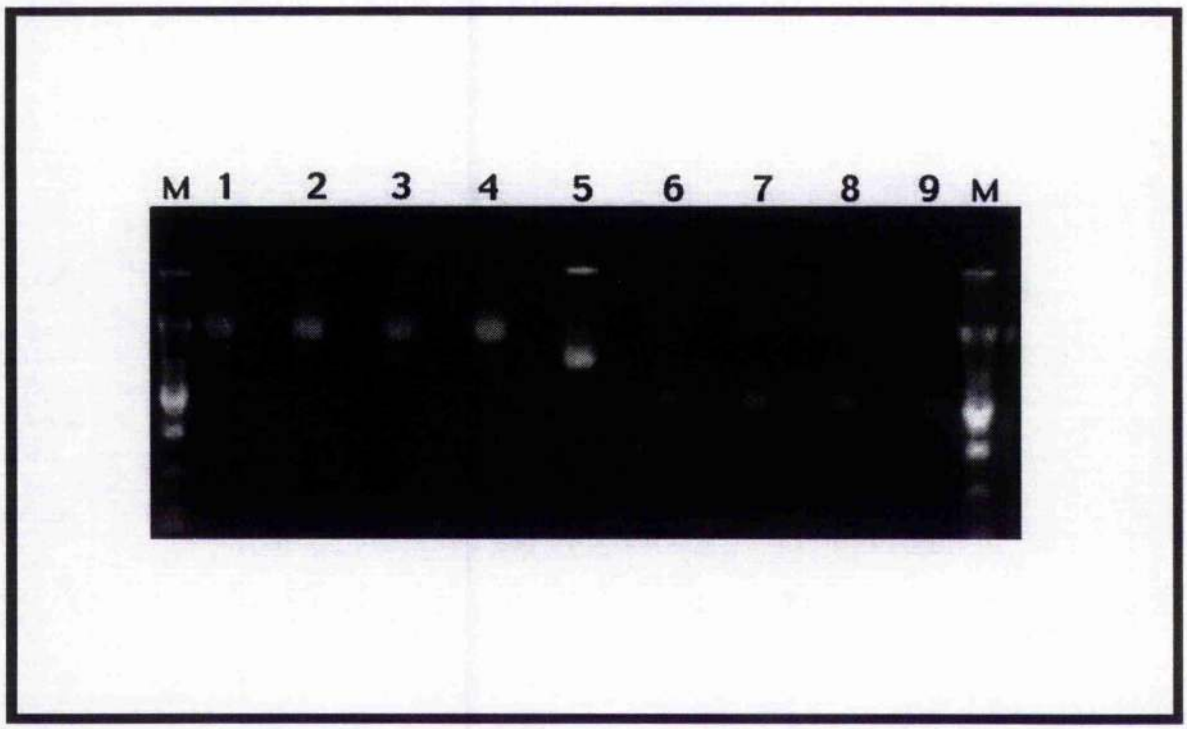

1
2
3
4
5
6

Figure 4.5 Gel analysis of GeneII and ExoIII digestion products.

After digestion with GeneII or GeneII-Exonuclease III, dsDNA was electrophoresed on an agarose gel as described (section 4.3.3). DNA was visualised under ultraviolet irradiation and photograph exposure time was 0.5-1.0 seconds.

Lanes 1-4: 500ng GeneII-treated human hypothalamic cDNA library.
Lane 5: 500ng undigested human hypothalamic cDNA library.
Lanes 6-9: 500ng Gene II-Exonuclease II treated human hypothalamic cDNA library.

Restriction digested DNA markers (M) were electrophoresed alongside reaction products. 1Kb ladder (Life Technologies).


M 1 2 3 4 5 6 7 8 9 M

single stranded DNA. After digestion with GeneII more than 50% of the supercoiled DNA has been nicked by the enzyme, and therefore migrates as open circle DNA, appearing as a slowly migrating band on the gel. Following digestion with ExoIII, the nicked DNA generated by GeneII has been completely converted to single stranded DNA, which migrates more quickly than the supercoiled DNA of the undigested sample.

4.4.4 Screening of the selected library for NPY family members.

The biotinylated oligonucleotides designed as described in section 4.1, were used in individual capture reactions as described in the Genetrapper™ methodology sections 4.3.3.
Replica filters of the selected library were screened, with the corresponding oligonucleotides used in the selection process, for members of the NPY receptor family and potential novel family members as described in section 4.3.4.
The number of colonies obtained following the electroporation of each oligonucleotide selected library and the positive colonies detected following hybridisation are shown in Table 4.1.

Oligonucleotide	Number of colonies obtained/screened	Number of positive clones detected
GT1 (Y1/y6 degenerate)	1152	-
GT2 (Y2 homologous)	2106	-
GT3(Y4 homologous)	4608	7
GT4 (Y5 homologous)	2592	2
GT5 (Y5 homologous internal)	4896	2

The positive clones identified by hybridisation screening of the replica filters using the appropriate radiolabelled oligonucleotide were isolated from the corresponding 96 well microtitre plate, grown overnight in deep well 96 well microtitre plates containing agar, and plasmid DNA isolated (as described in section 2.3.2.4.) for sequence analysis.

4.4.5 Sequence analysis of positive clones.

The DNA prepared from the positive clones obtained following screening of the replica filters was sequenced using an ABI automated sequencer. Bioinformatic analysis was carried out using the *blast* programme of GCG to determine whether the clones obtained displayed sequence homology with the known NPY receptors. However, following extensive bioinformatic analysis, the sequences of the isolated positive clones failed to demonstrate

homology with any of the NPY receptor subtypes. Further sequence analysis revealed that the positively selected clones were shown to contain a region with significant homology to the oligonucleotides used in the Genetrapper™ cDNA selection procedure suggesting that the selection procedure had been appropriate.

Further analysis of the sequence of the 11 positive clones revealed a pattern of sequence identity. The positive clone sequences appeared to correspond to either human FK506-binding protein (Maki, 1990), human P-glycoprotein (Chen, 1997) or the protein encoded by the clone with accession number g1935053. Following *blast* and *bestfit* alignments of the oligonucleotide sequences against these sequences found to encode the positive clones, regions of homology were shown to exist between the oligonucleotides and sequences obtained from positive clones.
The table below shows the percentage sequence identity between oligonucleotides and positive clone sequences.

Genetrapper™ oligonucleotide	% Identity observed with FK506 binding protein	% Identity observed with P-glycoprotein	% Identity observed with clone g1935053
GT3	80	90	94
GT4	70	90	68
GT5	65	80	65

Table 4.2 depicts the percentage sequence identity observed between the Genetrapper™ oligonucleotides and sequences of the positive clones targeted using these oligonucleotides following *Bestfit* analysis using the GCG package (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisc).

No NPY receptors were identified upon executing the Genetrapper™ technique as described. A recent paper (Shepard, 1997) has suggested that, for very rare mRNAs, one round of selection is insufficient to select the cDNA but that further rounds of selection are required. As the NPY receptors are known to be very rare even within the hypothalamus, a decision was made to take an oligonucleotide forward for a further selection round. To avoid problems at the 5' end of the cDNA, the oligonucleotide GT5 captured library was selected for further screening following the protocol as described in sections 4.3. Colonies were obtained upon

electroporation of the enriched GT5 captured library and subsequently picked and transferred to filters for screening as described in section 4.3.3.9. Following hybridisation screening with a human Y5 receptor 3' UT cDNA probe (figure 6.2), twenty positive colonies were detected. These twenty positive clones remain to be subjected to sequencing and bioinformatic analysis, in order to confirm whether sequence identity exists between these clones and the human Y5 receptor. This work is currently ongoing.

4.5 Discussion

The failure to isolate human NPY receptor clones from a human hypothalamic cDNA library using one degenerate oligonucleotide designed from a conserved region of the 5' region of the NPY Y1 and y6 receptors and a series of oligonucleotides homologous to the remaining cloned NPY receptors (Y2, Y4 and Y5) can be attributed either to the low levels of expression exhibited by the NPY Y5 receptor or to the known difficulties of the 5' end of these cDNAs. The mRNA encoding this receptor protein is a particularly rare message, and the expression of this receptor protein is not abundant. This is exemplified by the initial efforts employed to clone the Y5 receptor (Gerald,1996). The Y5 receptor was eventually cloned using an expression cloning strategy to detect functional NPY receptors in a rat hypothalamic cDNA library (Gerald, 1996). Following a laborious screening procedure of an entire rat hypothalamic cDNA library, a single Y5 receptor clone was identified. Thus, 3.4 X 10^6 clones were screened in 453 pools of 7500 clones each. Only 3 positive clones were identified, and of these 2 were subsequently shown to encode the Y1 receptor, and only one was a novel sequence, Y5. This demonstrates the extremely low level of expression exhibited by this NPY receptor. As the feeding receptor for NPY is undoubtedly hypothalamic, it is probable, that like the Y5 receptor any novel NPY receptor involved in regulating the feeding response to NPY will be encoded by a rare mRNA, making expression levels of the receptor extremely low.

The Genetrapper™ technology was successful as the positive clones detected and isolated were found to possess sequence homology to the oligonucleotides used in the capture procedure. Therefore, this proves that the technique was effective, as it enabled sequences with homology to the oligonucleotides to be preferentially targeted from the library. As discussed above, the feeding receptor for NPY is likely to comprise a very rare message in the hypothalamic pool of cDNA. Therefore, cDNAs that show some homology with the oligonucleotides but are more abundant within the cDNA pool are likely to demonstrate favourable hybridisation. As the expression levels of the clones targeted by the Genetrapper™ oligonucleotides are likely to be more abundant than NPY receptor subtypes present within the hypothalamus, the isolation of these clones is therefore more favourable.

As table 4.2 illustrates, the percentage identities observed between the sequence of the positive clones and the oligonucleotides used in the Genetrapper™ selection procedure suggest that considerable homology exists between these clones and the capture oligonucleotides.

In an attempt to favour the detection of novel NPY receptors from the human hypothalamic library, an enriching process was carried out following a publication in 1997 which described optimisation of identification of rare messages using a technique similar to Genetrapper™ technology (Shepard, 1997). Depending on the relative abundance of the desired cDNA clone in the library, two or more rounds of enrichment may be necessary. By transforming the entire first round captured DNA and preparing DNA for another round of capture hybridisation, the captured library becomes enriched thereby increasing the relative abundance of the clone of interest. The oligonucleotide GT5 was used to examine this. The cDNAs captured with this oligonucleotide from the first round of selection against the whole hypothalamic library were used in this enriching process. The reason for this is that this oligonucleotide was designed with homology to the human Y5 receptor and was also located in a more 3' position in the cDNA. The Y5 receptor appears to have considerable secondary structure at the 5' end of the cDNA, as demonstrated by the PCR studies described in chapter 2 and through personal communication (Dan Larhammar, University of Uppsala), and so it may be difficult to enrich through the Genetrapper™ approach. The presence of secondary structure within the 5' region of the Y5 receptor mRNA likely results in inefficient first strand cDNA synthesis making the presence of a full length Y5 cDNA improbable. Use of an oligonucleotide homologous to a region of the cDNA outwith this region may increase the likelihood of hybrid formation between the oligonucleotide used and the desired cDNA clone.

As this enriching stage will undoubtedly increase the proportion of sequences with similarity to the capture oligonucleotide identified in the initial screening step, a cDNA probe encompassing a region of the human Y5 3'untranslated region was used to screen the enriched library as this would increase the specificity of the probe as regards the NPY receptors, and diminish the detection of false positive clones. As expected, the enrichment procedure resulted in the detection of a greater number of positive colonies following hybridisation screening, suggesting that this enriching stage has successfully increased the relative abundance of the desired clone. Sequence analysis is required in order to confirm whether the positive clones obtained bear homology to the NPY Y5 receptor.

The Genetrapper™ technology is an effective novel receptor cloning system, as demonstrated by its success in the cloning of other novel members of receptor gene families (Mark Fidock personal communication). The use of a target specific library in this technique facilitates the identification of rare mRNA clones which can be targeted in a tissue specific manner, in a homology screening approach using a degenerate oligonucleotide based on conserved sequence

from existing gene family members. As the NPY receptor gene family exhibits an uncharacteristic lack of sequence identity between members within the same species (Larhammar, 1996), a homology screening strategy proved difficult due to the lack of a readily identifiable region of sequence containing reasonable degeneracy. Significantly greater sequence homology between NPY receptor gene family members may have enabled the hypothalamic receptor believed to mediate NPY elicited feeding to be isolated more readily, as the presence of a contiguous sequence of nucleotides characteristic of NPY gene family members would provide a highly selective tool.

The Genetrapper™ technology also optimises the cloning of full length receptor cDNAs. The size selection of the cDNA library above 1Kb prevents isolation of partial clones and the design of oligonucleotides in the 5' region of the gene also preferentially favours the isolation of full length clones, which are essential for subsequent functional studies. Identification of a full length clone using this technique therefore negates the need for subsequent cloning steps necessary to obtain a full length clone.

As the Genetrapper™ technique is a cloning technique based on the formation of hybrids between oligonucleotides and target cDNA sequence in solution, non-functional pseudogenes may be isolated using this technology. In contrast to expression cloning strategies, Genetrapper™ does not confer any functional analysis, therefore, novel clones isolated using this approach may represent a gene which does not express a functional receptor protein either *in vivo* or when transfected into mammalian cells. Functional studies are therefore essential following the cloning of a novel receptor subtype using this technique, in order to determine whether the isolated novel clone will contribute to the physiology of the relevant ligand.

Other factors which contribute to the success of the Genetrapper™ technique are the design of the oligonucleotides used in the capture hybridisation and the quality of the cDNA library used. Construction of the appropriate cDNA library is a crucial factor which may determine the success of the technique. Poor first strand cDNA synthesis from appropriate mRNA will result in low quality cDNA and the subsequent inability to readily detect the desired transcript. A degenerate oligonucleotide primer designed from a conserved region of sequence from receptor gene family members is an effective means of targeting novel family members. Therefore, receptor gene families which possess high percentage identities between members are more amenable for Genetrapper™ technology.

4.6 Summary

1)Degenerate oligonucleotide design proved difficult, as the low sequence identity exhibited between members of the NPY receptor family within the same species prevented identification of a readily identifiable region of reasonable degeneracy.

2)The novel receptor cloning technique Genetrapper™ was employed in an attempt to isolate novel NPY receptors, in particular the feeding receptor, using a human hypothalamic cDNA library and a series of oligonucleotides homologous to human NPY receptor subtypes and one degenerate oligonucleotide.

3)Eleven positive clones were identified following hybridisation screening of the colonies obtained after executing the Genetrapper™ strategy.

4)Sequence analysis of the positive clones revealed no NPY receptor sequence. However, upon further analysis these clones were shown to contain significant regions of homology with the oligonucleotides used in the Genetrapper™ procedure, proving that the technique is effective.

Chapter 5

Novel receptor cloning : COS cell expression.

Chapter 5

5.1 Introduction

The dramatic effects elicited by NPY on feeding behaviour and bodyweight have generated tremendous interest regarding the receptor subtype involved in the regulation of this physiologically fundamental behaviour. To date the identity of the receptor subtype believed to mediate this characteristic effect of NPY remains highly controversial. In 1996 a novel NPY receptor subtype was cloned from rat hypothalamus (Gerald, 1996) and attributed the role of the feeding receptor, as the pattern of distribution and pharmacology of this Y5 receptor matched the proposed profile and location of the feeding receptor. Subsequent findings have proved contentious, with the NPY Y1 receptor rapidly emerging as a candidate for the role of feeding receptor (Kanatani, 1996, 1998, Ishihara, 1998). A gene knockout approach whereby both NPY Y1 and Y5 receptors were targeted, furthered the existing controversy, suggesting that both receptor subtypes may play a part in NPY induced feeding (Marsh, 1998 and Pedrazzini, 1998). The identity of this long sought after receptor remains controversial to date, and the possibility that a novel, previously unidentified NPY receptor subtype may be responsible for mediating NPY elicited eating remains to be determined (O'Shea, 1997).

As discussed in detail in chapter 2, the NPY receptor gene family, unlike many other G-protein coupled receptor families, is extremely diverse. The cloned receptors for NPY (Y1, Y2, Y4, Y5 and y6) display significant structural diversity, with low percentage identities observed between family members even in transmembrane regions (Larhammar, 1996b). The lack of homology between NPY family members makes this gene family difficult to exploit using homology screening approaches, as discussed in chapter 4. As the sequence alignments in figure 2.8, and the difficulty encountered upon attempting to design a degenerate oligonucleotide for Genetrapper™ demonstrate, the lack of sequence identity exhibited between the NPY family members makes homology screening approaches unreliable for isolating novel family members. Therefore, an alternative cloning rationale was employed in an attempt to isolate new members of the NPY gene family, in particular the feeding receptor for NPY.

The experimental strategy employed in the experiments discussed in this chapter is a functional cloning approach whereby expression of receptor protein in mammalian cells is detected in a ligand binding assay. The lack of sequence homology between NPY receptor subtypes is inconsequential in this functional cloning approach, as the rationale behind this technique is based on the affinity of the ligand (NPY) for the receptor. Therefore this type of cloning strategy circumvents the need for sequence homology, as a functional clone bearing poor homology to other gene family members will be readily detected, through its affinity for the relevant ligand. As the NPY family of receptors are characterised pharmacologically by their

ability to bind members of the PP family of peptides, a functional cloning approach would enable novel NPY receptor subtypes to be identified, following NPY or PYY binding and subsequent screening.

The rare mRNA predicted for the hypothalamic NPY feeding receptor (Gerald, 1996) may also be overcome in a functional cloning approach, as the affinity of the ligand for the receptor will enable even rarely expressed receptor proteins to be detected. As the pharmacology of the NPY feeding receptor has been previously described (Kalra, 1991, Stanley, 1992 and McLaughlin, 1991), the ligand known to potently stimulate feeding behaviour can be used in the binding assay to enable the clone of interest to be readily detected.

It is also noteworthy that expression cloning strategies were successful in the cloning of other NPY receptor subtypes. Both the NPY Y2 receptor and the NPY Y5 receptor were cloned using a functional cloning assay to identify novel NPY receptor subtypes (Rose, 1995 and Gerald, 1996).

The experimental results of two different expression cloning strategies are described in this chapter. Both cloning strategies involved overexpression of a cDNA library or DNA prepared from a cDNA library in mammalian host cells, followed by detection of cells expressing the target cDNA and the isolation of cDNA's encoding novel proteins using a functional assay. The mammalian host cells used in the experiments described are COS cells, which are frequently used host cells derived from modified African green monkey kidney epithelial cells. COS cells constitutively express the simian virus 40 (SV40) large T nuclear antigen, which permits these cells to replicate transfected plasmids containing the SV40 origin of replication to high copy number. This high replication level results in a large number of plasmids in the transfected cells which enables plasmid DNA to be effectively recovered from selected positive COS cells (Seed, 1987 and Aruffo, 1987).

5.2 Experimental strategy

The expression cloning techniques described in this chapter differ in their screening procedures following ligand binding. The rationale behind each strategy employed in this chapter is described below.

5.2.1. Panning.

The panning technique employed in an attempt to isolate novel NPY receptor gene family members, is an immunoselection procedure based on the expression of desired clones in COS cells, followed by the physical selection of expressing cells (Seed, 1987). Following ligand binding with biotinylated ligand, the cells expressing positive clones were selected using a mouse monoclonal antibody against biotin and captured with paramagnetic beads coated with an anti-mouse antibody. Plasmid DNA was extracted from the captured cells by the Hirt procedure, and the cDNA obtained was transformed into bacteria or used in a PCR reaction. Diagnostic restriction analysis and PCR permitted further analysis of the selected cDNA to be carried out. The panning strategy employed in the experiments described in this chapter is summarised in figure 5.1.

5.2.1.1 Validation of the panning methodology.

Before employing this technique as a means of identifying novel NPY receptor subtypes, experiments were carried out in order to ensure the system was effective. A known receptor was diluted with a competing plasmid before transfecting into COS cells and carrying out the panning procedure. After applying this technique the receptor used was successfully re-cloned, proving that the selection system works. The known receptor used in this procedure was the human high affinity receptor for the immunoglobulin IgG; namely the FcγRI receptor which is a Type I single membrane spanning glycoprotein (Allen and Seed, 1989). A preparation of recombinant mammalian expression plasmid CDM containing the FcγRI receptor was used to transfect COS cells before executing the panning protocol.

5.2.1.2 Validation of the panning system using a G-protein coupled receptor.

As the known receptor used to validate the system was a single membrane receptor, a known G-protein coupled receptor was applied to this technique in order to determine whether the panning system would enable a seven transmembrane domain G-protein coupled receptor to be successfully cloned.

Figure 5.1 Schematic depicting experimental strategy for COS cell panning.

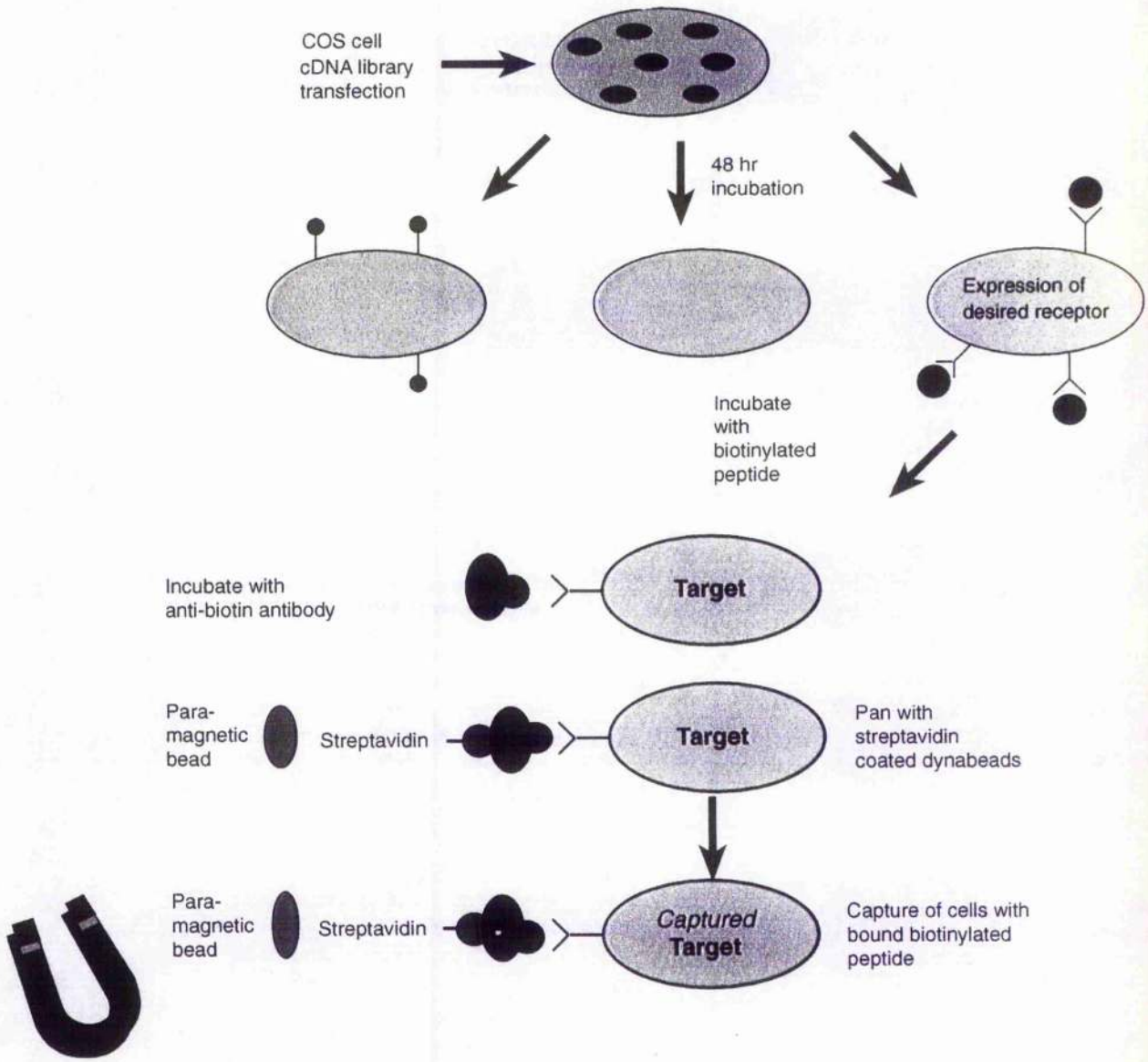
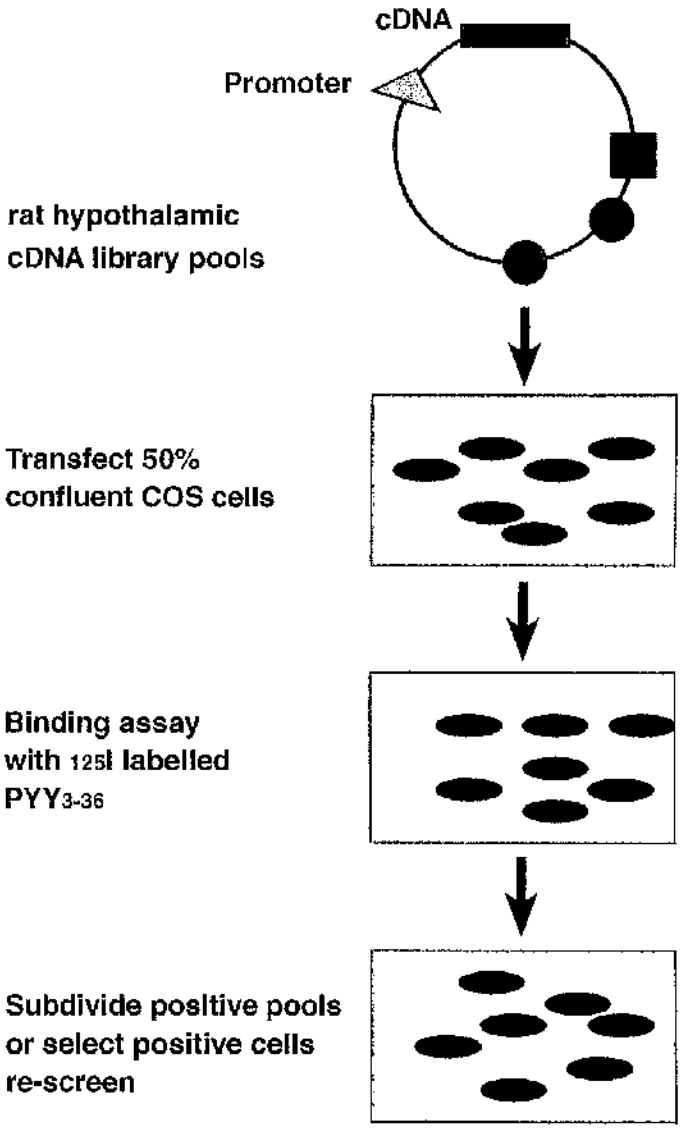

COS cell
cDNA library
transfection
48 hr
incubation
Expression of
desired receptor
Incubate
with
biotinylated
peptide
Incubate with
anti-biotin antibody
Target
Para-
magnetic
bead
Streptavidin
Target
Pan with
streptavidin
coated dynabeads
Para-
magnetic
bead
Streptavidin
Captured
Target
Capture of cells with
bound biotinylated
peptide

Figure 5.2 Schematic depicting experimental strategy for radioligand binding expression cloning.


cDNA
Promoter
rat hypothalamic
cDNA library pools
Transfect 50%
confluent COS cells
Binding assay
with 125I labelled
PYY3-36
Subdivide posltive pools
or select positive cells
re-screen

The G-protein coupled receptor used in these experiments was the rat NPY Y1 receptor, as a preparation of this receptor cDNA in the mammalian expression plasmid CDM had previously been constructed in the lab (J.C Bournat), and enabled validation of the technique using a G-protein coupled receptor.

5.2.2 Radioligand binding expression cloning

An expression cloning method where ^{125}I-labelled PYY$^{3-36}$ was used to screen transfected COS cells in a ligand binding assay was also employed in an attempt to isolate novel NPY receptor subtypes. The rationale behind this expression cloning strategy is determined by the proposed pharmacology for the NPY feeding receptor, whereby screening for the binding of a high affinity ligand at this receptor, namely ^{125}I-labelled PYY$^{3-36}$, would enable positive expressing cells to be detected. In this expression cloning approach, detection of a positive pool following initial screening, requires further subdivision and re-screening until an individual clone is identified. Figure 5.2 summarises the expression cloning strategy employed in the experiments described in this chapter.

5.3 Chapter specific methods

5.3.1 COS cell culture.

COS-7 cells were maintained on disposable polystyrene plates (Costar®) in Dulbecco's Modified Eagles Medium (DMEM, Imperial Laboratories Ltd) supplemented with 10% calf serum (Sigma, Appendix I). Cells were maintained in a Napco® Model 5410 tissue culture incubator set at 37°C and 6.5% carbon dioxide with 100% humidity. Cell manipulations were carried out under sterile conditions in Microflow Laminar Flow Cabinets (MDH Ltd.). Cells were split onto fresh plates every few days by aspirating, washing twice in 5ml 1X Phosphate buffered saline (PBS) (Appendix I) then adding 1ml of trypsin (Appendix I) to lift the cells. Cells were incubated at 37°C for 5 minutes before neutralising the trypsin by addition of DMEM supplemented with 10% calf serum and the cell suspension divided onto fresh plates (typically one plate was split on to four to six plates of the same size).

5.3.1.2 COS cell transfection.

COS-7 cells were plated at a density of approximately 4 X 10^7 cells per 100mm plate one day before transfection at a confluency of approximately 50%. NU medium (Appendix I) containing 100μM chloroquine was prewarmed to 37°C. Meanwhile 5μg (unless otherwise stated) cDNA plasmid was carefully mixed with 250μl TE buffer and 180μl 10mg/ml filter sterile DEAE-dextran in 1 X PBS. Care was taken upon addition of DNA to ensure the DNA

didn't precipitate, and in the event of this occurring, the mixture was heated to 70°C and vortexed to redissolve the DNA. Culture medium was aspirated from cells and replaced with 5ml of NU medium (100μM chloroquine prewarmed to 37°C) for each plate. The DNA was evenly added dropwise to the NU media and gently mixed. Plates were returned to the incubator for 2 to 4 hours. During this incubation large bright vacuoles appear and some cells detach and/or round up. At this time, the cells were subjected to a DMSO shock. For this the NU medium was replaced with 5ml 1 X PBS containing 10% DMSO for 2 minutes at room temperature. The cells were then washed twice in 5ml 1 X PBS and returned to their maintenance medium (DMEM supplemented with 10% calf serum). Cells were incubated overnight at 37°C/6.5% carbon dioxide. The following day the cells were subjected to trypsin (see 5.3.1), and all the cells were plated onto a fresh tissue culture dish. Experiments were carried out on one of the following two days when expression levels are maximal, i.e 48 to 72 hours post-transfection (Allen, 1989). Mock transfections, where no plasmid DNA was transfected were carried out when appropriate.

Transfection efficiency was determined by transfecting COS cells with β galactosidase (βGal) cDNA cloned in the expression vector CDM. All steps were performed similarly and the COS cells expressing βGal were revealed by incubating the glutaraldehye fixed slides in 0.2% XGal, 10mM Na phosphate, 150mM NaCl, 1mM $MgCl_2$, 3.3mM $K_4Fe(CN_6)$ trihydrate, 3.3mM $K_3Fe(CN)_6$ for 30 minutes at 37°C. The XGal solution was removed and the plates containing the cells were dried before microscopic examination. Transfected cells appeared blue, and the transfection efficiency was routinely determined as 40-50%.

5.3.2 Panning immunoselection procedure.

Forty eight hours post transfection with either rat hypothalamic cDNA or plasmid DNA in a mammalian expression vector, the panning procedure was carried out as described with the aim of identifying novel NPY receptor subtypes or confirming the effectiveness of the technique.

5.3.2.1 Panning protocol for COS cells transfected with rat hypothalamic cDNA library or NPY Y1 receptor plasmid DNA.

5.3.2.1.1 Binding assay.

The transfected COS cells were lifted from the 100mm plates with a solution of PBS/1mM EDTA. Following centrifugation for 5 minutes at 2,000 X g at 4°C, the cell culture media was removed by aspiration and the cells resuspended in 10mls of a solution containing ice-cold HEPES buffered RPMI (20mM HEPES, Imperial Laboratories Ltd) containing 1% BSA (Sigma). The cells were then subjected to a second centrifugation step as described above and resuspended in 1ml of ice-cold HEPES buffered RPMI containing 1% BSA.
To each 1ml aliquot of transfected cells 10µl of 1mM biotinylated PYY (Cambridge Research Biochemicals) was added to give a final working concentration of 10µM. The cells were then incubated with constant agitation at 4°C for 2 hours to ensure adequate contact between transfected COS cells and biotinylated peptide, thereby facilitating ligand binding.

5.3.2.1.2 Dynabead preparation.

During the incubation of biotinylated PYY to paramagnetic Dynabeads® (Dynal®) coated with sheep anti-mouse IgG1 (Fc) were prepared for use in the capture of cells expressing relevant receptor. A 300µl aliquot of Dynabeads® containing 1.2 X 10^8 beads was resuspended in 1ml of PBS/0.1% albumin and placed in a magnetic microcentrifuge holder to pellet the beads. The supernatant was removed and the beads resuspended in 1ml PBS/0.1% albumin before repeating the washing procedure. The beads were finally resuspended in 600µl of PBS/0.1% albumin.
The washed beads were then incubated with 1µl monoclonal anti-biotin (4.2µg, Sigma) in order to coat the sheep anti-mouse IgG 1 beads with the corresponding monoclonal anti-biotin antibody. The Dynabead® and antibody preparation was incubated at 4°C for 90 minutes to ensure adequate antibody binding occurred. When satisfied that the paramagnetic beads were sufficiently coated in monoclonal antibody, the beads were washed twice in PBS/0.1% albumin.

5.3.2.1.3 Capture of COS cells expressing desired receptor.

The isolation of COS cells expressing the desired receptor protein was achieved by incubating the cells bound to the biotinylated peptide with the antibody coated beads, in order to permit the targeted COS cells to be captured via the anti-biotin antibody and corresponding sheep anti-mouse IgG1 coated paramagnetic beads.

Before the cells were incubated with the antibody coated beads, a washing step was carried out to ensure unbound biotinylated peptide was removed from the cell preparation, as the presence of free biotinylated peptide would hinder the selection and capture of expressing cells. Following incubation with the biotinylated peptide, the cells were added to 10mls of ice-cold HEPES buffered RPMI containing 1% BSA and centrifuged at 4°C for 5 minutes at 3,000 rpm. The supernatant was carefully removed and the cells resuspended in 10ml of ice-cold HEPES buffered RPMI containing 1% BSA before centrifuging again at 4°C for 5 minutes at 2,000X g. The supernatant was again removed, and the cells were resuspended in 1ml of ice-cold HEPES buffered RPMI containing 1% BSA.

The cells expressing desired receptor protein were captured by adding 100µl of coated beads to the cells bound to the biotinylated peptide and incubating with constant agitation, at 4°C for 1 hour. Following incubation, the beads were repeatedly washed in ice-cold HEPES buffered RPMI containing 1% BSA, placing the mixture in a magnetic microcentrifuge holder to capture the beads, and removing the supernatant containing uncaptured cells not bound to beads through the antibody and peptide.

5.3.2.1.4 DNA extraction.

Episomal DNA was extracted using the Hirt procedure (Hirt, 1967). The captured cells were lysed by incubating in a solution of SDS (0.6%) and 10mM EDTA for 5 minutes at room temperature. The eppendorf tube was then placed in a magnetic microcentrifuge holder to capture beads, and the supernatant containing the targeted episomal DNA removed and transferred to a fresh tube. The extracted DNA was precipitated by adding 5M NaCl and incubating in an ice/water mixture at 4°C overnight.

5.3.2.1.5 Precipitation of extracted DNA.

Following overnight incubation at 4°C, the extracted DNA was centrifuged for 15 minutes at 14,000 X g and 4°C. The supernatant was removed and re-centrifuged for a further 15 minutes at 14,000 X g and 4°C before removing the supernatant and repeating the centrifugation step as described.

The supernatant was removed, and an equal volume of phenol:chloroform was added before vortexing thoroughly and centrifuging at room temperature for 5 minutes at 14,000 X g. The upper aqueous layer containing the episomal DNA was removed, and the DNA precipitated by adding 3 volumes of absolute ethanol and 40µg glycogen and storing at -20°C overnight after mixing. Following overnight incubation the DNA was pelleted by centrifuging at 4°C for 15 minutes at 14,000 X g, and the pellet washed in 70% ethanol and re-centrifuged as described. The DNA pellet was air-dried before resuspending in 100µl TE buffer.

The DNA was then amplified by chemically transforming bacteria as described in section 2.3.1.4 or used in a PCR reaction as described in section 2.3.2.2.

5.3.2.2 Panning protocol for COS cells transfected with FcγR I receptor.

5.3.2.2.1 Binding assay.

The transfected cells were removed from the 100mm cell culture dishes and centrifuged as described in section 5.3.2.1.1 As the transfected receptor is a high affinity receptor for IgG, a binding assay was carried out using biotinylated human IgG. The transfected COS cells were resuspended in 1ml of ice-cold HEPES buffered RPMI containing 1% BSA and incubated with 5µl biotinylated human IgG for 2 hours at 4°C with constant rotation to ensure binding of the biotinylated immunoglobulin and FcγR I took place.

5.3.2.2.2 Dynabead® preparation.

Paramagnetic Dynabeads® coated in streptavidin, which displays a high affinity for biotin, and therefore will enable COS cells expressing the desired receptor to be captured via the biotinylated immunoglobulin and magnetic beads. The beads were prepared as described in section 5.3.2.1.2 and resuspended in ice-cold HEPES buffered RPMI containing 1% BSA

5.3.2.2.3 Capture of COS cells expressing desired receptor.

The isolation of COS cells expressing the desired receptor protein was achieved by incubating the cells bound to the biotinylated antibody with the streptavidin coated beads, in order to permit the targeted COS cells to be captured via the biotinylated antibody and streptavidin coated magnetic beads.
Before the cells were incubated with streptavidin coated beads, a washing step was carried out to ensure unbound biotinylated antibody was removed from the cell preparation. Following incubation with the biotinylated human IgG, the cells were added to 10ml of ice-cold HEPES buffered RPMI containing 1% BSA and centrifuged at 4°C for 5 minutes at 2,000 X g. The supernatant was carefully removed and the cells resuspended in 10ml of ice-cold HEPES buffered RPMI containing 1% BSA before centrifuging again at 4°C for 5 minutes at 2,000 X g. The supernatant was again removed, and the cells were resuspended in 1ml of ice-cold HEPES buffered RPMI containing 1% BSA.

The cells expressing desired receptor protein were captured by adding 100µl of streptavidin coated beads to the cells bound to the biotinylated antibody and incubating with constant agitation, at 4°C for 1 hour. Following incubation, the beads were repeatedly washed in ice-cold HEPES buffered RPMI containing 1% BSA, placing the mixture in a magnetic microcentrifuge holder to capture the beads, and removing the supernatant containing uncaptured cells not bound to beads via the biotinylated antibody.

5.3.2.2.4 DNA extraction.

Episomal DNA was extracted as described in section 5.3.2.1.4 and precipitated for further analysis as described (5.3.1.2.5).

5.3.3. Radioligand binding expression cloning.

A rat hypothalamic cDNA library, size selected above 1Kb and directionally cloned into the mammalian expression vector pSPORT-CMV (Life Technologies Inc) was kindly supplied by Mark Fidock (Pfizer Central Research, UK.). The average insert size was calculated as 1.5Kb and the library was shown to contain 4×10^6 clones.

5.3.3.1 DNA preparation from rat hypothalamic cDNA library.

The rat hypothalamic library was plated on LB agar plates containing 100μg/ml ampicillin, in pools of 2.5 X 10^4 independent clones. Following overnight incubation at 37°C the bacteria from each pool were scraped, resuspended in 1.5ml LB medium and processed for plasmid purification by the alkaline lysis method (section 2.3.1.5).

5.3.3.2 COS cell transfection.

DNA prepared from the rat hypothalamic cDNA was transfected, in pools of 25,000 independent clones, into 50% confluent COS-7 cells by the DEAE dextran method described in section 5.3.1.2. Mock transfections where no DNA was transfected were carried out as negative controls. 48 hours post-transfection the cells were analysed in a ^{125}I-labelled PYY^{3-36} binding screening assay in an attempt to identify novel NPY receptor subtypes.

5.3.3.3 Binding assay.

48 hours post-transfection the cells were analysed in a ^{125}I-labelled PYY^{3-36} binding screening assay as described below.
Following aspiration of the maintenance medium (DMEM supplemented with 10% calf serum), the transfected cells were washed twice in an ice-cold solution of 1X PBS/1%NFM (non fat milk, Marvel), and five times in an ice-cold solution of HEPES buffered RPMI containing 1%BSA. Positive pools were identified by incubating the cells with 2nM porcine ^{125}I-labelled PYY^{3-36} (Amersham, specific activity 2000Ci/mmol) in binding buffer- HEPES buffered RPMI containing 1%BSA for 2 hours at 4°C to permit binding of the radiolabelled ligand to receptor proteins expressed on the surface of the transfected cells. Following incubation, the cells were washed three times in ice-cold binding buffer without ligand, and a further twice in a solution of 1 X PBS/1%NFM. The cells were then fixed in a solution of 4% formaldehyde for 15 minutes at room temperature, rinsed twice in 1ml of distilled water and allowed to dry overnight. The cells were then exposed to autoradiographic film (Kodak XO-Mat) for 14 days at room temperature before developing, with the intention of subdividing the corresponding DNA from any positive pools for cell transfection and re-screening.

5.4 Results

5.4.1 Validation of the panning methodology using the FcγRI receptor.

Following COS-7 cell transfection with 2μg FcγRI receptor cDNA, the panning procedure was carried out in order to target and capture transfected cells expressing the desired receptor (section 5.3.2.2). The plasmid DNA obtained following Hirt extraction was transformed into *E.Coli* (MC1061P3) as described in section 2.3.1.4 plated onto LB agar plates containing 12.5μg/ml ampicillin and 7.5μg/ml tetracycline and incubated at 37°C overnight. Colonies were obtained following transformation of panning DNA from the FcγRI transfected cells. No colonies were obtained from the mock transfected cells after applying the panning procedure. Plasmid DNA was isolated from selected colonies as described in section 2.3.1.5, and diagnostic restriction digest analysis was carried out in order to confirm that the DNA obtained corresponded to FcγRI. Miniprep DNA was digested with the restriction endonuclease *Xba* I to excise the FcγRI cDNA (as described in section 2.3.1.6). A plasmid maxiprep of FcγRI in the same mammalian expression vector CDM was digested under identical conditions in order to compare the restriction analysis pattern obtained following miniprep digestion. A 1% agarose gel was prepared and gel electrophoretic analysis carried out (2.3.1.7) on all the digested samples.

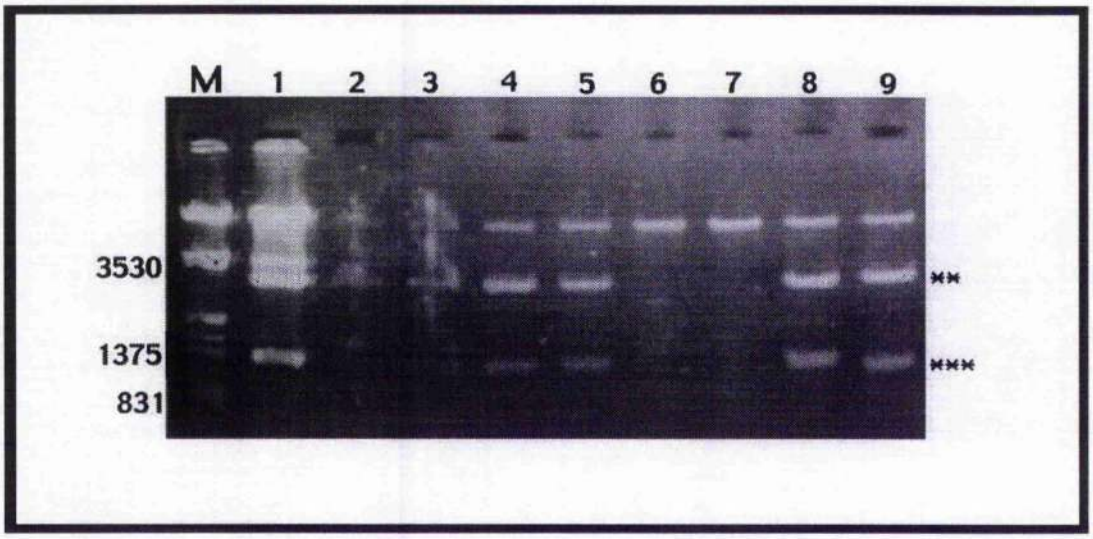
Gel electrophoretic analysis revealed that the DNA obtained upon executing the panning protocol corresponded to the FcγRI receptor, as the DNA fragments obtained upon restriction digest were the correct molecular weight predicted for the FcγRI receptor. Figure 5.3 shows the gel electrophoretic analysis of the digested minipreps and plasmid FcγRI DNA. As the results of this restriction digest analysis reveal, the panning protocol described in section 5.3.2.2 successfully allowed the transfected FcγRI DNA to be targeted and captured via the antibody and magnetic beads. This demonstrates that the panning immunoselection technique is an effective expression cloning method.

Figure 5.3 Validation of the panning methodology using the FcγR I receptor.

After performing the panning procedure described (section 5.3.2.2) plasmid DNA was isolated from selected colonies and diagnostic restriction digest analysis was carried out to confirm that the DNA obtained corresponded to the transfected FcγRI receptor.
A plasmid maxiprep of FcγRI in the same mammalian expression vector CDM was digested under identical conditions in order to compare the restriction analysis pattern obtained following miniprep digestion.
DNA was electrophoresed on an agarose gel as outlined in section 2.3.1.7 and visualised on an agarose gel under ultraviolet irradiation; photograph exposure time 0.5-1.0 seconds.

Lane 1: Plasmid maxiprep FcγRI digested with the restriction endonuclease *Xba* I
Lanes 2-9:Plasmid minipreps obtained from panning procedure digested with the restriction endonuclease *Xba* I

Molecular weight of FcγRI = 1321bp (***)
Molecular weight of CDM expression vector = 3500bp (**)
Restriction digested DNA markers (M) were electrophoresed alongside reaction products.


M
1
2
3
4
5
6
7
8
9
3530
1375
831
**
***

The panning technique was repeated in an attempt to determine the sensitivity of the technique with regards to the optimal concentration of transfected DNA required for effective recovery following panning. A series of dilutions of the FcγRI receptor cDNA were transfected into COS-7 cells as described (5.3.1.2). The dilutions were carried out by diluting 1μg FcγRI DNA with a competing plasmid (CDM). The following concentrations of DNA were transfected individually into 100mm plates of COS-7 cells : 1μg FcγRI DNA, 0.1μg FcγRI DNA, 0.01μg FcγRI DNA, 0.001μg FcγRI DNA.

The panning procedure was carried out 48 hours post-transfection as described in section 5.3.2.2. The DNA obtained following Hirt extraction was transformed as described above. The resulting colony numbers were assessed in order to determine the amount of transfected plasmid DNA required for successful amplification following panning. As expected, the colonies were most abundant following transformation with panning DNA obtained from the 1μg FcγRI DNA transfected cells. A proportional decrease in colony numbers was observed following transformation with panning DNA from cells transfected with 0.1μg FcγRI DNA and 0.01μg FcγRI DNA. No colonies were obtained following transformation with panning DNA from cells transfected with 0.001μg FcγRI DNA. A selection of colonies were picked from the plates and plasmid DNA isolated as described (2.3.1.5). Restriction digest analysis was performed as described above, and gel electrophoretic analysis confirmed that the panning DNA corresponded to the FcγR I receptor.

From the colony numbers obtained following transformation, the optimal concentration of DNA required for effective panning appears to be 0.01μg, as the presence of colonies following transformation of panning DNA from cells transfected with 0.01μg FcγRI DNA suggests that this technique is effective at targeting and capturing cells expressing low levels of receptor protein.

These results demonstrate that the panning immunoselection technique is an effective expression cloning method, which can be used effectively to re-clone cells expressing relatively low amounts of DNA.

5.4.2 Panning using COS cells transfected with rat hypothalamic cDNA library.

Cell were screened for expression of novel NPY receptor subtypes, using the panning technique described in section 5.3.2.1. COS-7 cells were transfected with 1μg of DNA prepared from a rat hypothalamic cDNA library and subjected to panning 48 hours post-transfection. Biotinylated peptides NPY, PYY and galanin were incubated with the transfected

cells, and panning was carried out to target expressing cells bound to the biotinylated peptides using the Dynabeads® as described in sections 5.3.2.1.2-5.3.2.1.5. Episomal DNA was extracted from the targeted, captured cells using the Hirt procedure as described (section 5.3.2.1.4).

The episomal DNA was transformed into *E.Coli* (TG1) as described in section 2.3.1.4, plated on agar plates containing 100μg/ml ampicillin and incubated overnight at 37°C. However following overnight incubation, no colonies were obtained. The transformation was repeated using a fresh stock of competent bacteria, however no colonies were obtained following overnight incubation. The entire experiment was repeated and panning DNA was transformed as described. However, colonies failed to be obtained following overnight incubation of transformed bacteria.

5.4.3 Panning using COS cells transfected with NPY Y1 receptor cDNA.

COS cells were transfected with 5μg NPY Y1 receptor cDNA in the mammalian expression vector CDM, and subjected to the panning protocol outlined in sections 5.3.2.1 48 hours following transfection.

The DNA extracted using the Hirt procedure as described (5.3.2.1.4), was used in a PCR reaction with oligonucleotide primers homologous to the rat NPY Y1 receptor cDNA (figure 5.4). As illustrated in figure 5.4, 2 sets of oligonucleotide primers were used, encompassing either the full length Y1 receptor cDNA, or a partial Y1 cDNA product encompassing the 3' region of the open reading frame. An identical PCR reaction was carried out using a sample of the Y1 receptor cDNA in CDM as a positive control, to ensure reaction conditions were optimal, and as a means of evaluating the PCR products obtained from the panning DNA. The PCR reaction was carried out as described in section 2.3.2.2, with an annealing temperature of 58°C and undergoing 40 cycles to ensure maximal amplification of the desired DNA.

The PCR products obtained were subjected to gel electrophoresis, and photographed under ultraviolet light to visualise the DNA. As figure 5.5 illustrates, the PCR products obtained using both sets of Y1 homologous primers on the Hirt extracted DNA, migrated to the molecular weight predicted for the Y1 receptor. The positive control PCR products obtained from the Y1 cDNA were identical to those obtained from the panning DNA, suggesting that the DNA recovered following the panning procedure corresponded to the transfected Y1 receptor cDNA. These results confirm that the panning procedure described is an effective expression cloning strategy for the isolation of G-protein coupled receptors.

Figure 5.4 Oligonucleotide primers schematic.

Schematic of cDNA sequences and the relevant oligonucleotide primers designed for PCR purposes. Hatched regions on the cDNA correspond to translated sequence, with wide bars representing oligonucleotide primers used in the PCR reaction.
FP = Forward primer
IP = Internal primer
RP = Reverse primer

Rat Y1 receptor: PCR fragments; FP & RP = ~1200bp
IP & RP = ~850bp

Rat Y1 receptor

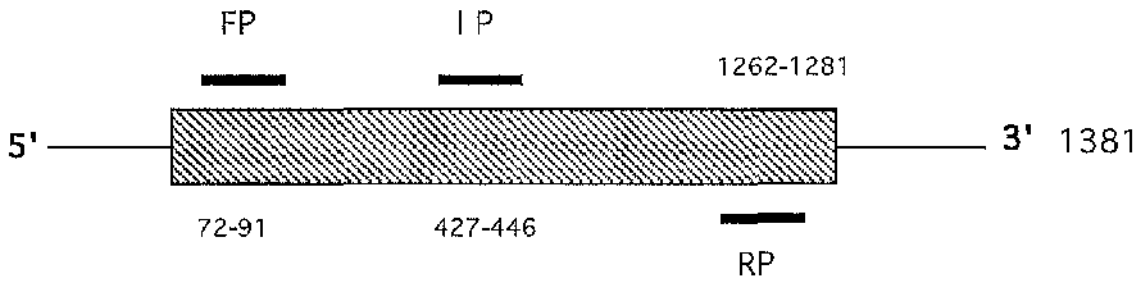


Figure 5.5 Validation of the panning system using the NPY Y1 receptor.

Paired oligonucleotide primers (see figure 5.4 and Appendix II) designed to the rat NPY Y1 receptor open reading frame were used to amplify corresponding cDNA fragments from the DNA extracted following the panning procedure. A plasmid maxiprep of the rat Y1 receptor was used as a positive control reaction, and as a means of evaluating the PCR products obtained with the panning DNA.

Aliquots of 10µl of 100µl total volume were electrophoresed on an agarose gel as described in section 2.3.1.7. DNA was visualised under ultraviolet irradiation; photograph exposure time 0.5-1.0 seconds.

Lane 1 PCR product obtained from panning DNA using Y1 homologous primers FP and RP. Product size = 1200bp (***)

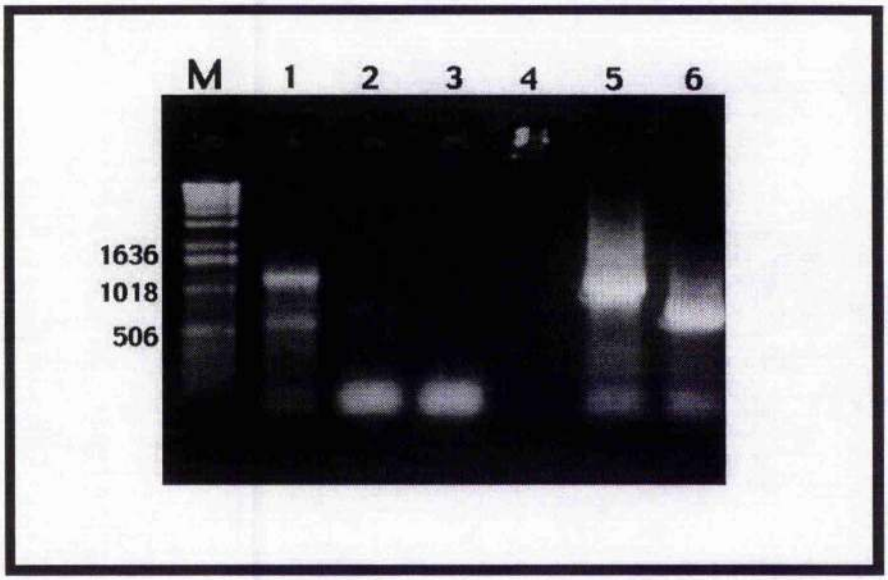
Lane 2: PCR product obtained from panning DNA using Y1 homologous primers IP and RP. Product size =800bp(**)

Lanes 3 and 4: Negative control PCR reaction containing no DNA template. No products obtained as expected.

Lane 5 : Control PCR product obtained with Y1 plasmid DNA and Y1 homologous primers FP and RP.

Lane 6: Control PCR product obtained with Y1 plasmid DNA and Y1 homologous primers IP and RP.

Restriction digested DNA markers (M) were electrophoresed alongside reaction products. 1Kb ladder (Life Technologies).


M
1
2
3
4
5
6
1636
1018
506

5.4.4 Radioligand binding expression cloning.

The radioligand binding expression cloning strategy was employed as described (sections 5.3.3) in an attempt to isolate the novel, hypothalamic feeding receptor for NPY. Screeing of COS-7 cells transfected with rat hypothalamic cDNA with radiolabelled ligand (^{125}I-labelled PYY$^{3-36}$) failed to reveal any positive pools of cDNA upon developing autoradiographic film. Half a million clones were screened in 20 pools, each containing 25,000 individual clones. The number of clones screened represented approximately one eighth of the clones present within the rat hypothalamic cDNA library. As the mRNA for this receptor is rarely expressed, levels of the mRNA encoding this receptor will not be abundant within the hypothalamic library, therefore screening of the entire library is necessary in order to optimise detection of the receptor. However, before further screening was accomplished, a paper was published in Nature in July of 1996, describing the cloning of the feeding receptor for NPY using an identical expression cloning method (Gerald, 1996). In this paper, the authors used a similar approach to isolate the Y5 NPY receptor. However, unlike the study described in this thesis, the authors screened 3.4 million clones using 453 pools of 7500 clones per pool. This is a highly labour intensive approach. Despite screening a large number of clones, only three "positive" clones were identified and subsequent sequence analysis revealed that two of these three positives were in fact the Y1 receptor. Only one clone of the 3.4 million screened was a novel receptor and is now known as the Y5 receptor. This indicates the extreme rarity of this cDNA in the hypothalamic tissue.

5.5 Discussion.

A functional cloning approach was employed in an alternative attempt to isolate a novel NPY receptor from the hypothalamus that may mediate feeding behaviour. The lack of sequence homology exhibited between NPY receptor gene family members renders homology screening potentially ineffective as an experimental rationale for novel receptor gene cloning. The predicted low abundance of the mRNA encoding the feeding receptor also makes a functional cloning approach more amenable, as a rarely expressed receptor protein can be detected following ligand binding and screening.

The COS cell panning strategy described (section 5.3.2 and figure 5.1) is an expression cloning technology which enables transiently transfected cells expressing a desired receptor protein to be targeted following binding of an appropriate ligand, and captured by screening for the ligand used. This expression cloning method enables a large number of clones to be effectively screened in one step, therefore enabling rapid screening of cDNA libraries constructed from tissues believed to possess a receptor of interest. The use of radiolabelled ligands is not necessary in this technique, as the panning strategy uses an immunoselection procedure to screen for positive cells expressing the desired receptor.

5.5.1 Validation of the panning technique using the FcγRI receptor.

Before applying the technique to screen a rat hypothalamic cDNA library for novel NPY receptor subtypes, the effectiveness of the system was validated. As the results obtained following the panning immunoselection procedure carried out on transiently transfected COS-7 cells expressing the FcγRI receptor demonstrate, the panning system is an effective means of isolating desired receptor clones from a population of cells. As the FcγRI receptor was originally cloned using a similar method (Allen, 1989), the use of this receptor enabled the steps involved in the panning system to be evaluated unequivocally.

5.5.2 Attempts to isolate novel NPY receptor subtypes using COS cell panning technology.

The hypothalamus is the brain region responsible for the regulation and co-ordination of energy and nutrient homeostasis, and is the site of action where NPY elicits powerful stimulatory effects on feeding behaviour (Stanley, 1985). The mRNA encoding this receptor is therefore likely to be found within the hypothalamus and as a result, a hypothalamic cDNA library was first constructed in an attempt to isolate the feeding receptor for NPY.

After screening COS-7 cells transfected with rat hypothalamic cDNA, and executing the panning procedure outlined in section 5.3.2, no colonies were obtained following transformation of the extracted panning DNA. This suggests that either the amount of DNA recovered following panning of cells transfected with rat hypothalamic cDNA library was minute and therefore not amplified by bacterial transformation or that no DNA was recovered after carrying out the panning technique. The expression levels of the hypothalamic feeding receptor for NPY are believed to be extremely low (Gerald, 1996), as the mRNA encoding this receptor is rarely expressed. As a result, it is increasingly likely that the quantity of DNA recovered from the COS cell panning experiment would be present in equally small quantities. Unlike a homology screening approach, where cDNA sequences unrelated to the receptor of interest may be targeted, this expression cloning technique confers specificity as regards

isolation of cells expressing the desired receptor, as the ligand used is selective for members of the NPY receptor gene family. Another factor which would prevent effective isolation of the desired cDNA clone, is the presence of secondary structure within the mRNA encoding this receptor. As demonstrated in Chapter 2, considerable difficulties were encountered when attempts were made to obtain full length Y5 receptor PCR products. The presence of secondary structure in the 5' region of the Y5 mRNA, would result in incomplete first strand cDNA synthesis during library construction and ultimately lead to the generation of a cDNA clone which would not be expressed following cell transfection. The presence of 5' secondary structure has been documented by other groups in the field (Dan Larhammar, personal communication) and can be attributed to the difficulties experienced in amplification of the cDNA by PCR technology.

5.5.3 Attempts to isolate novel NPY receptor subtypes using radioligand binding expression cloning.

As detailed above, the feeding receptor for NPY is believed to be located within the hypothalamus, and therefore a cDNA library constructed from this tissue is likely to contain the cDNA which encodes the feeding receptor. This expression cloning strategy has successfully enabled other NPY gene family members to be identified (Gerald, 1995, 1996).

Upon screening half a million clones using the radioligand binding expression cloning procedure described (section 5.3.3), no positive pool of cells expressing the feeding receptor for NPY was identified. As discussed above, the mRNA encoding this receptor does not confer abundance, and as only a proportion of the clones within the library were screened, the failure to isolate this receptor following screening with hindsight was not entirely surprising. Further screening was not carried out, as a *Nature* publication in July 1996 described the cloning of the feeding receptor for NPY (Gerald, 1996). The receptor was designated Y5, and was cloned using an expression cloning technology identical to the radioligand binding expression cloning described in this chapter. The extremely low expression levels exhibited by this receptor were clearly evident from the results of the expression cloning strategy used to clone this receptor. A rat hypothalamic cDNA library containing 3.4 X 10^6 individual clones was analysed in a ^{125}I-PYY-binding screening assay. Following screening of the entire library, a single clone corresponding to the rat NPY Y5 receptor was isolated from a positive pool identified by microscopic autoradiography (Gerald, 1996). The presence of a single Y5 clone within a library constructed from hypothalamic tissue demonstrates the incredibly low level of abundance exhibited by this receptor.

In addition to the likely rare nature of the mRNA encoding the feeding receptor an alternative explanation for the difficulty in cloning this receptor from the hypothalamus may lie in the lack

of an appropriate cofactor/accessory molecule to enhance surface expression. Recently, a family of proteins have been identified (RAMPS) which facilitate surface expression of the human calcitonin gene related peptide CGRP mRNA. RAMP is the acronym for receptor-activity-modifying-protein. These appear to be essential to transport the CGRP receptor to the plasma membrane and also modify the nature of ligand binding of the expressed receptor (McLatchie *et al* Nature, 1998).

5.5.4 Expression cloning of G-protein coupled receptors using the panning system.

As the receptor used to validate the panning system initially was a Type I single membrane spanning glycoprotein, the effectiveness of the panning technique in the expression cloning of G-protein coupled receptors remained to be established.

Validation of the panning system for use in the expression cloning of G-protein coupled receptors was carried out by transfecting COS-7 cells with NPY Y1 receptor cDNA, and carrying out the panning procedure as described (sections 5.3.2.1). The DNA extracted from the targeted cells was subjected to a PCR reaction, in order to maximise detection of the DNA obtained from the panning procedure. As the amount of DNA obtained from the panning experiments carried out with the rat hypothalamic cDNA library were extremely low, PCR technology was used in order to amplify the DNA obtained using oligonucleotide primers homologous to the Y1 receptor. As the results of the PCR reaction illustrated in figure 5.5 demonstrate, the panning technique enabled the Y1 receptor to be successfully re-cloned. Therefore, this suggests that the panning technology described in this chapter is efficient to clone G-protein coupled receptors. This technique could therefore be employed as a means of cloning novel receptors from a cDNA library using oligonucleotide primers homologous to the vector to amplify the desired DNA sequence in a PCR reaction.

The panning technology described has been demonstrated, by the results generated in this chapter to be an effective expression cloning technique. The demonstrated success of this technique in the cloning of G-protein coupled receptors, suggests that with further development this technique may prove to be an effective alternative to expression cloning which uses radiolabelled ligands to screen transfected cells. However, there are a number of reasons why panning may fail and these are impossible to predict in advance. It is an essential feature for functional expression approaches that the entire open reading frame of the mRNA is contained in the library. If the open reading frame is encoded by a long mRNA or there is marked secondary structure in the mRNA which prevents progression of the reverse transcriptase, only a partial cDNA will be synthesised and even though this is expressed, it will fail to be trafficked appropriately to the plasma membrane. In addition, if the receptor forms part of a complex which is required to be pre-formed within the Endoplasmic Reticulum, then again

even if a full length cDNA is present in the library, it cannot be expressed on the cell surface and therefore identified by ligand. However, despite the potential problems with this approach, it has been used successfully to identify a large number of receptors and when it works is a highly efficient method of obtaining a full length clone.

This technique may also have a role in validating the function of novel receptor genes cloned using a homology screening approach such as Genetrapper™. The use of the panning technology as a functional assay, would therefore permit a rapid and efficient assessment of the function of a novel receptor gene clone.

5.6 Summary

1) A functional expression cloning approach was employed in an attempt to clone the feeding receptor for NPY, as the structural diversity and lack of sequence homology exhibited by the NPY receptor gene family, make homology screening difficult.

2) A COS cell panning expression cloning technique was validated using a known receptor, in order to evaluate unequivocally that the steps involved in the panning system were effective for the isolation of desired receptor clones from a population of cells.

3) Attempts to isolate novel NPY receptor subtypes from a rat hypothalamic cDNA library using COS cell panning technology were unsuccessful. The rare mRNA encoding the hypothalamic feeding receptor for NPY, reflected in the extremely low expression levels exhibited by the hypothalamic feeding receptor for NPY, may result in the inability to detect the cDNA encoding this receptor using this technique.

4) Validation of the COS cell panning system using the NPY Y1 receptor demonstrated that the panning technology is an effective expression cloning strategy for the isolation of G-protein coupled receptors.

5) Radioligand binding expression cloning failed to reveal any positive clones. The rare nature of the mRNA encoding the feeding receptor can be attributed to the inability to detect a positive clone, as reflected by the results described in the cloning of the Y5 receptor describe din 1996 (Gerald, 1996) where the clone of interest was detected following the screening of 3.4 X 10^6 individual clones.

Chapter 6

Regulation of receptor expression: Analysis of NPY Y1 and Y5 receptor mRNA

Chapter 6

6.1 Introduction.

The regulation of nutrient balance and energy homeostasis is a complex procedure involving interactions between numerous neurotransmitters, neuropeptides and hormones known to elicit profound effects on feeding behaviour and energy expenditure. Neuropeptide Y plays an important role in the regulation of feeding behaviour, as demonstrated by the potent stimulatory effects on ingestive behaviour induced upon central administration of this peptide (Clark, 1984, Stanley, 1984). The past decade has witnessed a remarkable wealth of interest with regard to the role of NPY as a physiological mediator of food intake (Stanley, 1896 and Kalra, 1988). The paraventricular nucleus (PVN) of the hypothalamus has been attributed the site of action of this peptide (Stanley, 1985 and Li, 1994), and the receptor subtype responsible for mediating NPY elicited eating is believed to be hypothalamic (Leibowitz 1992). As discussed in chapter 1 section 1.9, NPY participates in numerous interactions with other neuropeptides, neurotransmitters, and hormones known to elicit stimulatory or inhibitory actions on feeding behaviour. The interactions between NPY and these mediators of feeding constitute the complex circuitry known to regulate this fundamental behaviour. The mechanism of action by which these interactions function is of particular interest, as by delineating the pattern of regulation, the intricate network which maintains nutrient and energy homeostasis may be further understood.

The identity of the receptor subtype responsible for mediating NPY elicited eating remains controversial to date. A hypothalamic NPY receptor designated Y5, was cloned in 1996 (Gerald, 1996), the pharmacology of which mimicked that known to stimulate NPY induced feeding. However, the role of the NPY Y5 receptor as the feeding receptor for NPY proved contentious, as increasing evidence emerged describing a role for the NPY Y1 receptor in the regulation of NPY induced feeding (Kanatani, 1996, 1998, and Ishihara, 1998). The identity of the feeding receptor for NPY remains controversial, with increasing evidence emerging in favour of both the Y1 and Y5 receptors in the control of feeding (Marsh, 1998, Pedrazzini, 1998).

As a result of the controversy surrounding the identity of the feeding receptor for NPY, the regulation of both Y1 and Y5 receptor subtypes is of particular interest, with regard to the control of feeding and interactions between NPY and other known mediators of ingestive behaviour. By examining the regulation of both the Y1 and Y5 receptors, a pattern of differential receptor regulation may be determined, thereby possibly facilitating an understanding of the role of each receptor.

The experiments described in this chapter were carried out in an attempt to examine the factors that regulate receptor mRNA levels. In order to investigate the regulation of expression of these receptor genes, mammalian cells constitutively expressing these receptor subtypes were used to examine changes in the levels of the mRNA encoding each receptor, following treatment with pharmacological agents believed to be involved in gene regulation.

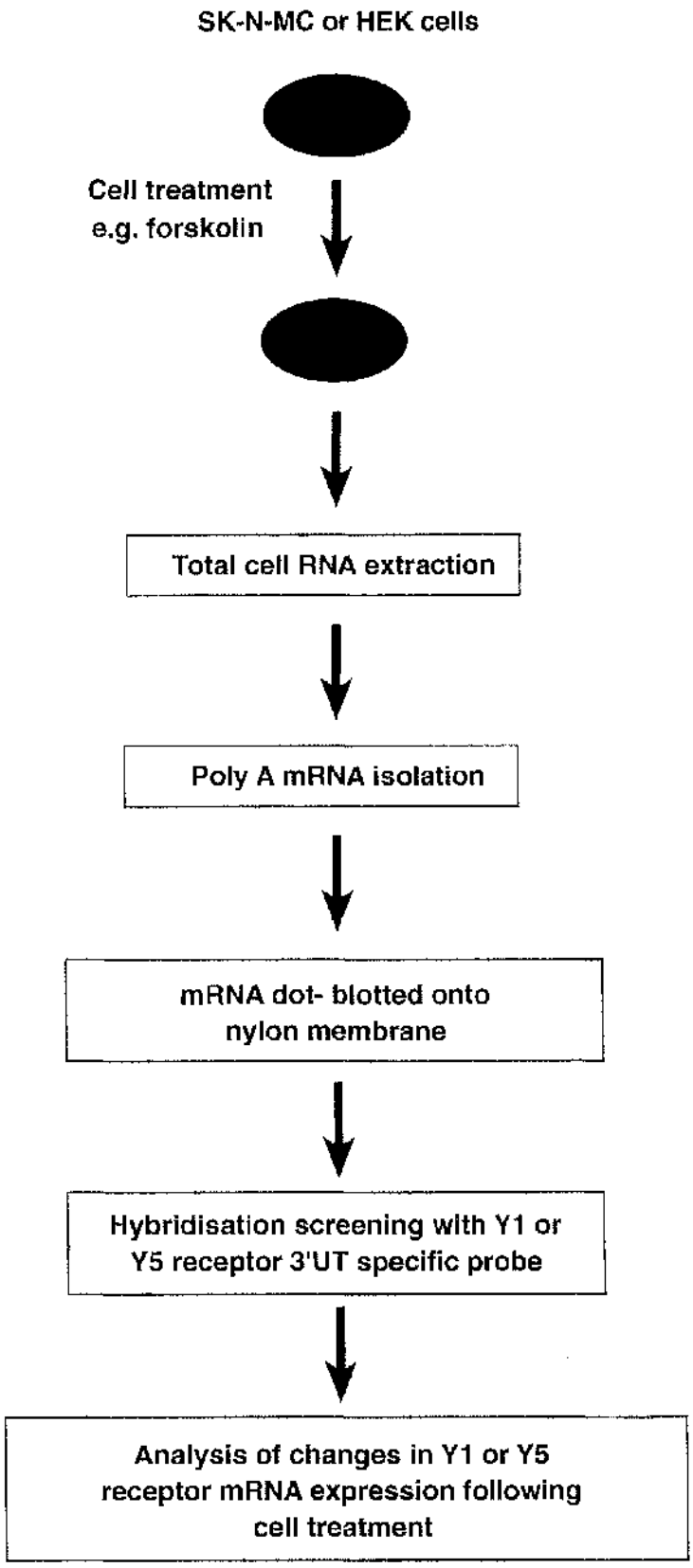
Human neuroblastoma cells, such as the SK-N-MC cell line, have been found to selectively bind Y1-receptor ligands (Sheikh, 1989 and Wahlestedt, 1991) and have subsequently been regarded as a model system for studies on the Y1 receptor. As these cells constitutively express the NPY Y1 receptor subtype, the transcriptional machinery of the Y1 receptor gene is present within the cell, and therefore changes in the levels of expression of this gene following treatment with potential regulators can be readily detected by examining the levels of the mRNA for the receptor. These cells therefore present an effective means of rapidly determining changes in receptor expression, and allow the regulation of the Y1 receptor to be investigated. To date, a cell line constitutively expressing the Y5 receptor has not been described. As the Y5 receptor has been identified peripherally in the kidney (Bischoff, 1997) and by Northern blot analysis described in chapter 2 (figure 2.12) it was reasoned that these cells may constitutively express the Y5 receptor. It is noteworthy that these cells constitutively express the leptin receptor and the galanin R2 receptor subtype (Fathi, 1998) both of which are known to be involved in regulating glucose homeostasis.

In order to investigate the regulation of Y1 and Y5 receptor expression, analysis of the mRNA encoding the receptor protein is necessary to enable transcriptional regulation and ultimately gene expression to be examined. As only between 1-5% of total cellular RNA is mRNA, the nuclear polyadenylation of mRNAs (whereby a homopolymer of 200-250 adenosine nucleotides known as the poly A tail are added to the RNA transcript) performed by eukaryotic cells provides a useful tool for the selective isolation of mRNA from total cellular RNA. Following hybridisation screening using a radiolabelled DNA probe complementary to either the Y1 or Y5 receptor cDNA, the expression levels of both receptors can be effectively examined, and a pattern of regulation determined.

6.2 Experimental Strategy.

A schematic representation of the experimental rationale employed in this chapter is illustrated in figure 6.1.

Figure 6.1 Schematic depicting regulation of receptor mRNA levels experimental rationale.


SK-N-MC or HEK cells
Cell treatment
e.g. forskolin
Total cell RNA extraction
Poly A mRNA isolation
mRNA dot- blotted onto
nylon membrane
Hybridisation screening with Y1 or
Y5 receptor 3'UT specific probe
Analysis of changes in Y1 or Y5
receptor mRNA expression following
cell treatment

6.2.1 Design of gene specific probes.

The 3'untranslated (3'UT) represents the most divergent region of the gene. Therefore the use of a 3'UT specific probe will enable Y1 and Y5 gene specific analysis of the isolated mRNA following cell treatment. Oligonucleotide primers were designed from the 3'UT region of the human Y1 and Y5 receptor genes in order to amplify by PCR technology, DNA fragments encoding a region of the 3'UT.

6.2.2 Northern analysis of SK-N-MC mRNA.

In order to confirm the presence of the human Y1 receptor mRNA in SK-N-MC cells, poly A^+ mRNA was isolated from total cell RNA, fractionated by electrophoresis through a 1% denaturing agarose gel, blotted onto nylon membrane and probed for the presence of the Y1 receptor using a Y1 specific 3'UT ^{32}P labelled probe.

6.2.3 Dot blot preparation of poly A^+ mRNA.

Following confirmation by Northern analysis that, under the hybridisation conditions defined, the Y1 specific probe only detected the Y1 mRNA transcript in poly A^+ mRNA extracted from SK-N-MC cells, further experiments were completed using dot blot analysis. Poly A^+ mRNA was extracted from SK-N-MC cells and bound to membrane using a dot blot apparatus to spot mRNA directly onto nylon membrane. This procedure was selected as it enabled rapid hybridisation analysis of poly A^+ mRNA following cell treatment.

6.2.4 Hybridisation analysis.

In order to examine changes in the level of expression of the Y1 and Y5 receptors, dot blots containing poly A^+ mRNA from treated cells were subjected to hybridisation screening with ^{32}P labelled 3'UT probes, corresponding to either the human Y1 or Y5 receptors. Changes in the level of receptor expression were observed as a change in the intensity of binding to the treated cell mRNA compared with control (untreated) cell mRNA.

6.3 Chapter specific methods.

General precautions and procedures (Blumberg, 1987) were followed to minimise RNase contamination in the laboratory. When working with solutions and equipment used for RNA analysis, gloves were worn at all times to prevent "finger nucleases" contamination. Sterile, disposable plasticware was used wherever possible and plastic disposable pipette tips and microcentrifuge tubes were autoclaved prior to use. All glassware and other bakeable

equipment, such as steel tweezers and foil, were baked at 270°C for at least eight hours to inactivate nucleases. Where possible, separate stocks were designated for RNA use only. Solutions for RNA use (with the exception of Tris-containing solutions) were treated with 0.05% diethyl pyrocarbonate (DEPC), a non-specific inhibitor of ribonuclease, and then autoclaved to destroy any remaining DEPC. Dilutions of stock chemicals were made with DEPC-treated water. Electrophoresis gel rigs, combs and bottles dedicated for RNA use were well-rinsed with DEPC-treated water, covered and stored away from other general laboratory equipment.

6.3.1. SK-N-MC cell culture.

SK-N-MC cells were maintained on disposable polystyrene plates (Costar®) in Eagles Minimum Essential Medium with Earles salts (MEM, Gibco BRL) supplemented with 10% foetal calf serum (Sigma) (Appendix I). Cells were maintained in a Napco® Model 5410 tissue culture incubator set at 37°C and 6.5% carbon dioxide with 100% humidity. Cell manipulations were carried out under sterile conditions in Microflow Laminar Flow cabinets (MDH Ltd). Cells were split onto fresh plates every few days as described in section 5.3.1.

6.3.1.2. SK-N-MC cell treatment.

SK-N-MC cells were treated with various agents for between 1-5 days before harvesting during initial experiments, and for 15 minutes, 30 minutes, 1, 2, 4 or 6 hours before harvesting in subsequent experiments. Treatments were added directly into cell medium 24 hours after cells had been replated. Forskolin (7β- Acetoxy-1α,6β, 9α-trihydroxy-8, 13-epoxy-labd-14-en-11-one, Calbiochem.), an activator of adenylyl cyclase (deSouza, 1983) was applied at a concentration of 10μM. Dexamethasone, (9α-Fluro-16α--methylprednisolone, Sigma), a synthetic glucocorticoid, was used to treat cells at a concentration of 1μM.

6.3.1.3 Cell quantitation.

Total cell counts were determined using a haemocytometer (Sigma). With the coverslip in place, a drop of cells suspended in growth medium were transferred to the haemocytometer by carefully touching the edge of the coverslip with the pipette tip and allowing the interface between the coverslip and the gridded markings on the haemocytometer to fill by capillary action. Cells were counted in the middle and four corner squares. The average number of cells per millilitre was calculated to be the average count per square multiplied by 10^4.

6.3.1.4 HEK cell culture.

HEK cells were maintained on disposable polystyrene plates (Costar®) in RPMI (Imperial Laboratories) supplemented with 10% foetal calf serum (Sigma) (Appendix I). Cells were maintained in a Napco® Model 5410 tissue culture incubator set at 37°C and 6.5% carbon dioxide with 100% humidity. Cell manipulations were carried out under sterile conditions in Microflow Laminar Flow Cabinets (MDH, Ltd.). Cells were split onto fresh plates every few days as described in section 5.3.1.

6.3.1.5 HEK cell treatment.

HEK cells were treated for two or four hours with forskolin (7β- Acetoxy-1α,6β, 9α-trihydroxy-8, 13-epoxy-labd-14-en-11-one, Sigma), an activator of adenylyl cyclase (deSouza, 1983) at a concentration of 10μM.

6.3.2 Oligonucleotide design and synthesis.

Oligonucleotide primers were designed from the 3'untranslated (3'UT) regions of the human NPY Y1 and Y5 receptors, as illustrated in figure 6.2. Each primer comprised 20 bases and was between 50-60% GC rich. These oligonucleotides were synthesised commercially by Oswel DNA Service (University of Southampton) and were dissolved in sterile water. (See Appendix II for sequences of the Y1 and Y5 3'UT oligonucleotide primers). All oligonucleotides were checked for complementary hybridisation with the DNA data bank using the *blast* or *fasta* for GCG (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisc).

6.3.3 PCR amplification of human Y1 and Y5 3'UT fragments from human genomic DNA.

The human NPY Y1 and Y5 3'UT oligonucleotide primers described in section 6.3.2 were used in a PCR reaction with human genomic DNA (section 2.3.2.2). The PCR products obtained using the thermostable *Taq* DNA polymerase were cloned into the pCR2.1 cloning vector using the TA cloning kit (Invitrogen) as described (sections 2.3.2.4 and 2.3.2.5). Plasmid DNA was isolated (2.3.2.6), and restriction digest and gel electrophoretic analysis carried out to confirm generation of recombinant plasmid DNA (section 2.3.1.6 and 2.3.1.7).

6.3.4 Extraction of Total RNA from SK-N-MC and HEK cells

Total cellular RNA was extracted and isolated using an RNeasy Midi kit (Qiagen) following manufacturer instructions as described. Cells were lysed directly in the culture dish by adding

1.9ml lysis buffer RLT (supplied) containing 10µl/ml 14.5M β-mercaptoethanol. Buffer RLT contains guanidium isothiocyanate which together with β-mercaptoethanol inactivate RNases, to ensure isolation of intact RNA. This lysis step is essential as the complete disruption of the cell walls and plasma membranes of the cells and organelles is an absolute requirement to release all the RNA contained in the sample. After 5 minutes incubation in lysis buffer, the lysed cells were collected with a rubber policeman (Costar®), and transferred to an RNase free polypropylene tube (Sarstetd). Cells were then homogenised using a Qiashredder™ (Qiagen) by applying the cell lysate to a Qiashredder unit and centrifuging for 2 minutes at room temperature and 14,000 X g. Homogenisation of the lysed cells is necessary to reduce the viscosity of the cell lysates produced by disruption, and complete homogenisation is required for efficient binding of the cell lysate to the RNeasy column used in this procedure. Following homogenisation, 1.9ml of 70% ethanol was added to the homogenised lysate to provide appropriate binding conditions, and mixed by vortexing. The sample was then applied to a RNeasy midi spin column (supplied), and centrifuged for 5 minutes at room temperature and 3,000 X g to facilitate binding of the RNA to the spin column. The flow-through obtained following centrifugation was discarded, 3.8ml wash buffer RW1 (supplied) were added to the spin column and the tube was centrifuged for 5 minutes at room temperature and 3,000 X g. The flow-through generated was discarded, and the spin column washed in 2.5ml buffer RPE (supplied) and centrifuged for 2 minutes at room temperature and 3,000 X g. A second wash in buffer RPE was carried out as described, and the spin column centrifuged for 5 minutes at room temperature and 3,000 X g. The washing steps carried out enable any contaminants to be effectively removed, while the total RNA remains bound to the RNeasy spin column. Total RNA was eluted, after transferring the RNeasy spin column to a fresh RNase free tube, adding 150µl RNase free water (supplied) directly onto the spin column and centrifuging for 3 minutes at room temperature and 3,000 X g, after standing for 1 minute prior to centrifugation. The elution step was repeated as described in a further 150µl RNase free water. Total RNA was routinely stored at -70°C.

Figure 6.2 Oligonucleotide primers schematic.

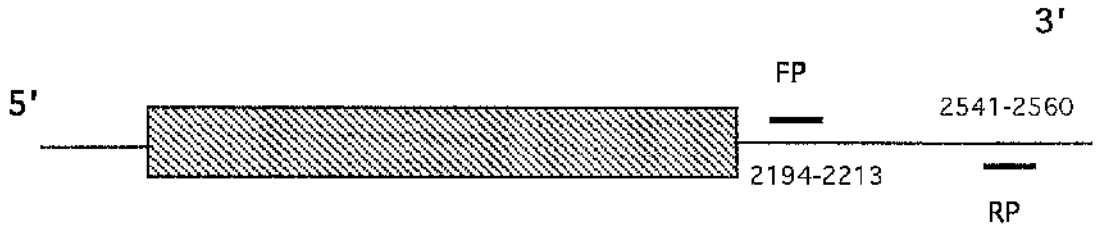
Schematic of cDNA sequences and the relevant oligonucleotide primers designed for PCR. Hatched regions on the cDNA correspond to translated sequences; wide bars represent oligonucleotide primers used for PCR.
FP = forward primer
RP = reverse primer
A: Human NPY Y1 receptor

3'UT PCR fragment; FP & RP = ~360bp

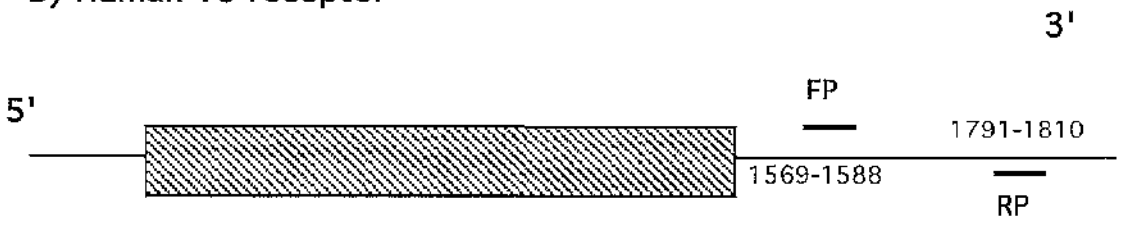
B: Human NPY Y5 receptor

3'UT PCR fragment; FP & RP = ~240bp

A) Human Y1 receptor



B) Human Y5 receptor



6.3.5 Purification of poly A^+ mRNA from total RNA extracted from SK-N-MC and HEK cells.

The total RNA isolated from the cells as described above was quantified (6.3.6) and prepared for poly A^+ mRNA isolation using a kit (Oligotex mRNA Midi kit, Qiagen). Total cellular RNA was mixed with 250µl DEPC treated water (diethyl pyrocarbonate, supplied) in an RNase free microcentrifuge tube, 500µl 2 X binding buffer (supplied) and 30µl prewarmed (37°C) Oligotex suspension (supplied) was then added. The contents of the tube were mixed by gentle flicking, and incubated at 65°C for 3 minutes to disrupt secondary structure in the RNA. Following incubation, the sample was incubated for 10 minutes at room temperature to allow hybridisation between the oligotex and the poly A tail of the mRNA. Oligotex consists of polystyrene latex particles covalently linked to dT_{30} oligonucleotides which form hydrogen bonds with the polyA tail of the mRNA, and in doing so selectively purify poly A^+ mRNA from a preparation of total RNA. The samples were then subjected to centrifugation for 2 minutes at room temperature and 14,000 X g in order to pellet the oligotex resin bound to the poly A^+ mRNA. The resulting supernatant was removed by careful aspiration and discarded.The oligotex pellet was resuspended in 400µl wash buffer QW2 (supplied) by vortexing. The resuspended pellet was then transferred to a spin column (supplied) and centrifuged for 30 seconds at room temperature and 14,000 X g, before discarding the flow through and washing in a further 400µl wash buffer QW2. The sample was subjected to further centrifugation at room temperature for 30 seconds at 14,000 X g, and the flow through discarded. To recover the poly A^+ mRNA from the oligotex particles, the spin column was transferred to a fresh RNase free microcentrifuge tube, and poly A^+ mRNA was eluted by adding 25µl preheated (70°C) elution buffer (supplied) to the spin column, resuspending the resin by pipeting several times and centrifuging as described above. The first eluate was pooled with a second elution to ensure maximal yield was obtained. The eluted poly A^+ mRNA was routinely stored at -70°C.

6.3.6 Quantification of RNA.

RNA was quantitated by spectrophotometry (DU-640B or DU-62, Beckman) at 260nm. RNA quantity was calculated, by assuming that an absorbance of 1 at 260nm corresponds to 40µg RNA per ml.

6.3.7 RNA formaldehyde gel electrophoresis.

Formaldehyde-denatured RNA was resolved on 1% agarose gels. RNA samples were denatured at 60°C for 20 minutes in 1X RNA electrophoresis buffer (Appendix I), 10% deionised formamide and 10% formaldehye and electrophoresed in gels containing 1 X RNA electrophoresis buffer, 25μg/ml ethidium bromide, 1.5% formaldehyde and 0.1M iodoacetamide. Equal amounts of denatured RNA were loaded (0.5-2μg poly A^+ mRNA, 20-40μg total RNA) in each lane. Electrophoresis was carried out at a constant 100V in 1 X RNA electrophoresis buffer, 25μg/ml ethidium bromide for 2-3 hours. After electrophoresis, RNA was visualised in the gel with ultraviolet light at 254nm to assess RNA integrity and loading precision.

6.3.8 RNA transfer to membrane.

Transfer of size separated, gel electrophoresed RNA to membrane was performed as outlined (Thomas, 1980). The blotting procedure was identical to that described for Southern transfer of DNA (section 3.3.5) except that the denaturation step was omitted and RNA handling procedures were used.

6.3.9 Dot blot preparation.

Poly A^+ mRNA was spotted directly onto nylon membrane (Hybond N, Amersham) after equilibrating the membrane and a sheet of Whatman 3MM paper in 4 X SSC solution (Appendix I) for a few minutes prior to blotter assembly. Typically 1μg poly A^+ mRNA was blotted onto the membrane following denaturation for 20 minutes at 60°C. Poly A^+ mRNA was bound to the membrane by passing 30μl poly A^+ mRNA (volume made up in 4 X SSC) through an RNA Dot Blot Manifold (BRL) connected to a vacuum pump. Care was taken to ensure the array of blotted samples was noted. Controls such as 70ng denatured Y1 3'UT and Y5 3'UT miniprep DNA (section 6.3.10) were blotted on to the same filter in a known array. After binding, the membrane was air-dried before cross-linking the RNA to the membrane by exposing the blot to ultraviolet light at 254nm for 30 seconds either side.

6.3.10 Denaturation of Y1 and Y5 receptor 3' UT miniprep DNA.

Before blotting on to nylon membrane, Y1 and Y5 receptor 3'UT miniprep DNA was denatured to enable effective hybridisation between the DNA and the probe. One microgram of Y1 3'UT or Y5 3'UT miniprep DNA was denatured by adding 2μl of a solution containing 2M NaOH/2mM EDTA and incubating at room temperature for 5 minutes. The denatured DNA

was neutralised by adding 2µl 2M ammonium acetate (pH4.5), and precipitated by adding 50µl ice-cold absolute ethanol and chilling to -70°C. Following centrifugation at 14,000 X g for 15 minutes at 4°C, the resulting pellet was rinsed in 100µl ice-cold 70% ethanol and centrifuged for a further 15 minutes at 4°C and 14,000 X g. The DNA pellet was air-dried before resuspending in 7µl water.

6.3.11 Radioactive labelling of Y1 and Y5 3'UT DNA.

The Y1 and Y5 3'UT PCR products generated as described in section 6.3.3 were radiolabelled with ^{32}P as described in section 2.3.3.2, and used in the hybridisation screening of the Northern and dot blots.

6.3.12 Hybridisation of Northern and dot blots.

Membrane was subjected to prehybridisation and hybridisation as described in section 2.3.3, using the prehybridising and hybridising solution described in section 3.3.3.4. Signal detection was carried out as described in section 2.3.3.1.

6.3.13 Rehybridisation of dot blots.

Blots were stripped of radioactive probe for rehybridisation by incubating blots in a solution of boiling 0.5% SDS (sodium dodecyl sulphate) until the solution reached room temperature. Stripped blots, were then equilibrated in 5X SSC solution for 20 minutes at room temperature, before storing in cling-film at -20°C.

6.4 Results.

6.4.1 Generation of Y1 and Y5 3'UT PCR products.

Figure 6.2 illustrates the regions of the human Y1 and Y5 receptor sequence where 3'UT oligonucleotides were designed. PCR reactions were carried out as described in section 6.3.3 using the primers designed to generate a 3'UT fragment of DNA. PCR products of the correct molecular weight were obtained from human genomic DNA (Promega) as illustrated in figure 6.3A and B. Subsequent subcloning into the vector pCR2.1 was confirmed by restriction digest analysis with the endonuclease *Eco*R 1 to excise the PCR product (data not shown). Sequence analysis of those minipreps containing the target sequence (section 2.3.1.9) revealed that the PCR products obtained were identical to the 3'UT of the human Y1 and Y5 receptors respectively.

6.4.2 Confirmation of Y1 and Y5 3'UT probe specificity

In order to confirm that both 3'UT probes were gene specific, and no cross-reactivity was observed between the Y1 3'UT probe and the Y5 3'UT miniprep DNA, or the Y5 3'UT probe and Y1 3'UT miniprep DNA, Southern blot analysis was carried out as described in section 3.3.5. Miniprep DNA prepared following transformation of Y1 and Y5 3'UT PCR products was digested with *Eco* RI before carrying out gel electrophoresis and preparing a Southern blot. Two identical Southern blots were prepared, and probed individually with either radiolabelled Y1 3'UT PCR product or radiolabelled Y5 3'UT PCR product. Following signal detection, the results were analysed, and it was concluded that each probe specifically hybridised to the digested miniprep DNA containing the 3'UT insert identical to the PCR product probe, i.e. the Y1 3'UT probe hybridised specifically to the digested miniprep DNA containing the Y1 3'UT insert, and the Y5 3'UT probe hybridised specifically to the digested miniprep DNA containing the Y5 3'UT insert. As figure 6.4 illustrates, no cross reactivity was observed, demonstrating that both probes are gene specific. These results ensured that subsequent hybridisation screening of Northern and mRNA dot blots would enable changes in the expression of the specific receptor mRNA to be detected in a receptor specific manner.

6.4.3 Detection of human NPY Y1 receptor mRNA in SK-N-MC cells.

In order to ensure that the human NPY Y1 receptor could be readily detected in poly A^+ mRNA isolated from SK-N-MC cells, Northern blot analysis was carried out. Four micrograms of poly A^+ mRNA isolated from SK-N-MC cells was run on a denaturing gel as described (section 6.3.7). Two micrograms of total RNA isolated from SK-N-MC cells was run alongside the poly A^+ mRNA as a molecular weight marker. The Northern blot constructed was probed with a ^{32}P labelled Y1 3'UT probe as described (section 2.3.3). As illustrated in figure 6.5, a discrete band corresponding to the molecular weight of the human NPY Y1 receptor transcript was detected in the poly A^+ mRNA isolated from SK-N-MC cells. The size of the single hybridising Y1 transcript in SK-N-MC cells was approximately 3.5 kb, in accordance with previous reports detecting the Y1 transcript in these cells by Northern analysis (Larhammar, 1992).

Figure 6.3 Analysis of human NPY Y1 and Y5 receptor 3'untranslated region PCR products.

A: Paired oligonucleotide primers (see figure 6.2A and Appendix II) designed to the human NPY Y1 receptor 3'UT were used to amplify corresponding cDNA fragments from human genomic DNA.
Aliquots of 10µl of 100µl total volume were electrophoresed on an agarose gel as outlined in section 2.3.1.7. DNA was visualised under ultraviolet irradiation; photograph exposure time 0.5-1.0 seconds.

Lanes 1 and 2: PCR products obtained from human genomic DNA using Y1 receptor 3'UT primers. Product size = 360bp (***)

Lane 3: Negative control PCR reaction containing no template. No product obtained as expected.

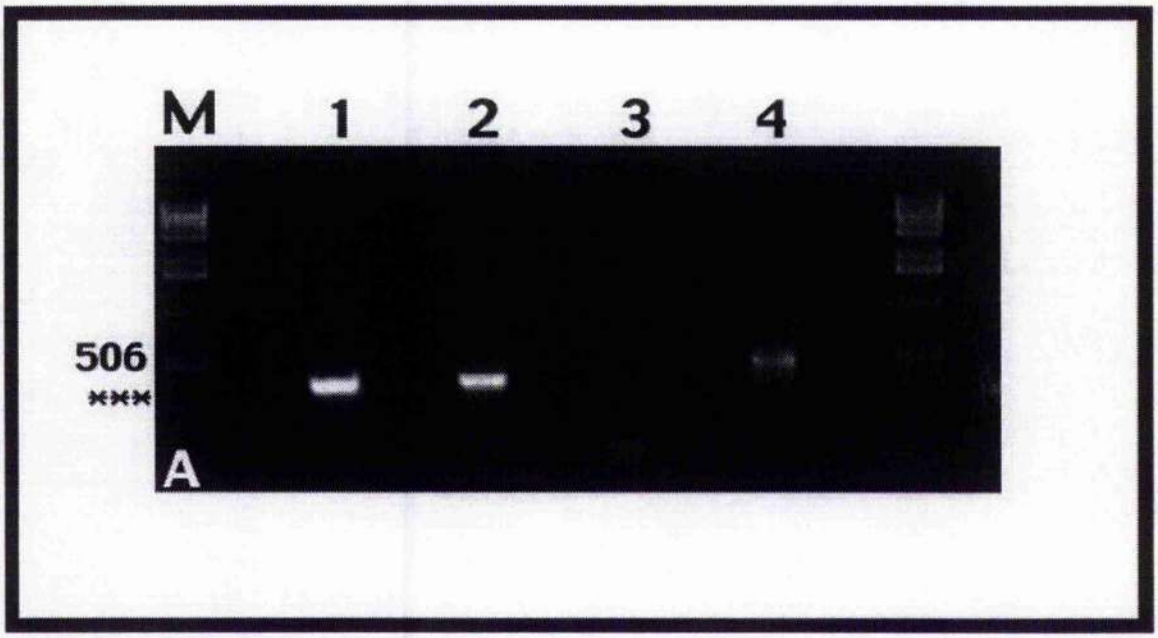
Lane 4: Positive control PCR product obtained from human genomic DNA using G3PDH primers. Product size = 500bp.
All other lanes were empty.

B: Paired oligonucleotide primers (see figure 6.2B and Appendix II) designed to the human NPY Y5 receptor 3'UT were used to amplify corresponding cDNA fragments from human genomic DNA.
Aliquots of 10µl of 100µl total volume were electrophoresed on an agarose gel as outlined in section 2.3.1.7. DNA was visualised under ultraviolet irradiation; photograph exposure time 0.5-1.0 seconds.

Lane 1: Positive control PCR product obtained from human genomic DNA using G3PDH primers. Product size = 500bp.

Lanes 2 and 3: PCR products obtained from human genomic DNA using Y5 receptor 3'UT primers. Product size = 240bp (***).

Lane 4: Negative control PCR reaction containing no template. No product obtained as expected.
All other lanes were empty.
Restriction digested DNA markers (M) were electrophoresed alongside reaction products. 1Kb ladder (Life Technologies).


M
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2
3
4
506
A

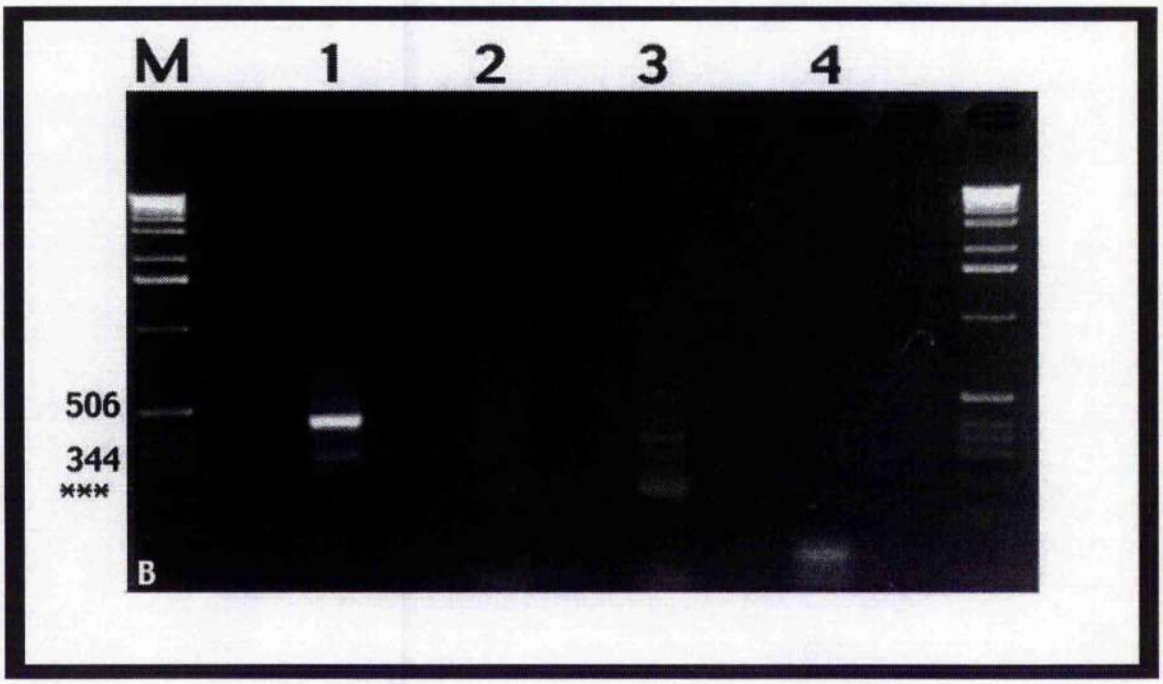
M
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Figure 6.4 Southern blot analysis demonstrating human Y1 and Y5 receptor 3'UT probe specificity.

A: Miniprep DNA prepared following transformation of Y1 and Y5 3'UT PCR products was digested with *Eco* RI to excise the Y1 or Y5 3'UT DNA insert. DNA was electrophoresed on an agarose gel as outlined in section 2.3.1.7 and visualised under ultraviolet irradiation; photograph exposure time 0.5-1.0 seconds. Two identical gels were prepared.

Lane 1: 2μg *Eco* RI digested human Y1 3'UT miniprep DNA. Insert size =360bp(***)

Lane 2: 2μg *Eco* RI digested human Y5 3'UT miniprep DNA. Insert size = 240bp (**)
All other lanes were empty.

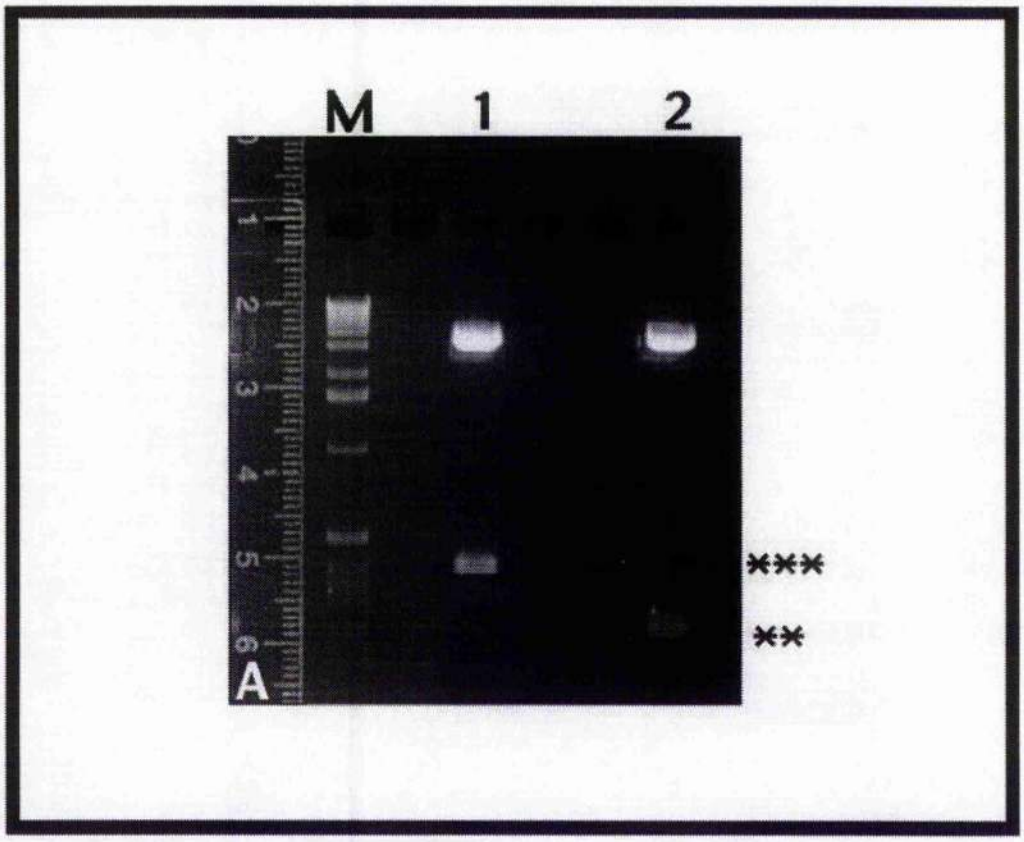
Restriction digested DNA markers (M) were electrophoresed alongside reaction products. 1Kb ladder (Life Technologies).

B: Two identical Southern blots were generated from the gel described above, and probed separately with either radiolabelled Y1 3'UT PCR product or radiolabelled Y5 3'UT PCR product.

Autoradiogram 1: Southern blot of gel in figure 6.4A hybridised to a ^{32}P-labelled human Y1 receptor 3'UT probe. *** denotes Y1 3'UT DNA insert.

Autoradiogram 2: Southern blot of gel in figure 6.4A hybridised to a ^{32}P-labelled human Y5 receptor 3'UT probe .** denotes Y5 3'UT DNA insert.

These results demonstrate that each probe specifically hybridised to the digested miniprep DNA containing the 3'UT insert identical to the PCR product probe.


M
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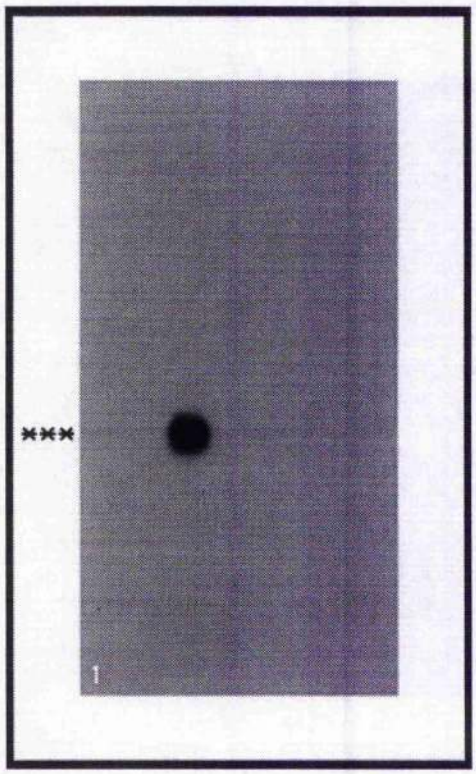
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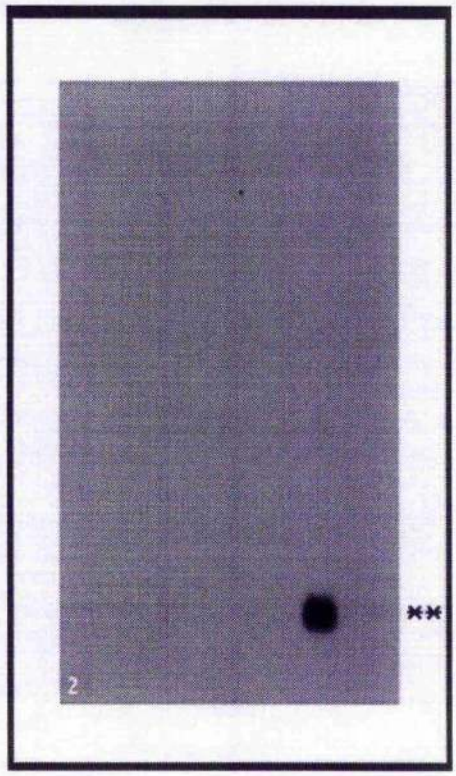

**
2

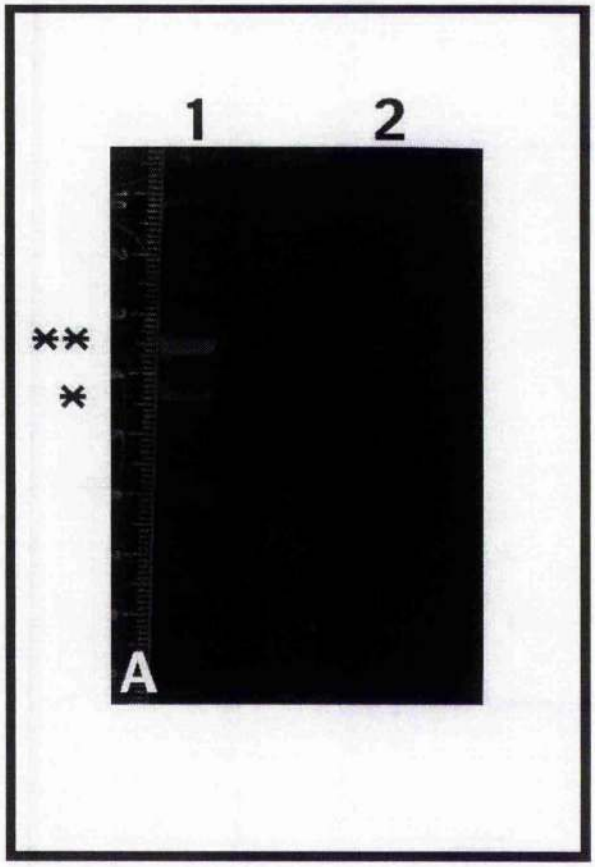
Figure 6.5 Detection of human NPY Y1 receptor mRNA in SK-N-MC cells.

A: 4 μg of poly A^+ mRNA isolated from SK-N-MC cells were denatured, electrophoresed on an RNA agarose gel and photographed under ultraviolet illumination, as described in section 6.3.7. 2μg of total RNA isolated from SK-N-MC cells was run alongside the poly A^+ mRNA as a molecular weight marker.

Lane 1: SK-N-MC cell total RNA.
Double and single asterisk denote the migration position of 28S and 18S rRNA respectively.

Lane 2: Poly A^+ mRNA isolated from SK-N-MC cells

B: A Northern blot was generated from the gel described above and analysed for the human NPY Y1 receptor by hybridisation with a ^{32}P labelled Y1 3'UT probe.
The asterisks (***) mark the position of hybridisation signal for the human NPY Y1 receptor.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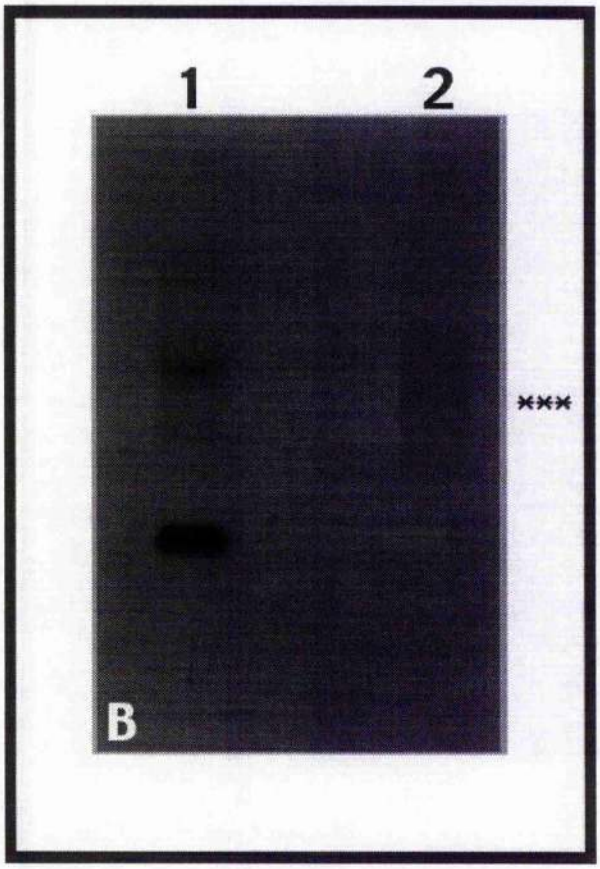

1
2
***
B

6.4.4 Detection of human NPY Y5 receptor mRNA in HEK cells.

In an attempt to determine whether the human NPY Y5 receptor could be detected in HEK cells, Northern blot analysis was carried out. Four micrograms of poly A^+ mRNA isolated from HEK cells was run on a denaturing gel as described, alongside an RNA molecular weight marker (Promega). The Northern blot constructed was probed with a ^{32}P labelled PCR product encompassing a region of the rat Y5 receptor cDNA. The oligonucleotides used to generate this PCR product are the internal primer and reverse primer illustrated in figure 2.4, and the size of the PCR product obtained was approximately 800bp.

As illustrated in figure 6.6, a discrete band corresponding to the molecular weight of the Y5 receptor transcript was detected in the poly A^+ mRNA isolated from HEK cells. The size of the single hybridising Y5 transcript in HEK cells was approximately 2.7 Kb, the same size as the Y5 transcript detected in whole brain mRNA described in the original Y5 cloning paper (Gerald, 1996).

6.4.5 Effect of forskolin treatment on Y1 receptor mRNA expression.

In the experiments described in this chapter, poly A^+ RNA dot blots were the selected method of transferring RNA to nylon membrane, as they present a rapid method for detecting changes in expression levels of Y1 and Y5 receptor mRNA following hybridisation screening.

The initial experimental approach employed to investigate regulation of Y1 receptor mRNA expression examined the effects of forskolin on Y1 receptor expression following treatment for 1-5 days. Forskolin was chosen as the stimulus as previous work in this laboratory has shown that the 5' flanking region of the gene contains two cyclic AMP responsive elements (CRE's) and that the rate of gene transcription is altered by cAMP This time course was selected initially as the effect of cAMP on mRNA levels was unknown. Therefore, by examining changes in the levels of Y1 receptor mRNA over the 5 day time course described, the effect on Y1 mRNA levels could be elucidated.

To ascertain the effect of cAMP (cyclic adenosine monophosphate) on Y1 receptor expression, SK-N-MC cells were treated with 10μM forskolin over a five day period. Forskolin is a diterpine which acts to activate constitutively adenylyl cyclase, resulting in the increased production of cAMP. As the human NPY Y1 receptor gene contains 2 cAMP responsive elements (CRE), application of forskolin to the cells will result in changes in the level of mRNA expression as a result of the action of cAMP on the cAMP responsive elements present on the Y1 receptor gene (Bournat, 1998).

Total RNA was extracted (section 6.3.4) from SK-N-MC cells following treatment with forskolin for 24 hours, 48 hours, 72 hours, 96 hours and 120 hours. Total RNA was also isolated from untreated SK-N-MC cells, following the time course outlined for treated cells. Poly A^+ mRNA was prepared (section 6.3.5) from the total RNA extracted from both treated and untreated cells and quantified (6.3.6) to ensure equal amounts were blotted onto nylon membrane. Following quantification, 1µg of poly A^+ mRNA from each of the forskolin treated cell time-points and 1µg poly A^+ mRNA from each of the untreated cell time-points were blotted in a known array onto nylon membrane (Hybond N, Amersham, section 6.3.9). Denatured Y1 and Y5 3'UT DNA were also blotted as control samples to act as positive and negative controls for hybridisation. The blot was then subjected to hybridisation screening using a ^{32}P labelled Y1 3'UT PCR product in order to examine the effects of forskolin treatment on Y1 receptor mRNA expression.

Figure 6.7 illustrates the results obtained following hybridisation screening of the SK-N-MC mRNA dot blot with a human NPY Y1 receptor 3'UT probe. The data obtained revealed that forskolin treatment surprisingly resulted in a decrease in the levels of Y1 mRNA over the time points 24-96 hours. Levels of Y1 receptor mRNA expression following 120 hours forskolin treatment were comparable to basal levels of Y1 receptor mRNA expression. To check for eveness of mRNA loading, blots were probed for the housekeeping enzyme G3PDH (data not shown).

6.4.6 Effect of forskolin treatment on Y5 receptor mRNA expression.

As the human Y1 and Y5 receptors are located on overlapping but opposite strands of the same chromosome (Herzog, 1997), it was reasoned that SK-N-MC cells may also constitutively express the Y5 receptor. In an attempt to ascertain whether the Y5 receptor is expressed in SK-N-MC cells, the blot prepared following SK-N-MC cell treatment with forskolin (section 6.4.5) was rehybridised following removal of radioactive probe (6.3.13), with a Y5 3'UT probe to determine whether the human Y5 receptor mRNA could be detected in SK-N-MC cells. Following hybridisation screening with the human NPY Y5 receptor 3'UT probe, no signal was detected in either the control or forskolin treated cells. The probe did however hybridise with its own control DNA. This data indicates that the Y5 3'UT probe failed to hybridise to the SK-N-MC cell mRNA. These results suggest that the Y5 receptor is not expressed in SK-N-MC cells, or that the level of expression is very low. The Y5 3'UT probe did hybridise to the Y5 3'UT DNA present on the blot, demonstrating that the hybridisation conditions were appropriate.

6.4.7 Effect of forskolin or dexamethasone treatment on Y1 receptor mRNA expression.

As the 5 day time course employed in the initial experiment carried out to investigate changes in Y1 receptor mRNA expression (6.4.5) revealed that levels of Y1 receptor mRNA expression were altered within 24 hours, a second time course of treatment was employed in an attempt to monitor changes in Y1 receptor mRNA expression in the period immediately following forskolin application. SK-N-MC cells were treated with 10μM forskolin for 2, 4 and 6 hours in order to ascertain the earlier effects of cAMP on Y1 receptor expression.

Total RNA was extracted (section 6.3.4) from SK-N-MC cells following treatment with forskolin for 2 hours, 4 hours and 6 hours. Total RNA was also isolated from untreated SK-N-MC cells, following the time course outlined for treated cells. Poly A^+ mRNA was prepared (section 6.3.5) from the total RNA extracted from both treated and untreated cells and quantified (6.3.6) to ensure equal amounts were blotted onto nylon membrane. Following quantification, 1μg of poly A^+ mRNA from each of the forskolin and dexamethasone treatment time-points and 1μg poly A^+ mRNA from control (untreated) cells were blotted in a known array onto nylon membrane (Hybond N, Amersham, section 6.3.9). Denatured Y1 and Y5 3'UT DNA were also blotted as control samples to act as controls for hybridisation . The blot was then subjected to hybridisation screening using a ^{32}P labelled Y1 3'UT PCR product in order to examine the effects of forskolin on Y1 receptor mRNA expression.

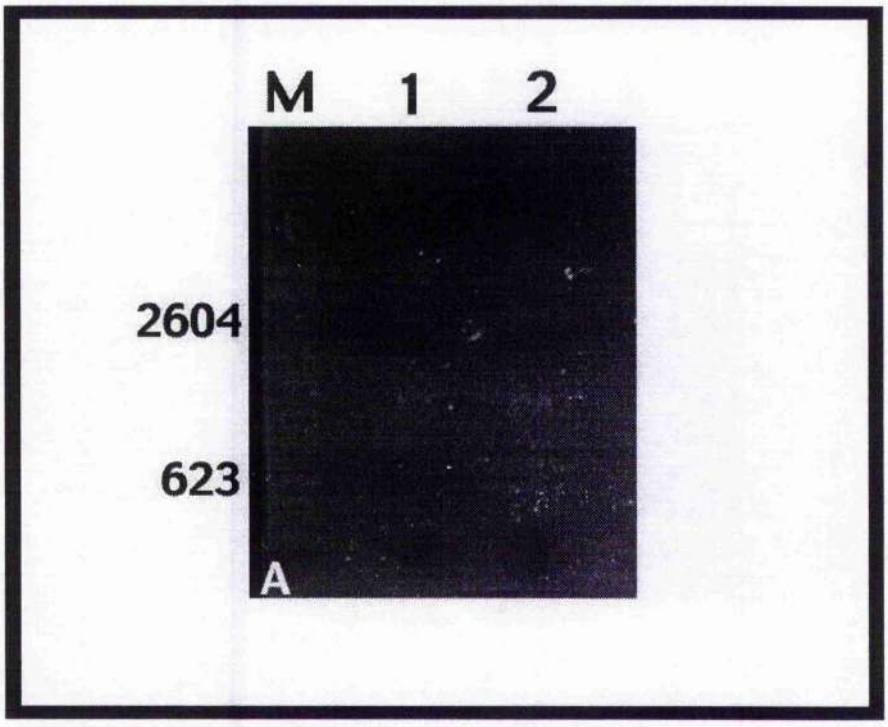
In parallel with these experiments with forskolin, SK-N-MC cells were treated with 1μM dexamethasone over similar time points. Dexamethasone is a synthetic glucocorticoid which mimics the actions of the steroid hormone hydrocortisone. As the human NPY Y1 receptor gene contains 4 glucocorticoid responsive elements (GRE) (J.C.Bournat, University of Glasgow, 1998), application of dexamethasone has been shown previously to alter NPY Y1 receptor gene transcription. However, little is known on the response of the mRNA.

Figure 6.6 Detection of human NPY Y5 receptor mRNA in HEK cells.

A: 4 µg of poly A^+ mRNA isolated from HEK cells were denatured, electrophoresed on an RNA agarose gel and photographed under ultraviolet illumination, as described in section 6.3.7. An RNA molecular weight marker (M) (Promega) was run alongside RNA samples.

Lanes 1 and 2: 4 µg HEK cell poly A^+ mRNA

B: A Northern blot was generated from the gel described above and analysed for the human NPY Y5 receptor by hybridisation with a ^{32}P labelled Y5 receptor cDNA probe encompassing a region of the rat open reading frame from TM5 to the C-terminus of the receptor protein.
The asterisks (***) mark the position of hybridisation signal for the human NPY Y5 receptor.


M
1
2
2604
623
A

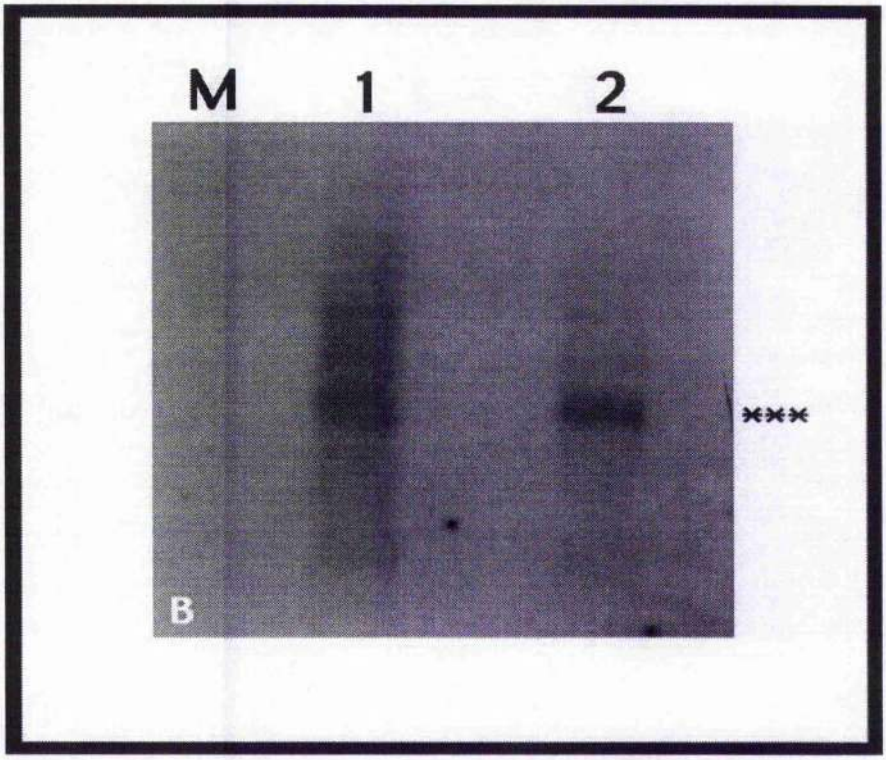

M
1
2
***
B

Figure 6.7 Effect of forskolin treatment on Y1 receptor mRNA expression.

1μg of poly A^+ mRNA from untreated SK-N-MC cells grown for 1-5 days (row B, samples 1-5) and 1μg of poly A^+ mRNA from cells treated with 10μM forskolin for 1-5 days (row A, samples 1-5) were blotted onto nylon membrane in a known array. Denatured Y1 and Y5 3'UT cDNA were also blotted onto the membrane as positive control samples.

The blot was then analysed for human Y1 mRNA expression by hybridisation with a ^{32}P labelled Y1 3'UT cDNA probe.

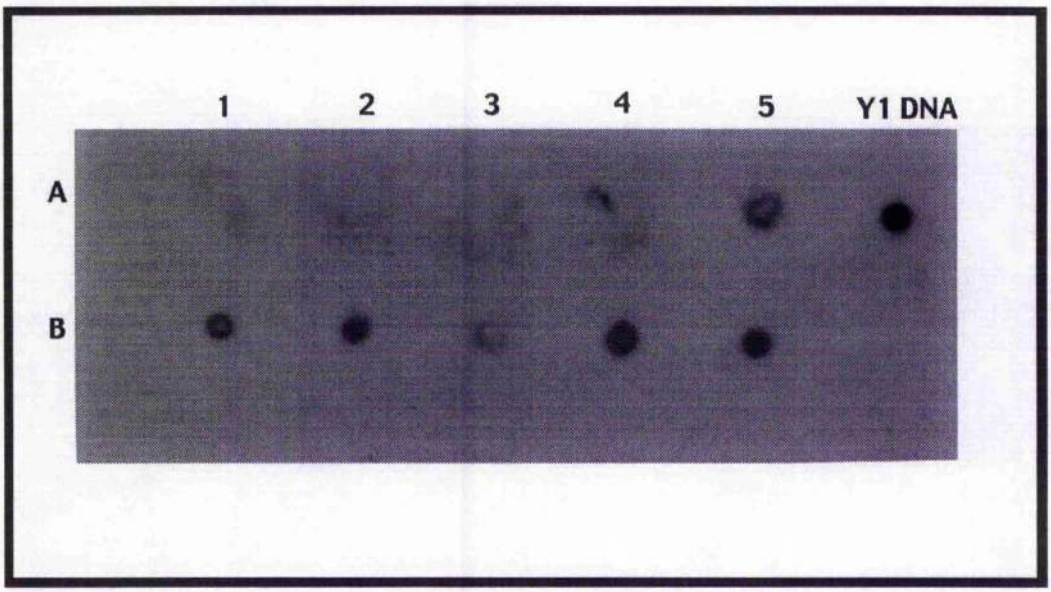

1
2
3
4
5
Y1 DNA
A
B

Figure 6.8 illustrates the results obtained following hybridisation screening of the SK-N-MC mRNA dot blot with a human NPY Y1 receptor 3'UT probe. The results obtained revealed that following treatment with forskolin for 2 and 4 hours, levels of Y1 receptor mRNA expression were elevated when compared with the basal levels of Y1 receptor mRNA expression observed in control (untreated) cell mRNA. 6 hours after addition of forskolin levels of Y1 receptor mRNA expression were comparable to basal levels of Y1 receptor expression as observed in control cell mRNA. Therefore, following forskolin treatment for 2-4 hours, Y1 receptor mRNA expression was elevated, but this response was reversed by treatment with forskolin for 6 hours.

Following treatment with dexamethasone only the 2 hour time point revealed an elevation in the levels of Y1 receptor mRNA expression when compared with basal levels of Y1 receptor mRNA in control cell mRNA. Treatment with dexamethasone for 4 or 6 hours decreased Y1 mRNA levels. Additional experiments were undertaken to confirm this transient increase in NPY Y1 mRNA levels. The two hour time point was repeated and in addition, earlier time points were included. This data confirmed that at 2 hours the mRNA levels of the Y1 receptor were elevated above control and this increase was observed within 30 minutes of treatment with forskolin (data not shown).
To check for eveness of mRNA loading, blots were probed for the housekeeping enzyme G3PDH (data not shown).

6.4.8 Detection of human NPY Y5 receptor mRNA in HEK cells by dot blot analysis.

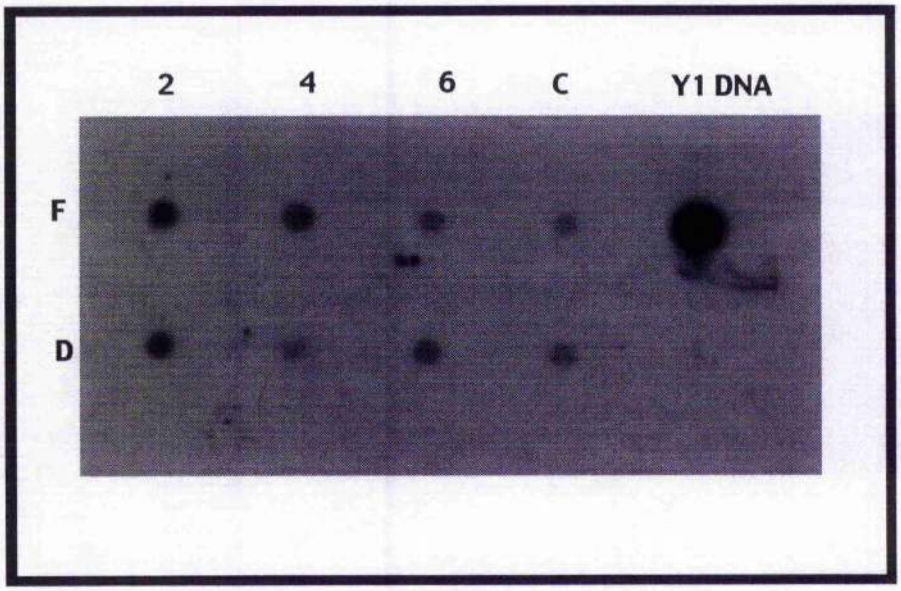
The following preliminary experiment was carried out in order to determine whether the human NPY Y5 receptor could be detected in HEK 293 cells and was similarly controlled by cAMP levels.

HEK cells were treated with 10μM forskolin for 2 hours, and RNA extracted from treated cells and untreated control cells. One microgram of isolated poly A^+ mRNA from treated and untreated cells was dot-blotted onto nylon membrane, with all steps performed similarly to those described in section 6.4.5. Denatured Y1 and Y5 3'UT DNA were also blotted as controls for hybridisation. The blot was then subjected to hybridisation screening using a ^{32}P labelled Y5 3'UT PCR product in order to determine whether HEK cells express the Y5 receptor. However, no signal could be detected as the Y5 3'UT probe failed to hybridise to the HEK cell mRNA isolated from either forskolin treated or untreated cells. The Y5 3'UT probe hybridised stongly to the Y5 3'UT DNA control sample on the blot, confirming that hybridisation conditions were favourable. This data indicates that 1μg poly A^+ mRNA

Figure 6.8 Effect of forskolin and dexamethasone treatment on Y1 receptor mRNA expression.

1μg of poly A^+ mRNA from untreated SK-N-MC cells (C), 1μg of poly A^+ mRNA from cells treated with 10μM forskolin for 2, 4 and 6 hours (row F, samples 2, 4 and 6) and 1μg of poly A^+ mRNA from cells treated with 1μM dexamethasone for 2, 4 and 6 hours (row D, samples 2, 4 and 6) were blotted onto nylon membrane in a known array. Denatured human Y1 and Y5 3'UT cDNA were also blotted onto the membrane as positive control samples.

The blot was then analysed for human Y1 mRNA expression by hybridisation with a ^{32}P labelled Y1 3'UT cDNA probe.


2
4
6
C
Y1 DNA
F
D

contains insufficient Y5 mRNA to be detected by the dot blot method and is further evidence for the rarity of the mRNA.

6.5 Discussion.

In this chapter the levels of expression of the mRNAs for the NPY Y1 and Y5 receptors have been determined using Northern blot analysis of poly A^+ selected RNA and subsequent dot blot analysis. To examine dynamic changes in the mRNA levels, dot blots provide a very rapid and convenient approach. However, it is essential to validate the hybridisation signal prior to interpreting data derived from the dot blot as the RNA is not fractionated in this procedure. A non specific signal in dot blots may arise from hybridisation of the probe to related mRNA species or to "bulk" effects of ribosomal RNA (28S and 18S). To avoid these issues, two decisions were made before embarking on these studies. First, Y1 and Y5 selective regions of the 3'UT region were obtained by PCR to use as probes for the study. The reason for this was to target the region displaying maximum diversity between these two genes. Because the 3'UT is not evolutionarily constrained by the need to conserve sequence to encode a protein, this region of the gene within families is recognised to be the most diverse. Thus, use of probes designed to this region of the mRNA, is likely to provide a higher level of specificity than use of probes within the open reading frame. The absolute specificity of the probes was determined by probing against the corresponding DNA of the Y1 and Y5 receptors. Thus, the Y1 probe only recognised Y1 DNA and the Y5 probe only recognised Y5 DNA.
The second factor employed to avoid non-specific signals in the dot blot experiments was use of poly A^+ selected RNA. Three RNA species are extracted and represented in the total RNA. These are the ribosomal RNAs (rRNA), transfer RNA (tRNA) and messenger RNA (mRNA). The vast bulk of the RNA is rRNA and this frequently binds to probe to give a non-specific signal. Messenger RNA can be selected away from the other RNA species by exploiting the fact that mRNAs are polyadenylated, thus oligodT will hybridise to mRNA. The selection procedure used here appears to be very effective as no ribosomal RNA could be detected in the poly A^+ selected lane by Northern analysis (figure 6.5).

Having made these two decisions, the specificity of the signal was validated by Northern analysis. Here unlike in dot blots, the mRNA is fractionated in a denaturing agarose gel and molecular weight of the positive signal can be estimated. These results demonstrated that the Y1 probe readily detected a signal in SK-N-MC mRNA (4μg) and that the band corresponded to a molecular weight of 3.5Kb consistent with previous reports (Larhammar, 1992). No other bands were observed. The mRNA for the Y5 receptor was more difficult to validate. Four micrograms of mRNA derived from HEK cells were fractionated and a weak band was

observed with an estimated molecular weight of 2.7Kb consistent with previous reports (Gerald, 1996).

The relative ability to detect mRNAs for the Y1 and Y5 receptor highlight a feature throughout this thesis that the Y5 receptor appears to be expressed at very low levels. Thus, the signal achieved by Northern analysis for the Y1 receptor meant that it was relatively simple to proceed to evaluate changes in expression using dot blot analysis, whereas the low signal observed in the Northern for the Y5 receptor predicted difficulties in detection by dot blot analysis.

Dot blots provide a convenient method of measuring changes in relative mRNA levels for a larger number of samples than can be handled by Northern analysis. Unlike Northerns where the number of wells (in our apparatus 8) restricts the number of samples that can be evaluated, dot blot manifolds allow the evaluation of a much larger number of samples. The major disadvantages of dot blots can be controlled. These are specificity of the signal as discussed above and the absolute requirement for an accurate measurement of mRNA loaded. This later problem can be controlled by probing for a housekeeping gene such as G3PDH a gene which is unlikely to change expression. Dot blots were used to evaluate factors which would change NPY Y1 receptor mRNAs, however the rarity of the Y5 receptor mRNA resulted in a failure to detect a hybridisation signal for the Y5 receptor in either SK-N-MC or HEK cell mRNAs. Thus this approach could not be used to measure the factors that alter the Y5 receptor mRNA. For rare mRNA species such as the Y5 receptor, it may be necessary to find alternative methods of measuring changes in mRNA levels. The most sensitive method conveniently available is to use quantitative RT-PCR. However, the need for an internal standard to provide an accurate level of quantitation means that this approach is not trivial, controversial and time consuming. In the time constraints of this thesis, it was not possible to consider using this approach.

6.5.1 Detection of human NPY Y1 receptor mRNA in SK-N-MC cells.

Northern analysis carried out on poly A^+ mRNA isolated from SK-N-MC cells and screened with a human NPY Y1 3'UT probe, revealed that the Y1 receptor transcript could be readily detected in these cells using the probe designed (figure 6.3). A discrete band corresponding to the predicted molecular weight of the human Y1 transcript was readily detected in the poly A^+ mRNA isolated from SK-N-MC cells. This confirmed that the Y1 3'UT probe effectively hybridised to the Y1 receptor mRNA, and that the Y1 transcript could be readily identified in SK-N-MC cells by this method. This result enabled subsequent experiments to be carried out whereby poly A^+ mRNA isolated from SK-N-MC cells was dot-blotted onto nylon membrane and probed with the Y1 3'UT probe to detect changes in Y1 receptor mRNA expression, as it

had been established that the Y1 receptor 3'UT probe only hybridised to the Y1 mRNA under these hybridisation conditions.

6.5.2 Regulation of expression of the mRNA encoding the Y1 receptor in SK-N-MC cells.

The studies undertaken in this chapter have examined the levels of mRNA for the Y1 receptor in SK-N-MC cells following pharmacological manipulations. SK-N-MC cells are derived from a human neuroblastoma and have previously been shown to express the Y1 receptor (Sheikh, 1989 and Wahlestedt, 1991). The studies undertaken here used this cell line as a model system to determine the response of the Y1 receptor mRNA to agents known to influence gene expression including activation of protein kinase A (PKA) following forskolin treatment, activation of protein kinase C (PKC) by phorbol esters and activation of the glucocorticoid receptor (GR) by dexamethasone. The 5' region of the Y1 gene contains consensus sequences able to respond to elevated cyclic AMP (CRE) activation of protein kinase C (AP1) and activation of the glucocorticoid receptor (GRE) and previous work in this laboratory has shown that these sequences are functional (Bournat, 1997). However the response of mRNA is not known.

6.5.2.1 Response to forskolin.

The promoter region of the human NPY Y1 receptor gene contains two CRE sites (Ball, 1995). The sequences encoding both CRE sites are located at positions -113 (conserved in human and rat Y1 receptor gene) and -493 (conserved in human, mouse and rat Y1 receptor gene). Forskolin is an activator of adenylyl cyclase, and by increasing the activity of this enzyme, augments the levels of intracellular cAMP. Elevation of intracellular cAMP levels can result in increased transcriptional activity of a gene harbouring CRE sites. The rat Y1 receptor gene is highly responsive to intracellular cAMP, as observed by stimulation of rat Y1 receptor transcriptional activity in PC12 cells following forskolin application (Bournat, 1997). In an attempt to investigate the effects of forskolin treatment on regulation of the human Y1 receptor gene in SK-N-MC cells, mRNA was isolated from SK-N-MC cells following treatment of cells with forskolin.

In view of the fact that the 5' flanking region of the gene contains functional CREs, the initial experiments measuring mRNA levels in response to forskolin revealed an unexpected finding. The mRNA for the Y1 receptor was easily detectable in poly A^+ RNA extracted from resting cells. However at all time points after forskolin treatment (24, 48, 72, 96 and 120 hours) the level of the Y1 receptor mRNA was reduced or undetectable compared to control cells. These results led to an examination of mRNA levels at earlier time points. Consistent with the presence of CREs in the 5' flanking region of the gene, forskolin resulted in an increase in the NPY Y1 receptor mRNA levels but their increase was very transient. It was observed at the earliest time point studied (2hours) and was maintained 4 hours after treatment but levels were suppressed 6 hours after forskolin treatment.

The results of these experiments highlight the potential differences between measuring gene transcription using a reporter function and mRNA levels expressed from the endogenous gene. Transcription studies use heterologous expression of a fusion gene where a reporter function is placed under the control of the 5' flanking region of the gene of interest. Reporter functions such as chloramphenicol acetyl transferase (CAT), firefly luciferase or alkaline phosphatase, are chosen on the basis of ease of assay. A function in this selection is that the mRNA and protein of the reporter function are relatively stable and so, after gene activation, the product of the fusion gene can accumulate in the cell and be measured. Reporter functions have been widely used to provide valuable information on factors that regulate gene transcription. However, reporter function can only give a read out of transcriptional activity. Levels of the mRNA for the endogenous gene and protein are influenced by a large number of additional factors. These include for mRNA factors that regulate post-transcriptional processing and mRNA stability. For protein, translational control and post-translational processing play a role.
The results shown here demonstrate that forskolin results in a transient increase in mRNA followed by an apparent sustained suppression of the mRNA species.

6.5.2.2 Response to dexamethasone.

The NPY Y1 receptor gene contains four tandem GREs and dexamethasone treatment increases NPY Y1 receptor gene transcription. The data shown here demonstrates that the mRNA for the Y1 receptor is increased after dexamethasone treatment but again this response is very transient in nature.

6.5.2.3 Y1 receptor mRNA regulation.

Taken together, this data indicates the Y1 receptor mRNA can be measured in SK-N-MC cells but that factors known to influence gene transcription have a very transient effect on the Y1

receptor mRNA. This data implies that the mRNA encoding the receptor is very tightly regulated and that the mRNA is relatively unstable and short lived.

The manipulations used to investigate mRNA levels were pharmacological and avoided the need for cell surface receptor coupled mechanisms. Clearly it will be valuable to extend these studies to examine the effects of factors known to influence feeding behaviour on levels of the Y1 receptor mRNA. These studies may help resolve the controversy surrounding the role of receptor subtypes in mediating NPY induced feeding behaviour, as changes consistent with physiological effectors of feeding would re-inforce the potential role of the Y1 receptor in the control of feeding behaviour.

6.5.3 Failure to detect the Y5 mRNA in SK-N-MC cells.

No Y5 mRNA could be detected in poly A$^+$RNA from SK-N-MC cells. This either means that the Y5 gene is not expressed in these cells or that the Y5 mRNA was below the detection limit of the procedure used in these studies. The Y1 and Y5 receptors are encoded on opposite strands of the same region of the gene (Herzog, 1997). The structure of these two genes indicates a complex pattern of alternative splicing of the first exon for the gene, and potentially could lead to overlapping RNA transcripts. As duplex RNA species are rapidly eliminated from cells, it seems possible that such structural organisation may prevent the appearance of the two RNA species within the same cell. However, it remains a possibility that the mRNA species is very rare and this is suggested by the results obtained for the HEK cells.

6.6 Summary.

1) SK-N-MC cells express the Y1 receptor subtype as demonstrated by the presence of a band of the correct molecular weight in poly A$^+$ RNA extracted from these cells.

2) Forskolin elicits a very transient increase in Y1 mRNA levels and this is followed by sustained suppression of the mRNA levels.

3) No hybridisation signal could be detected for the Y5 mRNA in SK-N-MC cells indicating either that this mRNA species is very rare and below the detection limit of the procedure or that the gene is not expressed in these cells.

Chapter 7

General discussion

Chapter 7

General Discussion

The pancreatic polypeptide (PP-fold) family of peptides comprises the endocrine peptides, pancreatic polypeptide (PP) and peptide Y (PYY), and the neuronally derived peptide, neuropeptide Y (NPY). These three peptides constitute a family of regulatory peptides, characterised by a common distinct tertiary structural feature, the PP-fold (Glover, 1985). Since PP, the first member of the family, was identified in 1968 (Kimmel, 1968), the PP-fold family of peptides have emerged as a physiologically active family of peptides, with widespread functions in both the central and peripheral nervous system. The work carried out in this thesis was aimed at identifying a receptor subtype involved in mediating a characteristic response to the neuronally derived family member NPY. Neuropeptide Y was discovered in 1982 (Tatemoto, 1982), and has since emerged as a neuropeptide with widespread distribution and numerous functions. The physiological activity of NPY is not confined to the CNS, where NPY mediates numerous behavioural functions, but is also evident in the periphery, where NPY elicits numerous effects, including a role as a potent vasoconstrictor (Wahlestedt, 1990a). The diverse effects of the PP-fold family of peptides are mediated by G-protein coupled receptors, which bind the members of the PP-fold family of peptides with differing affinities, depending on the receptor subtype.

NPY has a diverse functional role within the central nervous system, where it is involved in the regulation of blood pressure, circadian rhythm, anxiety and depression, endocrine function and metabolism, memory retention and feeding and obesity (Gehlert, 1998). The characteristic effects of NPY are each mediated by specific subtypes of G-protein coupled receptors. The initial aim of the experimental research undertaken in this thesis was directed at cloning the NPY receptor subtype involved in mediating NPY induced feeding.

As the pharmacology of the cloned NPY receptor subtypes, failed to mimic the pharmacology known to stimulate NPY-induced feeding, the receptor subtype responsible for mediating the feeding response to NPY was believed to be a novel, previously unidentified receptor subtype. (Kalra, 1991and Stanley, 1992). When the experimental work described in this thesis began, four receptor subtypes had been described for the PP-fold family of peptides. Each receptor subtype has a distinct pharmacology, and exhibits a specific pattern of tissue distribution (see figure 1.8). The first NPY receptor subtype to be described was the Y1 receptor, which was cloned in 1991(Eva, 1991), and is the best characterised receptor for NPY. This receptor was identified pharmacologically by its requirement for the whole NPY or PYY molecule, as demonstrated by studies where carboxyl terminal fragments of the peptide were shown to be ineffective at activation of this receptor subtype (Wahlestedt, 1986). The substituted analogue

of NPY, Pro^{34}NPY is a selective agonist at the NPY Y1 receptor (Fuhlendorf, 1990). Receptor heterogeneity in the PP-fold family was suggested in 1986, following pharmacological identification of a second NPY receptor subtype (Wahlestedt, 1986). The NPY Y2 receptor was cloned in 1995 (Rose, 1995), and is distinguished pharmacologically from the Y1 receptor, by its ability to bind carboxy terminal fragments of NPY such as NPY^{13-36} with similar affinity to NPY or PYY (Wahlestedt, 1986). The Y3 receptor is distinguished from other PP-fold family receptor subtypes by its high affinity for NPY, and relatively low affinity for PYY (Grundemar, 1991a & b). This receptor has yet to be cloned, and remains a pharmacologically distinct receptor subtype. The Y4 receptor was cloned in 1995 (Lundell, 1995) and is characterised by an unusual pharmacology, in that this receptor possesses extremely high affinity for PP with somewhat lower affinity for NPY and PYY (Gehlert, 1996).

The receptors responsible for mediating the actions of the PP-fold peptides exhibit significant structural differences. The structural diversity described for the NPY receptor gene family (Larhammar, 1996b) is exemplified in the alignments described in chapter 2 (figures 2.5 and 2.6). Initial attempts to design a degenerate oligonucleotide primer from conserved regions of the Y1, Y2 and Y4 receptor sequence were hindered by the lack of homology exhibited between these members of the NPY receptor family. Unlike most G-protein coupled receptor families, molecular cloning of the Y1, Y2 and Y4 receptors revealed an extremely low sequence identity between the Y2 receptor and both the Y1 and Y4 receptors, and between the Y1 and Y4 receptor (Larhammar 1996b). The sequence alignments illustrated in figures 2.5 and 2.6 mirror previous findings, in that they exhibit clearly the low percentage identities displayed between receptors even in transmembrane regions, normally highly conserved within receptor gene families (Larhammar, 1996b). As discussed above, the receptor subtypes for NPY are readily identified by the markedly different pharmacology exhibited by each subtype. The structural differences displayed by the Y1, Y2 and Y4 receptor subtypes may therefore result in differing modes of interaction of the receptors with their ligands.

The cloning of the NPY Y5 and y6 receptors in 1996 (Gerald, 1996., Weinberg, 1996) perpetuated further the heterogeneity exhibited by this receptor family. As the sequence alignments in figure 2.8 illustrate, there is a clustering of sequence similarity between the Y1, Y4 and y6 receptors, while the Y2 and Y5 receptors, clearly define another family (Gehlert, 1998). The structural diversity of this gene family is illustrated further in Chapter 4, where attempts to design a degenerate oligonucleotide primer for the Genetrapper™ cloning experimental strategy proved unsuccessful due to the lack of homology exhibited between the NPY gene family members. The clustering of amino acid sequence between the Y1 and y6 receptors is also evident, as the only oligonucleotide primer designed with reasonable

degeneracy was constructed from conserved regions of the Y1 and y6 receptor sequence (figure 4.2). The lack of sequence homology exhibited between the PP-fold receptor subtypes constitutes the principal difficulty encountered in attempts to clone novel family members using a homology screening approach. As the alignments in figures 2.8 and 4.2 illustrate, design of an oligonucleotide primer with reasonably low degeneracy, based on conserved regions of NPY receptor family sequence is extremely difficult, and as a result render homology screening ineffective as a cloning strategy for identifying novel PP-fold family members.

One of the many interesting aspects of this peptide family is the varying degrees of genetic conservation exhibited. The evolution of the PP-fold family of peptides has been studied extensively (Larhammar, 1996a), and as a result has enabled a detailed analysis of the evolutionary events that have led to the present PP-fold family of peptides, and the receptor subtypes responsible for mediating the physiological effects of this peptide family. NPY displays a remarkable degree of sequence conservation, and is considered to be one of the most conserved peptide sequences known (Larhammar, 1993). This peptide exhibits very few differences in sequence across a wide variety of species, with twenty-two positions identical in all NPY sequences known (Larhammar, 1996a). PYY is more variable, exhibiting more divergent amino acids within the peptide sequence across species. The peptides NPY and PYY appear to have evolved from a gene duplication event, with a common ancestral NPY/PYY sequence diverging to give rise to the respective peptides (Larhammar, 1996a). Several lines of evidence suggest that the third member of the PP-fold family, PP, arose as a copy of the PYY gene relatively late in evolution (Larhammar, 1996a). This peptide exhibits striking sequence differences between species, with only seven amino acids conserved across a variety of species (Larhammar, 1996a). The evolutionary rate for these peptides is reflected in the degree of conservation between species exhibited by each individual receptor subtype. Between man and rat the Y1 receptor displays 94% identity. A similar degree of conservation is shown by the Y2 receptor in man and rat where 94% of the amino acids are identical. This suggests that the evolutionary rate for these receptors is in the slower range for G-protein coupled receptors, and that the gene duplication which generated the Y1 and Y2 receptors from a common ancestral gene took place long ago, in correlation with the evolutionary rate of the receptor ligands NPY and PYY, which both exhibit a high degree of species conservation (Larhammar, 1996b). The great variability between species for the pancreatic polypeptide receptor (Y4) is evident in the modest 75% sequence identity exhibited between man and rat, which is one of the lowest percentages reported for G-protein coupled receptors (Larhammar, 1996b). The low Y4 receptor conservation and rapid evolutionary rate of the Y4 receptor correlate with that of its ligand PP, which is also divergent between rat and man, suggesting that the receptor and its ligand have co-evolved at a rapid pace (Larhammar, 1996b).

The past decade has witnessed the characterisation and cloning of receptors for the PP-fold family of peptides. The availability of new pharmacological tools and the advent of molecular biology enabled new receptors to be identified and their physiological role established. The structural diversity of this receptor gene family, suggests that further advances in molecular biology and development of pharmacological tools may result in the cloning and characterisation of previously unidentified PP-fold receptors with new physiological roles. At this stage it is difficult to estimate how many receptor subtypes constitute this family, and as this gene family is not amenable to a homology screening approach, as illustrated by the Genetrapper™ cloning strategy employed in chapter 4, the diversity of sequences observed with the present members would suggest a plethora of as yet "uncloned" subtypes which are more closely related.

The fascinating complexity intrinsic to this receptor family is tempered with the apparent loss of functionality observed with the y6 receptor in humans and primates (Gregor, 1996). The y6 cDNA encodes a functional receptor in mice and rabbits but not in primates. The presence of a functional y6 receptor in mice and rabbits, suggests that the y6 receptor emerged as a non-functional pseudogene in humans during evolution (Rose, 1997). The loss of function of this PP-fold receptor subtype furthers the speculation regarding unidentified PP-fold receptor subtypes, as the receptor(s) responsible for the physiological role played by the y6 receptor in mice and rabbits may be as yet undiscovered. The y6 receptor phenomenon poses numerous questions regarding the role of this receptor in mice, as the functionality of this receptor subtype appears to be species dependent. The physiological reason for the functional role of the y6 receptor subtype in mice remains unclear, and whether a functional equivalent exists in humans and primates remains to be determined. However, as exemplified by the y6 receptor, it may be that many potential members of this receptor gene family have been lost during evolution, and the search for additional human subtypes will prove futile.

As the PP-fold peptides NPY and PP, both have receptor subtypes which display a favourable affinity for the respective PP-fold peptide, the existence of a PYY-preferring receptor subtype, distinguished by a preference for PYY over NPY and PP represents another potential unidentified PP-fold receptor, with a role in mediating selectively the physiological functions of PYY.

Evidence in support of a PYY-preferring receptor has been suggested from reports outlining a preferential PYY response in several systems such as rat small intestine, where PYY has a 5-10 fold higher affinity over NPY (Larburthe, 1986), adipocytes (Castan, 1993), and in a kidney proximal tubule cell line (Voisin, 1993). The cloning and characterisation of such a receptor is essential in order to establish the physiological role attributed to this novel PYY-preferring receptor subtype. As discussed above, the structural divergence exhibited by this receptor gene

family render a homology screening approach to novel receptor cloning ineffective, and the identification of pharmacologically distinct novel receptor subtypes such as the PYY preferring-receptor, or the human functional equivalent of the mouse y6 receptor would be more favourably achieved using a functional expression cloning technology. As discussed in chapter 5, the lack of sequence homology intrinsic to this gene family is inconsequential when a functional cloning strategy is employed, as the affinity of the receptor subtype for the PP-fold ligand enables effective identification and isolation. By exploiting the demonstrated pharmacology of a potential novel PP-fold receptor subtype in a functional cloning assay, additional members of this peptide gene family, bearing relatively low percentage identities to the cloned PP-fold receptors, may be identified. The panning immunoselection procedure described in chapter 5, is demonstrably an effective functional cloning technique for the isolation of G-protein coupled receptors, and is arguably a valuable technique for the isolation and identification of novel PP-fold receptors. Further experiments employing the panning technique with tissue specific cDNA libraries believed to harbour novel receptor clones, and ligand binding with a specific PP-fold peptide, should enable the effectiveness of this technique to be demonstrated further, while simultaneously identifying novel receptor clones.

The structural similarity exhibited by the PP-fold peptides (Glover, 1985) lends support to the existence of a receptor subtype which is capable of binding the currently identified PP-fold peptides; NPY, PYY and PP. In contrast to the receptor subtypes implicated in mediating the effects of an individual PP-fold family member, a receptor subtype believed to mediate the functions of all three peptides has been described in the rat, and named the PP-fold receptor as a result of the ability of this receptor to bind NPY, PYY and PP with equal affinity (Nata, 1990).

Further evidence in support of as yet unidentified PP-fold receptor subtypes has come from the cloning and identification of multiple NPY receptors in the zebrafish (*Danio rerio*) (Lundell, 1997 and Ringvall, 1997). The zebrafish is a widely used model organism for the study of vertebrate development (Eisen, 1996), and the cloning of PP-fold receptor subtypes from the zebrafish has enabled the evolutionary events that have generated the various PP-fold receptor subtypes to be studied. The cloned zebrafish subtypes appear to be distinct from known mammalian subtypes with regard to primary sequence data, which suggests that the low percentage identity exhibited between the zebrafish receptors and the cloned mammalian PP-fold receptors is lower than expected for zebrafish orthologues of the mammalian receptors. Detailed studies on the sequence divergence exhibited between the cloned zebrafish receptor subtypes (Ringvall, 1997) suggest that at least two of the zebrafish receptors arose prior to the divergence of ray-finned fishes and the mammalian lineage, implying that mammals may possess orthologues of the zebrafish receptors (Ringvall, 1997). Therefore, it remains to be

determined whether mammalian homologues exist, and further studies will be required before a role for these receptors in the large family of PP-fold receptors is established.

Neuropeptide Y, peptide YY and pancreatic polypeptide are firmly established as important mammalian peptides, but it is also likely that, like the receptor subtypes for these peptides, evolution may have provided additional members of this peptide family. The evidence in support of additional PP-fold receptor subtypes would suggest that there are more receptor subtypes in the PP-fold family than there are ligands. Therefore, the complete elucidation of this peptide family constitutes another important area of research, with the potential for interesting findings in the next decade, regarding the identity and physiological role of new PP-fold family members. The presence in selected species of fish of a peptide, PY (Larhammar 1996a), which appears to have been derived from a gene duplication of the PYY gene, which has not been found in any mammalian species to date, and whose functional role has not been described, suggests that several gene duplications of the PP-fold peptide sequences may have occurred during evolution.

Another copy of the PYY gene has been described in cows (Herzog, 1995). This gene, which exhibits greater than 95% nucleic acid sequence identity to the PYY gene (Larhammar, 1996a), encodes a protein which was originally found in bull semen and given the name seminalplasmin. However, the amino acid sequence has diverged considerably and lacks many of the PYY features such as the characteristic PP-fold structure (Larhammar, 1996a). The functional role of this protein is unknown, and whether seminalplasmin binds to a receptor with similarity to the PP-fold family members is unknown. The expansion of the CRF-like peptide family with the recent discovery of urocortin (Vaughan, 1995), the mammalian orthologue of the fish peptide urotensin I, suggests new peptides can even be identified in relatively well-established peptide families. Therefore, it is possible that additional members of the PP-fold family exist and remain to be discovered.

Further speculation regarding the presence of unidentified members of the PP-fold family has evolved from closer examination of PP binding within the brain. As the PP-fold peptide family member PP is a pancreatic peptide involved in the regulation of gastrointestinal and pancreatic function, and is not distributed in the brain, the presence of the PP binding sites within the brain (Gehlert, 1997) suggest that a novel undiscovered PP-like molecule may be present within the brain. As intracerebroventricular injection of PP has been shown to elicit an increase in feeding behaviour (Clark, 1984), it is possible that central administration of PP may result in the occupation of receptor sites normally occupied by a PP-like molecule distributed within the brain. Recent reports detailing the presence of PP binding sites in two principal brain structures (Gehlert 1997) which fail to bind ligand with high affinity for Y1 and Y4 receptors, imply PP receptor heterogeneity. This favours the existence of a PP-like molecule present

within the brain, as the lack of Y1 and Y4 binding may be the result of the presence of a receptor subtype selective for a PP-like molecule, which can readily bind PP but not NPY or PYY.

The role of any additional members of the PP-fold family remains to be resolved, and a comprehensive understanding of the physiological role of such peptides can only be accomplished when the relevant receptor subtypes are cloned and characterised, and selective agonists and antagonists are developed.

The wealth of controversy surrounding the identity of the feeding receptor for NPY is tempered further by the implications discussed above which favour the existence of novel PP-fold receptors and PP-fold family members. The completion of the Human Genome project (currently expected to be fully elucidated in 2003) should resolve this issue unequivocally. As the aim of this world-wide collaboration is to obtain the complete sequence of the entire human genome, bioinformatic analysis will enable all open reading frames to be readily identified, therefore the full complement of PP-fold receptors and family members may be revealed.

The work presented in this thesis has been aimed at the identification of the feeding receptor for NPY, and concentrated initially on novel cloning techniques employed in an attempt to isolate such a receptor. As the current literature illustrates (chapter 1, section 1.8), the identity of the feeding receptor for NPY is a highly contentious issue, implicating both the Y1 and Y5 receptors, and with a strong emphasis on the role of a novel NPY receptor subtype in the regulation of NPY elicited eating. The evidence in favour of a role for both the Y1 and Y5 receptors in the control of NPY induced feeding has arisen as a result of studies using selective Y1 receptor antagonists and receptor gene knockouts. The inhibition of feeding behaviour observed following administration of Y1 receptor selective antagonists (Kanatani, 1996, 1998), generated considerable interest in this NPY receptor subtype, with regard to NPY mediated feeding behaviour. Prior to the findings implicating the Y1 receptor subtype in the control of feeding behaviour, the cloning of the Y5 receptor from hypothalamic tissue, coupled with the pattern of distribution and pharmacology displayed by this receptor (Gerald, 1996) had resulted in the consensus that the Y5 receptor was the NPY receptor responsible for mediating NPY induced feeding behaviour. It is somewhat remarkable that the genes encoding both NPY receptors implicated in the control of ingestive behaviour reside on the same chromosome, and are transcribed in opposite orientations, with a degree of overlap exhibited between the Y5 receptor gene and a distant promoter of the Y1 receptor gene (Herzog, 1997). The Y1 and Y5 receptors display considerable structural diversity, exhibited by the lack of sequence homology between these NPY receptor subtypes. As illustrated in the sequence alignments in chapter 2, the clustering of amino acid sequence observed within the PP-fold receptor gene family, whereby two distinct subfamilies are defined, does not include the Y1

and Y5 receptor subtypes, as these receptor subtypes appear to belong to different, diverse PP-fold receptor subfamilies. A role for both receptors in the regulation of feeding behaviour was substantiated further following the findings of a recent genetic knock out study. Both Y1 and Y5 receptors were demonstrated to play a part in the regulation of feeding, with fasting induced feeding, but not NPY induced feeding impaired in Y1-deficient mice (Pedrazzini, 1998), and feeding induced by high doses of NPY impaired in Y5 deficient mice (Marsh, 1998). A co-operative relationship between these receptors in the regulation of ingestive behaviour has been corroborated by studies with receptor specific antagonists, as administration of the Y1 selective antagonist 1229U91 was capable of inhibiting feeding in Y5 deficient rats (Marsh, 1998). The work presented in chapter 3 of this thesis was directed at the cloning and characterisation of a rat Y5 receptor genomic clone, with the ultimate aim of investigating the regulation of the Y5 receptor gene. As feeding behaviour is undoubtedly complex, involving numerous interactions between neurotransmitters, neuropeptides and hormones, determining the pattern of Y5 receptor gene regulation would enable part of the intricate circuitry which modulates ingestive behaviour to be elucidated. By examining the regulation of both the Y1 and Y5 receptor genes, the role of both receptors in the control of NPY elicited eating could be delineated. Future work carried out would be directed at elucidating further the role of the Y1 and Y5 receptors in the regulation of NPY induced feeding behaviour. Characterising the promoter region of the rat Y5 receptor gene, and identifying transcription factor binding sites within this region, would enable promoter studies to be carried out using a reporter gene construct to detect changes in the level of expression of the Y5 gene following interactions with other known mediators of feeding. By comparing the regulation of the Y1 and Y5 receptor genes, the role of each receptor in the modulation of feeding may be determined, and the role of the co-ordinate regulation of both Y1 and Y5 receptor genes in the control of NPY induced feeding may be established.

The work presented in chapter 6 of this thesis was carried out with the intention of investigating the regulation of both Y1 and Y5 receptors, by examining changes in the levels of receptor mRNA expression. Cell lines constitutively expressing Y1 and Y5 receptor subtypes were used as model systems for the analysis of transcriptional activity following cell treatment with pharmacological agents believed to be involved in gene regulation. Future work would enable the regulation of the Y1 and Y5 receptor genes to be investigated by reporter gene promoter studies, and by examining changes in the levels of Y1 and Y5 receptor mRNA expression.

The role of a novel, previously undiscovered NPY receptor in the regulation of NPY induced feeding remains speculative. As demonstrated by the work presented in this thesis, the lack of homology exhibited by this PP-fold receptor family renders this receptor gene family difficult to exploit in a homology screening approach to receptor cloning. Screening potential target

tissues for additional PP-fold receptors is therefore difficult, as the structural diversity characteristic of this gene family, and the implications that another structurally diverse set of PP-fold receptor may exist, make identification of novel receptor subtypes increasingly challenging. As discussed above, and illustrated by the work presented in this thesis, a functional cloning approach is a more amenable strategy for isolating novel PP-fold receptors, as the affinity of the PP-fold ligand for the receptor subtype effectively overcomes the lack of homology between family members. However, the rarity of the mRNA encoding the feeding receptor for NPY (Gerald, 1996) makes the screening of target cDNA libraries laborious, and the success of the technique relies heavily on the quality of the cDNA library used in the technique. As demonstrated in chapter 2 of this thesis, the presence of secondary structure at the 5' end of the Y5 receptor open reading frame, make cloning by PCR technology difficult. The presence of secondary structure also has implications for a functional cloning approach, as secondary structure present in the receptor mRNA will result in inefficient cDNA synthesis, and the inability of the cDNA to be expressed when transfected into appropriate cells. Therefore, the presence of secondary structure within the mRNA of a novel feeding receptor for NPY, will make isolation of the clone from a cDNA library by a functional cloning approach difficult. The isolation of novel PP-fold receptor subtypes is therefore a challenging pursuit, the lack of homology displayed by this receptor gene family making homology screening an ineffective strategy, while a functional cloning approach may be hindered by mitigating factor such as cDNA quality. An ideal approach for the identification of additional members of this receptor gene family would utilise both cloning techniques, as the function of a clone identified by homology screening could be assessed using an expression cloning technique to ensure that any novel member isolated did not encode a pseudogene, and thereby represented a physiologically functional receptor. Therefore, the potential disadvantages of both cloning techniques may be overcome by combining two different, experimental strategies with essentially identical aims.

As discussed above, the entire number of PP-fold receptors that constitute the family remains to be fully identified, and it is possible that a previously unidentified PP-fold receptor subtype is responsible for the regulation of NPY elicited eating. As the role of PP in the modulation of feeding behaviour remains to be established, with regard to the potential existence of a PP-like peptide member of the PP-fold family, the identity of the feeding receptor may be more complicated. The increase in food intake elicited by PP appears to be mediated by the Y5 receptor, as a Y5 selective antagonist has been demonstrated to attenuate PP induced feeding, with no effect on NPY induced feeding (Kanatani, 1997). The findings described following genetic knock out of the Y5 receptor in mice, also suggest a role for the Y5 receptor in the control of PP feeding, as PP feeding was completely abolished in Y5-deficient mice, while NPY feeding in these animals was unaffected (Marsh, 1998). Therefore it is possible that a novel NPY receptor mediates NPY induced feeding, while the Y5 receptor is responsible for

regulating feeding elicited by an undiscovered PP-like molecule within the brain. The role of a novel receptor subtype with high affinity for a PP-like molecule in the regulation of feeding behaviour remains to be established. The role of a novel PP-like molecule present in the brain in the regulation of feeding behaviour also remains to be delineated. The potent stimulation of feeding observed following PP administration (Clark, 1984), and the clear cut role of this peptide in the regulation of feeding, as illustrated in knock out studies (Marsh, 1998) suggest that the stimulation of feeding elicited by NPY, may be the result of the combined actions of NPY with a novel, undiscovered PP-like molecule.

The cloning of the entire family of PP-fold receptors, and the complete elucidation of the PP-fold peptide family may provide the answers required to determine the receptor subtype involved in NPY induced feeding. Whether a novel PP-fold family member exists, and has a role to play in the regulation of ingestive behaviour also remains to be delineated. For the time being, the PP-fold family of peptides, and the receptor subtypes responsible for mediating their physiological actions, remain an intriguing and incredibly provocative area of research, which undoubtedly presents considerable "food for thought".

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APPENDIX

Appendix I

SOLUTIONS

Ampicillin

100mg/ml stock in distilled water, filter sterilised, stored at -20°C

10% foetal calf medium (COS cells)

Dulbeco's modified Eagles Medium (DMEM) (Imperial) supplemented with :-

New born calf serum (Gibco BRL)	10%
Glutamine	2.0mM
Gentamicin	15µg/ml
Penicillin (Gibco BRL)	100IU/ml
Streptomycin (Gibco BRL)	100µg/ml

10% foetal calf medium (SK-N-MC cells)

Minimum Essential Medium (MEM) (Gibco BRL) supplemented with :-

Foetal calf serum (Sigma)	10%
Non-essential amino acids (10 X)	5ml
Sodium pyruvate (100mM)	5ml
Glutamine	2.0mM

10% foetal calf medium (HEK cells)

RPMI (Imperial) supplemented with :-

Foetal calf serum (Sigma)	10%
Glutamine	2.0mM
Penicillin (Gibco BRL)	100IU/ml
Streptomycin (Gibco BRL)	100µg/ml

5 X Denaturing solution

NaOH	40g
NaCl	146.1g

Make up to 1 litre with distilled water, autoclave to sterilise.

50 X Denhartds reagent

Ficoll	5g
Polyvinylpyrrolidone	5g

Bovine serum albumin	5g

make up to 500ml with distilled water and filter sterilise.

Dexamethasone

10mM stock in dimethly sulfoxide, stored at -70°C

Ethidium bromide stock

10mg/ml in TE, stored away from light

Forskolin

10mM stock in dimethyl sulfoxide, stored at -70°C

Genetrapper™ solutions

Information on the components of the supplied reagents with the Genetrapper™ cDNA positive selection system are not available.

Buffer I

Tris-HCl pH8.0	15mM
EDTA	10mM
RNase A	100µg/ml
RNase T1	1,200 units/ml

Buffer II

NaOH	0.2M
SDS	1%

GTE

Glucose	50mM
Tris-HCl pH8.0	25mM
EDTA	10mM

Hybridisation/Prehybridisation buffer

Denhardts solution (5X)	10ml
SSC (5X)	25ml
20mM NaH_2PO_4	0.7g
10% SDS	1ml
Denatured salmon sperm DNA (10mg/ml)	100µl
Distilled water	65ml

Iodoacetamide

0.5M dissolved in EtOH, stored at 4°C

IPTG

0.1M stock in distilled water, filter sterilised, stored at -20°C.

LB agar plates (Lennox L agar)

SELECT Peptone 140	10.0g	
Yeast extract		5.0g
NaCl		5.5g
Agar		12.0g

premixed (Gibco BRL) ; make up to 1 litre with distilled water.

Lennox L broth base

SELECT Peptone 140	10.0g	
Yeast extract		5.0g
NaCl		5.5g

premixed (Gibco BRL) ; make up to 1 litre with distilled water

10 X Low salt buffer (Boehringer Mannheim)

Tris-HCl		10mM
$MgCl_2$	10mM	
Dithioerythritol (DTE), pH 7.5		1mM

NaCl

5M in distilled water, sterilised by autoclave

4 X Neutralisation solution

Tris-HCl	264.4g
Tris-base	38.8g
NaCl	116.8g

make up to 1 litre with distilled water, autoclave to sterilise

NU medium

DMEM supplemented with :-	
NU serum (Colloborative Research)	10%

Glutamine	2.0mM
Penicillin (Gibco BRL)	100IU/ml
Streptomycin (GibcoBRL)	100µg/ml

Chloroquine was added immediately prior to use at a final concentration of 100µM

PBS (Phosphate buffered saline) (20 X stock)

NaCl	80g
KCl	2g
$Na_2HPO_4.2H_2O$	11.5g
KH_2PO_4	2g

Make up to 500ml with distilled water, sterilise by autoclaving;pH7.4

Potassium Acetate solution (minipreps)

5.0M KOAc	60ml
Glacial acetic acid	11.5ml
Distilled water	28.5ml

This solution is 3.0M with respect to potassium and 5.0M with respect to acetate (~pH 4.8)

10 X RNA electrophoresis buffer

MOPS	0.2M
EDTA	10mM

SOC medium

Distilled water	250ml
Bacto-tryptone	5g
Bacto-yeast extract	1.25g
NaCl	0.125g
250mM KCl	2.5ml

Autoclave to sterilise; immediately prior to use add 1.25ml sterile 2.0M $MgCl_2$ and 5.0M sterile glucose

20 X SSC

NaCl	3M
Citric acid	0.3M

pH to 7.0 with NaOH.

SM buffer

NaCl		5.8g
$MgSO_4.7H_2O$	2.0g	
Tris-HCl pH 7.5 (1M)	50ml	
2% gelatin solution		5ml

Make up to 1 litre with distilled water, sterilise by autoclaving.

TAE (50 X stock)

Tris-base	242g
Glacial acetic acid	57.1ml
0.5M EDTA	100ml

Make up to 1 litre with distilled water

TE

Tris-HCl pH8.0		10mM
EDTA	1mM	

Tetracyclin

Tetracyclin	30mg/ml
Ascorbic acid	15mg/ml

Trypsin (10 X stock)

Trypsin (13,000-20,000 units per mg)	50mg
0.5M EDTA	1ml
1X PBS	99ml

Diluted to working concentration in 1X PBS

X-Gal

2% stock in N' N'-dimethyl formamide; stored at -20°C.

Appendix II

SYNTHETIC OLIGONUCLEOTIDES

Human Y1, Y2 and Y4 receptor degenerate oligonucleotide primers

Sequence title: EC1 forward

5' T A (C/T) A C N (A/C/T)T N A T G G A (C/T) (T/C) A (C/T) T G G (A/G) T (A/G) T T (C/T) 3'=nts 435-464

sequence title: TM7 reverse

5' G G (G/A) T T N A A (G/A) C A N G T N G A N G C C A T N G C 3'= nts 930-956

n= equal mix of A/T/G/C.

Rat NPY Y5 receptor specific primer sequences

(Gerald, 1996)

Sequence title: rat Y5 forward

5' C G T G C G AT C G T T C T T C A A G C T G C T A 3' = nts 1-24

T_m=75.8°C

Sequence title: rat Y5 forward primer (-11)

5' A T G G A G T T T A A G C T T G A G G A G 3' = nts 58-80

T_m=66.9°C

Sequence title: rat Y5 forward primer (MP)

5' C G G A A T G C A G C C T T C C C T G C C 3' = nts 125-146

T_m=76.7°C

Sequence title: rat Y5 internal

5' G G C T C A G C A C T G C T G A G T A G 3' = nts 621-640

T_m=72.4°C

Sequence title: rat Y5 reverse

5' G T G C A C A G A G A G A A T C A T G A C A T G T 3' = nts 1385-1410

T_m=72.5°C

Human NPY Y 5 receptor specific primer sequences

(Hu, 1996)

Sequence title: human Y5 forward

5' G C A G T G T A G A T G A C T T A C A G 3' nts = 121-141

T_m=66.2°C

Sequence title: human Y5 internal

5' C T G A T A G C T A C T G C T T G G A C 3' nts = 492-512

T_m= 68.3°C

Sequence title: human Y5 reverse

5' A G A C A A C A G G A C A T C A T G C C3' nts = 1255-1275

T_m=68.3°C

Genetrapper™ primer sequences

Human Y1/y6 receptor degenerate primer

Sequence title:GT1

5' T (C/T) A C C A(A/G) C A T (A/C) C T G A T T G (C/T) (G/C)3'

Human Y2 receptor homologous primer

Sequence title: GT2

5' G C C T A C T G C T C C A T C A T C T T 3'

Human Y4 receptor homologous primer

Sequence title: GT3

5' C G T G A T G G T C T T C A T C G T C A 3'

Human Y5 receptor homologous primer

Sequence title: GT4

5' G C A G T G T A G A T G A C T T A C A G 3'

Human Y5 receptor (internal) homologous primer

Sequence title: GT5

5' C T G A T A G C T A C T G T C T G G A C 3'

Rat NPY Y1 receptor specific primer sequences

(Eva, 1990)

Sequence title: rat Y1 forward primer

5' A T T C A A C T C T G T T C T C C A G G 3' = nts 72-91

T_m= 66.2°C

Sequence title: rat Y1 internal primer

5' G C T T A C A T A G G C A T T A C T G T 3' =nts 427-446

T_m= 64.2°C

Sequence title: rat Y1 reverse primer

5' A C C A T G T G A C A G G C T GT G C T 3' = nts 1262-1281

T_m= 70.3°C

Human Y1 receptor 3' UT specific primer sequences

(Ball, 1995)

Sequence title: human Y1 3' UT forward primer

5' C A G A C T T G T T C A G T G T T T G T C 3' =nts 2194-2213

T_m= 66.2°C

Sequence title: human Y1 3' UT reverse primer

5' G A T T C T G A G G C T C A A T C A T G 3' = nts 2541-2560

T_m= 66.2°C

Human Y5 receptor 3' UT specific primer sequences

(Herzog, 1997)

Sequence title: human Y5 3' UT forward primer

5' G T G C A G T C A G T G T C A A T C C A 3' = nts 1569-1588

T_m= 68.3°C

Sequence title: human Y5 3' UT reverse primer

5' T A A G A A G C C T A T G G A G T A G A 3' = nts 1791-1810

T_m= 64.2°C