The Role of Insulin Receptor Substrate Signalling Pathways in the Regulation of Energy Homeostasis

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Thesis submitted to the University of London for the degree of Doctor of Philosophy

June 2007
Acknowledgments

I would like to thank Professor Dominic Withers for giving me the opportunity to work in his laboratory and for his guidance and encouragement throughout. I would also like to express my gratitude to the Medical Research Council for sponsoring this research.

I wish to thank all members of the Withers laboratory, past and present, for their support. The positive and friendly atmosphere created by all has contributed greatly to my experiences over the last three years. I am especially grateful to those individuals who have contributed to this work, and particularly thank Dr. Marc Claret, Dr. Melanie Clements, Helen Heffron, Steve Lingard, Dr. Irina Neganova and Dr. Colin Selman for their help and expertise. I would also like to thank my dear friends Sarah Ahmed and Danielle Aw, for their encouragement, support and friendship.

Finally, I am eternally grateful to my parents, Hisham and Nahida, and my sisters Sura and Nuha. Without the love and support of my family, I would be lost and so I dedicate this work to them.
Abstract

Leptin and insulin act as adiposity signals signalling in the brain to regulate energy homeostasis. However, in contrast to leptin, the precise details of the cell types and signalling pathways involved in the actions of insulin have not been well defined. The dominant view in the field at the commencement of this work was largely extrapolated from studies on leptin action. It was therefore suggested that insulin exerted its effects on energy balance by inhibiting orexigenic NPY/AgRP and activating anorexigenic POMC/CART neurons of the arcuate nucleus region of the hypothalamus, thereby coordinately regulating energy homeostasis.

Mouse genetic studies have demonstrated that insulin receptor substrate (IRS) 2, a major downstream effector of insulin signalling, plays a key role in the regulation of glucose and energy homeostasis. Mice lacking Irs2 in all tissues exhibit insulin resistance, hyperglycaemia and β-cell failure. In addition, Irs2 null female mice are hyperphagic, obese and infertile. However, the precise contribution of CNS Irs2 signalling in the actions of insulin and leptin, and the identity of the neuronal circuits in which IRS2 acts to regulate energy homeostasis, are unclear. The PI3K signalling pathway has been implicated in mediating the effects of insulin and leptin, in part acting downstream of IRS signalling. However, the precise hypothalamic cell types in which PI3K signalling acts also remain to be defined.

To address these issues, mice with deletion of Irs2 in all neurons (NesCreIrs2KO), POMC neurons (POMCCreIrs2KO) and AgRP neurons (AgRPCreIrs2KO) were generated. Animals lacking the PI3K p110β catalytic subunit in POMC (POMCCre110βKO) and AgRP neurons (AgRPCre110βKO) were also generated. NesCreIrs2KO animals were obese, hyperphagic and long, suggesting altered melanocortin function. Despite hyperleptinaemia, NesCreIrs2KO animals were leptin sensitive suggesting that IRS2 pathways are not required for leptin action. Reproductive function in NesCreIrs2KO females was normal. In addition, NesCreIrs2KO mice
displayed hyperglycaemia, mild glucose intolerance and hyperinsulinaemia. In contrast, *POMCCreIrs2KO* and *AgRPCreIrs2KO* mice exhibited normal hypothalamic function and glucose homeostasis. *AgRPCreI10βKO* mice were lean and hypophagia. Conversely, *POMCCreI10βKO* animals demonstrated increased adiposity and are hyperphagia.

Taken together, these studies highlight a key role for CNS IRS2 pathways in the regulation of energy homeostasis but demonstrate that CNS IRS2 pathways act in neuronal populations distinct from POMC and AgRP/NPY neurons to regulate energy homeostasis. In contrast, p110β-mediated signals in POMC and AgRP neurons play a key role in the regulation of energy homeostasis. Overall, these studies have provided new insights into the role insulin receptor substrate signalling mechanisms in the hypothalamic regulation of energy homeostasis.
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# Abbreviations

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<td>2-AG</td>
<td>2-arachidonoylglycerol</td>
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<td>α-MSH</td>
<td>Alpha-melanocyte stimulating hormones</td>
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<tr>
<td>3V</td>
<td>Third ventricle</td>
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<td>AgRP</td>
<td>Agouti-related protein</td>
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<td>AMP</td>
<td>Adenosine monophosphate</td>
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<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<td>ARC</td>
<td>Arcuate nucleus</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>BAT</td>
<td>Brown adipose tissue</td>
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<td>Blood brain barrier</td>
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<td>Body Mass Index</td>
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<td>Cyclic adenosine monophosphate</td>
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<td>Cocaine-amphetamine-regulated transcript</td>
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<td>CCK</td>
<td>Cholecystokinin</td>
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<td>CDNA</td>
<td>Complementary DNA</td>
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<td>CNF</td>
<td>Ciliary neurotrophic factor</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CRH</td>
<td>Corticotrophin releasing hormones</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>DEXA</td>
<td>Dual-energy x-ray absorptiometry</td>
</tr>
<tr>
<td>DMH</td>
<td>Dorsomedial hypothalamus</td>
</tr>
<tr>
<td>DMV</td>
<td>Dorso-motor nucleus of the vagus</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunoabsorbance assay</td>
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<td>Extracellular signal-regulated kinase</td>
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<td>FBG</td>
<td>Fasted blood glucose</td>
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<td>Free Fatty Acid</td>
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<td>Fox</td>
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<td>FSH</td>
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<td>Glucose 6-phosphatase</td>
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<td>GTT</td>
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<td>HFD</td>
<td>High fat diet</td>
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<td>HGP</td>
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<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal</td>
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<td>HPO</td>
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<td>I.p.</td>
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<td>Intracerebroventricular</td>
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<td>Igf-1</td>
<td>Insulin growth factor-1</td>
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<td>Immunoglobulin G</td>
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<td>Interleukin 2</td>
</tr>
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<td>Insulin receptor</td>
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<td>IRS</td>
<td>Insulin receptor substrate</td>
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<tr>
<td>JAK2</td>
<td>Janus-kinase 2</td>
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<td>JNK</td>
<td>c-Jun-N-terminal kinase</td>
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<tr>
<td>KATP channel</td>
<td>ATP-activated potassium channel</td>
</tr>
<tr>
<td>Kcal</td>
<td>Kilocalorie</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
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<td>LH</td>
<td>Luteinising hormones</td>
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<td>LHA</td>
<td>Lateral hypothalamus</td>
</tr>
<tr>
<td>LoxP</td>
<td>Locus of 'cross(x)-over'</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MC-R3</td>
<td>Melanocortin 3 receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>MC-R4</td>
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<td>MCH</td>
<td>Melanin-concentrating hormone</td>
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<td>Median eminence</td>
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<td>MEFs</td>
<td>Mouse embryonic fibroblasts</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<td>Melatonin II</td>
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<td>MTOR</td>
<td>Mammalian target of rapamycin</td>
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<td>NIRKO</td>
<td>Neuron-specific insulin receptor knockout</td>
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<td>Neuropeptide Y</td>
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<td>NTS</td>
<td>Nucleus of the solitary tract</td>
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<td>Leptin long-form receptor</td>
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<td>OXM</td>
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<td>p70s6k</td>
<td>P70 ribosomal s6 protein kinase</td>
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<td>PBS</td>
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<td>PC2</td>
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<td>Pck1</td>
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<td>PP</td>
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<td>PTB</td>
<td>Phosphotyrosine binding</td>
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<td>Paraventricular nucleus</td>
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<td>-----------</td>
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<tr>
<td>pY</td>
<td>Tyrosine phosphorylated</td>
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<td>R26R</td>
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<td>Radioummunoassay</td>
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<td>RIP</td>
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<td>RMR</td>
<td>Resting metabolic rate</td>
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<td>Rotations per minute</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<td>SEM</td>
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<td>Src homology-2</td>
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<td>SH2-containing protein</td>
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<td>Shp2</td>
<td>SH2 domain phosphatase-2</td>
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<td>Suppressor of cytokine signalling</td>
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<td>Son of sevenless</td>
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<td>STAT3</td>
<td>Signal transducer and activators of transcription 3</td>
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<td>STZ-DM</td>
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<td>Type 2 diabetes</td>
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<td>Thyrotropin-releasing hormone</td>
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<td>TSC1</td>
<td>Tuberous sclerosis complex 1, hamartin</td>
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<td>UCP</td>
<td>Uncoupling protein</td>
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<td>VMH</td>
<td>Ventromedial hypothalamus</td>
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<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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<tr>
<td>WAT</td>
<td>White adipose tissue</td>
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<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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</table>
Chapter 1

Introduction
1 Introduction

1.1 Obesity: a worldwide epidemic

Obesity is defined medically as a state of increased body weight, more specifically adipose tissue, of sufficient magnitude to produce adverse health consequences (Kopelman, 2000). Traditionally viewed as an affliction solely of Western society, this is no longer the case - the global incidence of obesity has increased by over 75% since 1980 (Flegal et al., 1998). In 2005, approximately 1.6 billion people in the world were overweight, with at least 400 million of these obese, leading the World Health Organisation (WHO) to proclaim obesity as a global epidemic (Cummings and Schwartz, 2003). The WHO further projects that by 2015, approximately 2.3 billion adults will be overweight and more than 700 million will be obese. In the UK alone, approximately 64% of the population is overweight with 23% of these classified as obese (WHO, 2007).

The prevalence of obesity is commonly assessed by using body mass index (BMI), defined as the weight in kilograms divided by the square of the height in metres (kg/m²). A BMI over 25 kg/m² is defined as overweight, and a BMI of over 30 kg/m² as obese (Kopelman, 2000).

1.2 How does excess body fat impact health?

Increased adiposity is a major risk factor for a number of diseases, and is particularly linked with the development of insulin resistance, type 2 diabetes mellitus (T2DM), hypertension, dyslipidaemias, coronary heart disease (CHD), increased incidence of certain forms of cancer, respiratory complications and osteoarthritis (Kopelman, 2000). Although it is difficult to estimate the impact on public health due to its complex interactions with factors such as age and smoking, it has been estimated that in
developed countries 2-7% of total health care costs are attributable to obesity (Seidell et al., 1996). Thus, understanding the mechanisms that regulate energy homeostasis, and the nature of any defects in these systems in obese subjects, has both significant health care and economic implications.

1.3 Regulation of energy balance – a complex homeostatic process

In order for an organism to maintain energy balance it must ensure that energy intake matches energy expenditure. To do so requires the integration of numerous signals such as those indicating the levels of internal energy stores and dietary nutrient content, and adjusting factors such as energy expenditure, hormone levels, and feeding behaviour accordingly.

The finding that body fuel stored in the form of adipose tissue remains relatively constant over time, despite short-term variations in energy intake and expenditure, demonstrates the existence of precise regulatory mechanisms that maintain energy balance (Keesey and Powley, 1986; Stallone et al., 1991). For example, the average individual consumes approximately 900,000 kcal over the course of a year with the estimated age related drift in weight being less than 0.25 kg per year for males between the ages of 20 and 60 years. Such a weight gain implicates an excess energy intake of less than 0.2 % over the whole year. It is highly unlikely that such a precise level of regulation could be maintained without the existence of a complex system that continually matches energy intake and expenditure (Weigle, 1994). Additional evidence for the existence of precise mechanisms aimed at the maintenance of body fuel stores come from various experimental approaches designed to alter body fat content. In all studies, individuals were able to induce compensatory responses that resulted in the restoration of baseline adiposity levels (Drenick and Johnson, 1978; Edholm, 1977; Faust et al., 1977a; Faust et al., 1977b).
1.4 Energy homeostasis and ‘The Lipostatic Theory’

The biological system that regulates body adiposity was hypothesised more than 50 years ago by Gordon Kennedy (Kennedy, 1953). He postulated that humoral signals generated in proportion to body fat stores have the ability to provide negative feedback to brain areas involved in the regulation of energy homeostasis. Thus, when the stability of body fat stores is threatened, the brain receives afferent input in proportion to the current level of body fat, and is then able to engage appropriate mechanisms to maintain fat stores.

Several criteria must be met by an afferent humoral signal (McMinn et al., 2000). To be considered as such a signal, a compound must be secreted in proportion to body fat stores and enter the brain in proportion to its circulating level. In addition, a signal transduction system that mediates the effects of the signal should be located in key CNS regions involved in energy homeostasis regulation. Finally, administration of the putative signal into the circulation or directly into the brain should result in a concordant reduction in food intake and body weight. Currently only two hormones, insulin and leptin, satisfy all the criteria required of such a signal.

1.5 Insulin and leptin: roles in the CNS control of energy homeostasis

1.5.1 Insulin and the central regulation of energy homeostasis

Following Kennedy’s lipostatic theory (Kennedy, 1953), it was Woods and Porte who first hypothesised that insulin represented an afferent signal to the brain that had the ability to couple changes in adiposity to compensatory changes in food intake (Woods et al., 1979). Many lines of evidence, summarised below, have strongly supported this initial hypothesis.
Firstly, levels of plasma insulin correlate directly with body weight and especially with total body adiposity, with higher basal insulin levels found in obese individuals (Bagdade et al., 1967; Polonsky et al., 1988a). In addition, obese animals and humans secrete more insulin in response to a meal than lean individuals (Bagdade et al., 1967; Woods et al., 1974), with the degree of glucose-stimulated secretion a direct function of body fat (Polonsky et al., 1988b).

Secondly, insulin receptors (IR) and IR messenger RNA (mRNA) are widely distributed in key CNS regions involved in the regulation of food intake and body weight. For example, a high density of insulin receptors are found in the arcuate nucleus (ARC) of the hypothalamus, an important candidate region in energy homeostasis regulation by adiposity signals. Consistent with its role as a putative adiposity signal, insulin has been found to enter the brain via a saturable transport process that allows transport from the plasma to brain interstitial fluid (Hachiya et al., 1988).

Finally with regards to the final criterion of a putative adiposity signal, the administration of exogenous insulin directly into the brain has been shown to reduce food intake and increase energy expenditure (Air et al., 2002; Woods et al., 1996; Woods et al., 1974; Woods et al., 1979). Even peripheral administrations of insulin, in amounts that do not cause hypoglycaemia, have been associated with a decrease in food intake (McGowan et al., 1990; Nicolaides and Rowland, 1976; Woods et al., 2003). Consistent with these findings, the administration of antibodies against insulin directly into the brain, also result in an increase in food intake (McGowan et al., 1992; Strubbe and Mein, 1977).

1.5.2 Leptin and the central regulation of energy homeostasis

Twenty years after Kennedy first proposed his lipostatic theory of energy homoeostasis, parabiosis experiments by Coleman added considerable weight to his arguments. Obese
(ob/ob) and diabetes (db/db) animals are two spontaneously occurring murine models of obesity, diabetes and hyperinsulinaemia. When normal mice were surgically coupled to ob/ob animals to create a common circulation, this resulted in reductions in the food intake and body weight of the ob/ob mouse (Coleman, 1973). In contrast, when normal mice were coupled to db/db animals, this resulted in a wasting effect on the parabiotic normal mice (Coleman and Hummel, 1969). Finally, when ob/ob and db/db mice were coupled to one another, ob/ob mice displayed reduced food intake and body weight (Coleman, 1973).

Taken together, these results led to the hypothesis that the ob locus is necessary for the production of a humoral satiety factor, whereas the db locus encoded a molecule required for the response to this factor. As ob/ob and db/db mice are hyperinsulinaemic as expected to their degree of obesity, the missing adiposity signal was unlikely to be insulin.

The hormone leptin (Greek: lepto = thin), a 167 amino acid polypeptide, was discovered in 1994 by positional cloning of the ob locus by Zhang and colleagues, and was heralded as a previously unknown adiposity signal (Zhang et al., 1994). The identification of leptin led rapidly to the cloning of its cognate receptor, encoded by the db locus (Tartaglia et al., 1995).

Leptin, like insulin, meets all the criteria required of a putative adiposity signal. Firstly, it is secreted by adipocytes (and at lower levels in gastric epithelium and the placenta) and circulates in the periphery in direct proportion to total adiposity. The actual stimulus for secretion is related more to the metabolic activity of the fat cell than to actual fat storage (Woods and Seeley, 2000) such that dissociations can occur between stored fat and leptin release (Ahren et al., 1997; Wisse et al., 1999). Nonetheless, over the course of a day, plasma leptin levels are a reliable indicator of body fat.
Secondly, a signal transduction system has also been shown to exist in the brain with leptin being transported into the CNS via a saturable process (Banks et al., 1996; Schwartz et al., 1996b). Leptin receptors (LepRs) have been localised to many brain areas, including within key hypothalamic nuclei implicated in the regulation of energy homeostasis such as the ARC (Banks et al., 1996; Baskin et al., 1999; Schwartz et al., 1996c).

Thirdly, the administration of leptin locally into the brain has been extensively shown to decrease food intake and body weight (Campfield et al., 1995; Pelleymounter et al., 1995; Seeley et al., 1996). This response is dose dependent and not secondary to illness or incapacitation (Thiele et al., 1997).

1.5.3 Other signals involved in the regulation of food intake and body weight

It should be noted that in addition to insulin and leptin, a wide range of other hormones have been shown to affect food intake and body weight (Figure 1.1). These include adipose tissue derived hormones, pancreatic hormones and hormones of the gastrointestinal tract (Stanley et al., 2005). These peripheral signals, involved in the short-term regulation of meal initiation, meal size and satiety, are proposed to interact with the longer-term acting adiposity signals to affect food intake and body weight. Nutrients (for example amino acids, free fatty acids and glucose) cytokines (for example interleukin-6, tumour necrosis factor-alpha) and monoamines (noradrenaline, serotonin, dopamine) have also been implicated as acting in the CNS to regulate food intake and body weight (Havel, 2001).

An in-depth discussion of these hormonal and non-hormonal signals is beyond the scope of this thesis. However the properties of the various signals and their effect on energy homeostasis are summarised in Table 1.1 and Table 1.2 below.
Figure 1.1: Energy homeostasis is controlled by peripheral signals from adipose tissue, pancreas, and the gastrointestinal tract. Peripheral signals from the gut include peptide YY (PYY), oxyntomodulin (OXM), ghrelin, pancreatic polypeptide (PP), glucagon-like peptide 1 (GLP-1), and cholecystokinin (CCK). These gut-derived peptides and adiposity signals influence central circuits in the hypothalamus and brain stem to produce a negative (−) or positive (+) effect on energy balance. Consequently, the drive to eat and energy expenditure can be adjusted to ensure that over a set period of time, the stability of body weight is maintained.
<table>
<thead>
<tr>
<th>Location of main synthesis</th>
<th>Molecule</th>
<th>Function</th>
<th>Effect on energy balance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipose tissue</td>
<td>Adiponectin</td>
<td>Major antidiabetic and antiatherogenic functions. Insulin-sensitising effects, mediated by gluconeogenesis inhibition and the stimulation of fatty acid oxidation.</td>
<td>Reduces body weight. Increases oxygen consumption. Improves insulin sensitivity. Decreases lipid levels.</td>
<td>Berg et al., 2001; Fruebis et al., 2001; Yamauchi et al., 2001; Qi et al., 2004; Yamauchi et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Resistin</td>
<td>Increases insulin resistance in adipose tissue.</td>
<td>Reduces food intake when administered centrally.</td>
<td>Tovar et al., 2005; Wynne et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Amylin</td>
<td>Glucose homeostasis and gastric motility regulation.</td>
<td>Reduces food intake.</td>
<td>Wynne et al., 2005; Wielinga et al., 2007</td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td>Cholecystokinin (CCK)</td>
<td>Promotes gallbladder contraction. Increases secretion of pancreatic enzymes. Inhibits gastric acid secretion. Delays gastric emptying.</td>
<td>Reduces meal size and duration. No effect on food intake due to increased meal frequency.</td>
<td>Gibbs et al., 1973; Kissileff et al., 1981; Stanley et al., 2005</td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td>Ghrelin</td>
<td>Bombesin</td>
<td>Pancreatic Polypeptide YY3-36 (PYY)</td>
<td>Oxyntomodulin (OXM)</td>
</tr>
<tr>
<td>------------------------</td>
<td>---------</td>
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<td>---------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td></td>
<td>Tschop et al., 2000; Wren et al., 2001a; Wren et al., 2001b</td>
<td>Gibbs et al., 1979; Smith et al., 1981</td>
<td>Adrian et al., 1985; Hoentjen et al., 2001; Batterham et al., 2002; Challis et al., 2003; Pittner et al., 2004</td>
<td>Dakin et al., 2001; Dakin et al., 2002; Cohen et al., 2003; Dakin et al., 2004; Wynne et al., 2005</td>
</tr>
</tbody>
</table>

Table 1.1: Peripheral hormonal signals and the regulation of energy balance. Table summarising the location of synthesis of peripheral hormones, and the effect on energy balance upon peripheral administration.
<table>
<thead>
<tr>
<th>Signals acting in the CNS to regulate food intake and body weight</th>
<th>Effect on energy balance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monoamines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serotonin (5-HT)</td>
<td>Anabolic</td>
<td>Blundell, 1984; Leibowitz et al., 1988; Jackson et al., 1997</td>
</tr>
<tr>
<td>Dopamine</td>
<td>Catabolic (LHA) and anabolic (VMH)</td>
<td>Fetissov et al., 2000</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>Catabolic</td>
<td>Leibowitz and Miller, 1969; Leibowitz, 1988</td>
</tr>
<tr>
<td><strong>Amino acid neurotransmitters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gamma-aminobutyric acid (GABA)</td>
<td>Anabolic</td>
<td>van den Pol, 2003</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Catabolic</td>
<td>van den Pol, 2003</td>
</tr>
<tr>
<td><strong>Endocannabinoids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anandamide, 2-arachidonoylglycerol (2-AG), noladin ether, NADA, and virodhamine</td>
<td>Anabolic</td>
<td>Di Marzo and Matias, 2005</td>
</tr>
<tr>
<td><strong>Endogenous opioids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-endorphin, dynorphin, and enkephalins</td>
<td>Anabolic</td>
<td>Cooper et al., 1985; Levine and Billington, 1989; Lambert et al., 1993; Kotz et al., 1997; Levine and Billington, 1997; Kalra et al., 1999</td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumour necrosis factor-alpha (TNF-α), Interleukins-1 (IL1), Interleukin-6 (IL6) and Ciliary neurotrophic factor (CNF)</td>
<td>Anabolic</td>
<td>Plata-Salaman, 1995</td>
</tr>
<tr>
<td>Glucose</td>
<td>Anabolic</td>
<td>Smith and Epstein, 1969; Thompson and Campbell, 1977; Woods et al., 1984; Novin et al., 1985</td>
</tr>
<tr>
<td>Protein (amino acids)</td>
<td>Catabolic</td>
<td>Bray, 1997</td>
</tr>
<tr>
<td>Fat (triglycerides, fatty acids and apolipoproteins)</td>
<td>Catabolic</td>
<td>Woods et al., 1984</td>
</tr>
<tr>
<td>Metabolites (lactate, pyruvate and ketones)</td>
<td>Catabolic</td>
<td>Fisler et al., 1995; Nagase et al., 1996</td>
</tr>
</tbody>
</table>

Table 1.2: Table summarising a variety of non-hormonal signals (of both peripheral and CNS origin) and their effect upon energy balance.
1.6 Roles of insulin and leptin as adiposity signals

The physiological roles that insulin and leptin play in the regulation of food intake and body weight, involves their ability to engage and regulate key neurochemical pathways or ‘central effector’ pathways, located in the brain that influence energy intake and energy expenditure. Central effectors can be divided into two broad categories: anabolic effectors that elicit increased food intake, decreased energy expenditure, and consequently increased stored energy in the form of adipose tissue and catabolic effectors that have the opposite effects (Kaiyala et al., 1995; Schwartz, 1997; Schwartz et al., 2000).

Insulin and leptin are proposed to activate catabolic effector pathways and suppress anabolic effectors pathways (Figure 1.2). For example, in response to fasting and weight loss, adiposity and thus adipose derived factor signalling is decreased. This results in the activation of CNS anabolic effectors and the inhibition of catabolic effectors, resulting in an overall increase in food intake and decrease in energy expenditure (Woods et al., 1998).

Many key details of these catabolic and anabolic effector systems have emerged in the past few years. These systems have been shown to be an interconnected series of discrete neurotransmitter systems and axonal pathways in the brain. The best known of these CNS systems are in the ventral hypothalamus, and there has been extensive investigation into their specific locations over the last century.
Figure 1.2: Model showing how a change in body adiposity is coupled to compensatory changes of food intake. Leptin and insulin are adiposity signals, secreted in proportion to body fat content, which act in the hypothalamus to stimulate catabolic, while inhibiting anabolic, effector pathways. These pathways have opposing effects on energy balance, and it is the balance between these two pathways that determines the amount of body fuel stored as fat. Adapted from Woods et al., 1998.

1.7 Identification of the hypothalamic nuclei involved in the regulation of energy homeostasis

Brain lesioning and electrical stimulation studies, performed over six decades ago, first implicated the hypothalamus as a major centre controlling food intake and body weight (Hetherington, 1940). The hypothalamus consists of many nuclei involved in the regulation of food intake, including the ventromedial hypothalamus (VMH), the dorsomedial hypothalamus (DMH), the lateral hypothalamic area (LHA), the paraventricular nucleus (PVN) and the arcuate nucleus (ARC) (Figure 1.3).

Hetherington and Ranson observed that medial hypothalamic lesions within the ARC, VMH, PVN and DMN nuclei resulted in uncontrolled hyperphagia and obesity (Hetherington, 1940). In contrast, destruction of the lateral hypothalamus resulted in
decreased food intake and starvation. From these observations, the ‘Dual Centre Model’ for regulation of feeding emerged, in which the lateral and ventromedial hypothalamic areas were believed to be the brain centres that controlled hunger and satiety respectively, working in a reciprocal fashion to maintain energy homeostasis (Stellar, 1954). Recent work, discussed in further detail below, has added considerably to our knowledge of the distinct roles of these various nuclei.

Figure 1.3: Main hypothalamic regions involved in the regulation of food intake. A: Longitudinal view of mouse brain, with olfactory bulb at anterior end on the left and the caudal hindbrain on the right. Cross-sections of the brain at two levels (indicated by the vertical lines) are shown as B and C. First order neurons are found in the arcuate nucleus (ARC) and project anteriorly to the paraventricular nucleus (PVN), the perifornical area (PFA) adjacent to the fornix (Fx) and lateral hypothalamic area (LHA). Other regions implicated in regulating food intake include the ventromedial nucleus (VMN) and the dorsomedial nucleus (DMN). Abbreviations of other brain structures: AM, amygdala; CC, corpus callosum; CCX, cerebral cortex; HI, hippocampus; ME, median eminence; OC, optic chiasm; SE, septum; TH, thalamus; 3V, third ventricle. Adapted from Barsh and Schwartz, 2002.
1.7.1 The paraventricular nucleus

The paraventricular nucleus (PVN) lies beside the top of the third ventricle in the anterior hypothalamus. This nucleus is an integrating centre on which a number of neural pathways that influence energy homeostasis converge. It is rich in terminals containing numerous appetite modifying neurotransmitters including; neuropeptide Y (NPY), alpha-melanocyte stimulating hormone (α-MSH), serotonin (5-HT), galanin, noradrenaline and the opioid peptides (Elmquist et al., 1998a; Elmquist et al., 1999). Corticotrophin releasing hormone (CRH) and thyrotrophin releasing hormone (TRH) are also expressed by neurons in the PVN that project to the median eminence (ME) (Arase et al., 1988).

A key role for neurons of the PVN in the regulation of food intake has been suggested by a number of studies (Michaud et al., 2001; Sawchenko, 1998; Weingarten et al., 1985). When administered specifically into the PVN, α-MSH suppresses feeding (Audinot et al., 2002). The recent work of Balthasar and colleagues supports this and suggests an important role for melanocortin 4 receptors (MC4-R) of the PVN in the control of food intake (Balthasar et al., 2005).

1.7.2 The ventromedial nucleus

The ventromedial hypothalamic nucleus (VMH) is one of the largest hypothalamic nuclei. Recent studies have shown a high abundance of leptin receptors in neurons of the VMH, and evidence indicates that this region may be an important target for circulating leptin (Meister et al., 1989). The VMH has direct connections with the DMH, PVN and the LHA (Williams et al., 2001).

1.7.3 The dorsomedial nucleus

The dorsomedial hypothalamus (DMH) is located immediately dorsal to the VMH. It has extensive direct connections with other medial hypothalamic nuclei such as the PVN, the
lateral hypothalamic area (LHA) and the brainstem. Neurons within the DMH have been shown to contain a high number of both insulin and leptin receptors on their surface (Kalra et al., 1999).

1.7.4 The lateral hypothalamic area

The lateral hypothalamic area (LHA) is vaguely defined and comprises a large diffuse population of neurons including defined subpopulations that express orexins and melanin-concentrating hormone (MCH) (de Lecea et al., 1998; Marsh et al., 2002). NPY, AgRP (agouti-related protein) and α-MSH terminals are abundant in the LHA, and are in contact with the orexin and MCH cells (Broberger et al., 1998a; Elias et al., 1998; Horvath et al., 1999).

1.8 The arcuate nucleus: a primary sensor of peripheral signals

The arcuate nucleus (ARC), which consists of an elongated collection of neuronal cell bodies, is located in the mediobasal hypothalamus above the base of the third ventricle (3V). This nucleus has extensive reciprocal connections with other hypothalamic regions, including the PVN, DMH, VMH and LHA (Figure 1.3).

The ARC is uniquely positioned to act as a site of metabolic sensing and to integrate endocrine information regarding the supply and demand of energy in the body. This is because the ARC-ME area is one of the ‘circumventricular’ organs in which the blood-brain barrier (BBB) is specially modified. Thus, neurons within this area are accessible to circulating hormones and nutrients (Broadwell and Brightman, 1976). It has been proposed that nutrients and hormones may also gain access to the ARC by diffusion across the ependyma from the cerebrospinal fluid (CSF) in the third ventricle (Elmquist et al., 1998b).
Two primary adjacent populations of neurons within the ARC are hypothesised to integrate signals of nutritional status and influence energy homeostasis (Cone et al., 2001). The first of these neuronal populations co-express the orexigenic NPY and AgRP neuropeptides that affect energy balance by their ability to stimulate food intake and decrease energy expenditure. The second neuronal population co-expresses the anorexigenic POMC and cocaine-amphetamine-regulated-transcript (CART) neuropeptides, which affect energy balance by their ability to inhibit food intake and stimulate energy expenditure.

Projections between ARC POMC/CART and NPY/AgRP neurons are thought to allow cross-talk and hence a co-ordinated response (Cowley et al., 2001). These neuropeptide systems located in the ARC are discussed in further detail below.

1.9 POMC/CART anorexigenic neurons

The POMC gene encodes a 31-36 kDa pre-pro-hormone from which seven mature peptide hormones are derived via post-translational cleavage by various prohormone convertases, such as pro-hormone convertases 1 and 2 (PC1 and PC2) (MacNeil et al., 2002). Of all the POMC derived peptides, α-MSH is considered the most important regulator of feeding (Hillebrand et al., 2002).

CART is an anorexigenic neuropeptide, originally identified by differential display polymerise chain reaction (PCR) as a novel mRNA, stimulated after acute administration of psychomotor stimulants (Douglas et al., 1995). In the rat, the CART precursor is spliced into two products of 116 and 129 amino acids. These two splice products are further processed in a tissue specific manner (Douglas et al., 1995). Currently five forms of CART are known; CART\textsubscript{55-86}, CART\textsubscript{54-102}, CART\textsubscript{55-102}, CART\textsubscript{61-102} and CART\textsubscript{62-102} (Thim et al., 1999).
1.9.1 Neuroanatomy of POMC/CART neurons

Within the CNS, the major site of POMC expression originates in neurons of the ARC. In the mouse brain there are approximately 3,000 POMC positive cells. These neurons also co-express CART. POMC/CART cell bodies are found throughout the rostrocaudal extent of the ARC and periarcurate area of the hypothalamus (Jacobowitz and O'Donohue, 1978; Joseph et al., 1983; Nilaver et al., 1979; Watson et al., 1978). From the ARC, POMC/CART neurons project broadly to other hypothalamic nuclei including the PVN, DMN, LHA and VMH (Bagnol et al., 1999; Elias et al., 1999; Jacobowitz and O'Donohue, 1978). These neurons also send descending sets of projections to the brainstem (Cone, 2005). Approximately half of the α-MSH immunoreactivity in the brainstem is thought to derive from hypothalamic POMC/CART neurons and the other half from a smaller (~300) population of POMC neurons in the brainstem (Joseph and Michael, 1988; Pilcher et al., 1988).

1.9.2 Effect of POMC and CART on energy balance

Both chronic infusions and acute intracerebroventricular (ICV) administration of α-MSH or synthetic analogues such as melatonin II (MTII) have been shown to result in strong reductions in food intake, body weight and adiposity in wild type and genetically obese rodents (Li et al., 2003; Pierroz et al., 2002; Williams et al., 2001; Yaswen et al., 1999). It has been demonstrated that administration of these peptides also increases metabolic rate and sympathetic outflow (Dunbar and Lu, 2000) and partially compensates for the alteration in body temperature regulation in leptin deficient ob/ob mice (Forbes et al., 2001). When injected into the third ventricle α-MSH also significantly inhibits food intake of food-deprived rats (Tsukamura et al., 2000).

Both acute and chronic ICV injection of CART peptide dose dependently reduce food intake, body weight and NPY-induced food intake in lean animals (Kristensen et al., 1998; Lambert et al., 1998) and also obese fa/fa rats (Larsen et al., 2000). Consistently, injections
of CART antibodies increase food intake in rodents (Kristensen et al., 1998; Lambert et al., 1998). The ICV administration of CART has also been shown to induce activation of the hypothalamic-pituitary-adrenal (HPA) axis (Stanley et al., 2001) and inhibit gastric acid secretion and gastric emptying (Okumura et al., 2000). An important role for CART in energy metabolism is also suggested by the finding that CART injection in the PVN results in increased expression of uncoupling proteins (UCPs) I, II and III in adipose tissue and muscle (Kong et al., 2003; Wang et al., 2000).

1.9.3 Melanocortin and CART receptors

The effect of the melanocortins is mediated by the melanocortin receptors (MC-Rs) of which five are currently known. These are MC-R1, MC-R2, MC-R3, MC-R4 and MC-R5. They are G-protein-coupled receptors (GPCRs). When activated, GPCRs function through adenylate cyclase to increase intra-cellular cyclic adenosine monophosphate (cAMP) concentrations. These receptors have differing affinity for POMC products and are expressed at varying levels in different tissues (Mountjoy et al., 1994). Both MC-R3 and MC-R4 are expressed in hypothalamic nuclei implicated in energy homeostasis including the VMH, DMH and ARC (Harrold et al., 1999; Roselli-Rehfuss et al., 1993). Interestingly, POMC and AgRP neurons in the ARC selectively express MC-R3 but not MC-R4, suggesting a role for this receptor in feedback regulation of the melanocortinergic circuitry (Bagnol et al., 1999).

Genetic studies in mice suggest that both MC-R3 and MC-R4 mediate pathways regulating energy expenditure and adiposity, but only the MC-R4 mediates the anorectic/feeding effects of the melanocortins axes (Butler et al., 2000; Butler et al., 2001; Chen et al., 2000; Huszar et al., 1997).

The mechanisms that mediate the effects of CART are still poorly understood, and so far no receptor has been identified.
1.9.4 Evidence for the role of POMC and CART from murine models

Further evidence for a key role for α-MSH in the central control of feeding and energy balance comes from genetic studies in rodents and humans. Humans with α-MSH deficiency due to either a mutation in the Pomp sequence or processing of the POMC polypeptide demonstrate hypopigmentation, adrenal insufficiency and early onset obesity (Carroll et al., 2005; Krude et al., 2003; Obici et al., 2001).

Targeted deletion of the Pomp gene in mice also results in obesity. This has been shown to be due to reductions in metabolic rate, alterations in lipid metabolism and hyperphagia (Challis et al., 2004; Yaswen et al., 1999). The loss of even one copy of the Pomp gene in mice has been demonstrated to be sufficient to render these animals highly susceptible to diet induced obesity (DIO) (Challis et al., 2004).

Despite the described anorexigenic effects of CART on energy homeostasis, Cart null mice display normal body weight and food intake on a normal chow diet but do appear to be less sensitive to pain (Bannon et al., 2001). On a high fat diet (HFD), these animals exhibit an increase in food intake, body weight and fat mass (Wortley et al., 2004).

1.10 NPY/AgRP orexigenic neurons

NPY, first isolated in 1982, is a 36 amino acid neurotransmitter belonging to the pancreatic polypeptide (PP) family (Tatemoto et al., 1982). Processing of the pro-NPY peptide results in the formation of six NPY fragments, with the effect of NPY on feeding thought to be mediated by the NPY(1-36) intact peptide (Flood and Morley, 1989).

Agouti is normally expressed within the hair follicle where it antagonises α-MSH on the MC-R1, inducing a colour switch from eumelanin (black/brown) to phaeomelanin (red/yellow) (Barsh et al., 1999; Ollmann et al., 1998; Wolff et al., 1999). The yellow coat,
hyperphagia and obesity of the A\textsuperscript{y} mouse is known to be due to widespread ectopic expression of this protein (Bultman et al., 1992; Morgan et al., 1999). The existence in the brain of an "agouti-like" protein was suggested by the fact that though only normally expressed in hair follicles, the agouti protein is a highly specific MC-R4 antagonist (Lu et al., 1994). This hypothesis was confirmed when the \textit{agouti-related protein (Agrp)} gene was isolated in 1997 and found to encode a 12 amino acid peptide with 25% homology to the agouti protein (Shutter et al., 1997).

1.10.1 Neuroanatomy of NPY/AgRP neurons

The main site of NPY expression in the hypothalamus is within the ARC, where it is co-localised with agouti-related protein (AgRP) in 90% of neurons (Broberge et al., 1998b). ARC NPY/AgRP neurons project to adjacent hypothalamic nuclei including dorsally and anteriorly to the PVN, DMH, VMH, LHA, perifornical area (PFA) and also ventrally to the ME. The ARC-PVN projection is particularly dense (Bai et al., 1985). In some hypothalamic nuclei such as the PVN and DMH, AgRP is thought to be released from NPY endings in the same synaptic complex as \textit{\textalpha}-MSH and acts as a competitive antagonist at both the MC-R3 and MC-R4 (Ollmann et al., 1997).

1.10.2 Effects of NPY and AgRP on energy balance

Several studies have shown NPY\textsubscript{(1-36)} to be a potent orexigenic peptide with the ability to over-ride both short and long term mechanisms of satiety and body weight regulation (Akabayashi et al., 1994; Stanley et al., 1986). A single bolus ICV injection of NPY\textsubscript{(1-36)} results in the potent stimulation of food intake in rodents, sheep and monkeys (Clark et al., 1984; Larsen et al., 1999; Miner et al., 1989). NPY administration has also been shown to decrease energy expenditure (Billington et al., 1991), suppress sympathetic nerve activity (Egawa et al., 1991), inhibit the thyroid axis (Fekete et al., 2002) and increase lipogenesis of white adipose tissue (WAT) (Billington et al., 1991; Stanley et al., 1986).
Similarly to NPY, injections of AgRP and synthetic AgRP analogues have been shown to stimulate food intake, increase body weight, decrease oxygen consumption and decrease the capacity of brown adipose tissue (BAT) to expend energy (Fan et al., 1997; Rossi et al., 1998). The stimulatory effect of a single injection is apparent within 4 hours and lasts up to 6 days, which is a longer duration than seen with NPY.

A physiological role for the hypothalamic NPY/AgRP system in energy homeostasis is also suggested by the fact that the production and release of these neuropeptides is affected by changes in energy balance and nutritional state (Kalra et al., 1999). For example, in a state of negative energy when leptin levels are low (e.g. during fasting) or deficient (ob/ob mouse), expression levels of NPY are increased (Schwartz et al., 1996c; Wilding et al., 1993). In contrast, in states of positive energy balance, both leptin and insulin decrease NPY expression in the ARC (Krysiak et al., 1999; Schwartz et al., 1992). With regards to AgRP, an 18-fold increase in AgRP mRNA is observed after a 48-h fast in mice (Hahn et al., 1998). In contrast, levels are reduced by DIO in rats (Harrold et al., 1999).

1.10.3 Receptors

Recently six different NPY receptors have been characterised. These are GPCRs and have been designated Y₁–Y₆ (Larhammar, 1996). These not only mediate the effects of NPY but also other members of the pancreatic polypeptide (PP) family such as peptide YY (PYY) and pancreatic polypeptide (PP). Cloning studies have confirmed the existence of NPY-Y1, -Y2, -Y4 and Y5 receptors as distinct entities in the hypothalamus (Williams et al., 2001), where as the NPY-Y3 receptor subtype has been mapped mainly to the nucleus tractus solitarius (NTS) of the brainstem (Grundeman et al., 1991).

The C-terminal region of AgRP, which is sufficient for receptor binding, shares ~40% sequence homology with agouti, although they bind to distinct sets of receptors. Agouti binds to MC-R1 and MC-R4 while AgRP acts as a high affinity antagonist to MC-R3 and
MC-R4 (Ollmann et al., 1997; Yang et al., 1997). By being able to bind to MC-R3 and MC-R4 in the ARC, AgRP acts as an antagonist to α-MSH at these receptors.

1.10.4 Evidence for role of NPY and AgRP from murine models

Although a large body of evidence indicates that NPY is an important orexigenic neuropeptide, mice on a mixed genetic background lacking NPY show no abnormalities in food intake or body weight regulation (Qian et al., 2002; Thorsell and Heilig, 2002). This is possibly due to the presence of compensatory mechanisms or alternative orexigenic pathways, as well as redundancy in orexigenic signalling (Wynne et al., 2005). Npy null animals do however demonstrate an increase in fast-induced feeding (Bannon et al., 2000).

Interestingly, on a C57BL/6 background, Npy ablation is associated with an increase in body weight and adiposity and a significant defect in refeeding after a fast. This indicates that genetic background must be taken into account when the biological role of NPY (and indeed all genes) is studied (Segal-Lieberman et al., 2003). When Npy is genetically knocked out in ob/ob mice, this results in reduction of the hyperphagia and obesity associated with these animals, indicating the full response to leptin deficiency requires intact NPY signalling (Erickson et al., 1996).

The importance of AgRP in the regulation of appetite was confirmed with the development of transgenic mice over-expressing AgRP in the hypothalamus (Ollmann et al., 1997). These transgenics, although retaining their wildtype coat colour, were found to phenotypically resemble both the A' and MC-R4 null mice. They exhibited hyperphagia, obesity, increased body length, hyperinsulinaemia, late onset hyperglycaemia and pancreatic-islet hyperplasia (Ollmann et al., 1997).

At around 2-3 months of age, Agrp null animals exhibit subtle changes in response to feeding, following both a fast and treatment with MTII (Wortley et al., 2005). From 6
months of age onwards, AgRP null animals exhibit robust reductions in body weight and adiposity, increased metabolic rate, increased body temperature, and increased locomotor activity. In addition, Agrp null animals display increased levels of circulating thyroid hormone and increased expression of UCP-1 in BAT. These results provide further proof of the importance of the AgRP neuronal system in the regulation of energy homeostasis. With regards to the role of the actual neuron, four different approaches have been used to selectively target AgRP neurons for death, with all studies reporting a lean and hypophagic phenotype (Bewick et al., 2005; Gropp et al., 2005; Luquet et al, 2005; Xu et al, 2005a).

1.11 A model for the central control of food intake and body weight by insulin and leptin

Prior to the commencement of this work, the dominant view held in the field was that insulin and leptin regulated energy homeostasis via their actions on the POMC/CART and NPY/AgRP neurons of the ARC. This model is outlined below.

Insulin and leptin in the circulation enter the ARC region of the hypothalamus via the ME where the BBB is not present. They bind to their corresponding receptors present on the first-order NPY/AgRP and POMC/CART neurons. NPY/AgRP neurons are inhibited by leptin and insulin and are consequently activated in conditions where insulin and leptin are low. POMC/CART neurons are stimulated by leptin and insulin and are consequently activated in conditions where insulin and leptin are high (Schwartz et al., 2000). These first order neurons in the ARC integrate information concerning the energy balance of the individual and relay this to second-order neurons located in other hypothalamic regions involved in the control of food intake and body weight (Figure 1.4). For example, ARC NPY/AgRP and POMC/CART neurons project to TRH neurons of the PVN (Fekete et al., 2000). This ensures the transduction of peripheral signals into behavioural and metabolic responses that attempt to maintain body fat stores at a constant level (Barsh and Schwartz, 2002). Table 1.3 below summarises the different second-order neuronal populations of the various 'downstream' hypothalamic nuclei involved in transduction of the insulin/leptin adiposity signal into a response which effects the overall energy homeostasis status of the individual.
Figure 1.4: Summary of the control of energy homeostasis by arcuate nucleus neurons. The adiposity signals, insulin and leptin, circulate in proportion to body adipose stores and inhibit NPY/AgRP neurons and stimulate the adjacent POMC/CART neurons. Lower insulin and leptin levels are therefore predicted to activate NPY/AgRP neurons, while inhibiting POMC/CART. Ghsr, growth hormone secretagogue receptor; Lepr, leptin receptor; Mc3r/Mc4r, melanocortin 3/4 receptor; Y1r, neuropeptide Y1 receptor. Adapted from Barsh and Schwartz, 2002.
<table>
<thead>
<tr>
<th>Hypothalamic Nucleus</th>
<th>Neuropeptide</th>
<th>Effect on energy Balance</th>
<th>Regulation by adiposity signals</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARC</td>
<td>Neuropeptide Y (NPY)</td>
<td>Anabolic</td>
<td>↓</td>
<td>Barsh and Schwartz, 2002</td>
</tr>
<tr>
<td></td>
<td>Agouti-related protein (AgRP)</td>
<td>Anabolic</td>
<td>↓</td>
<td>Barsh and Schwartz, 2002</td>
</tr>
<tr>
<td></td>
<td>Pro-opiomelanocortin (POMC)</td>
<td>Catabolic</td>
<td>↑</td>
<td>Barsh and Schwartz, 2002</td>
</tr>
<tr>
<td></td>
<td>Cocaine-amphetamine-regulated transcript (CART)</td>
<td>Catabolic</td>
<td>↑</td>
<td>Barsh and Schwartz, 2002</td>
</tr>
<tr>
<td>LHA</td>
<td>Melanin concentrating hormone (MCH)</td>
<td>Anabolic</td>
<td>↓</td>
<td>Qu et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Orexin A and B</td>
<td>Anabolic</td>
<td>↓</td>
<td>Sakurai et al., 1998</td>
</tr>
<tr>
<td>PVN</td>
<td>Thyrotropin releasing hormone (TRH)</td>
<td>Catabolic</td>
<td>↑</td>
<td>Lechan and Fekete, 2006</td>
</tr>
<tr>
<td></td>
<td>Corticotrophin releasing hormone (CRH)</td>
<td>Catabolic</td>
<td>↑</td>
<td>Rothwell, 1990; Mercer et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Galanin-like peptide</td>
<td>Anabolic</td>
<td>↓</td>
<td>Gundlach, 2002</td>
</tr>
</tbody>
</table>

Table 1.3: Summary of the main first-order and second-order neuronal populations found in the various hypothalamic nuclei implicated in energy homeostasis. The effect of the neuropeptides on energy balance and the regulation of their expression by adiposity signals are also summarised.
1.12 Role of the CNS in the regulation of peripheral glucose homeostasis

In addition to its role in energy balance regulation, recent work has also highlighted a role for the brain in the regulation of peripheral glucose homeostasis and compensatory responses to hypoglycaemia (such as pancreatic glucagon release and sympathoadrenal activation). Lesions in the VMH of rats have been shown to suppress counter regulatory responses to hypoglycaemia (Borg et al., 1994) consistent with previous findings implicating a key role for this nucleus in regulating peripheral glucose homeostasis (Oomura et al., 1969). Specific neurons in both the VMH and LHA have also been shown to have the capability of sensing changes in ambient glucose concentrations (Funahashi et al., 1999; Oomura et al., 1969).

Such a role for the CNS is not a novel concept and in fact was proposed more than 150 years ago by the French physiologist Claude Bernard. He showed in rabbits that “piqûre” (pricking) of the floor of the fourth ventricle of the brain resulted in glucosuria (Bernard, 1855), and thus hypothesised that the CNS plays an essential role in the control of peripheral blood glucose levels.

Such evidence implicating hypothalamic pathways as key regulators of glucose homeostasis has renewed interest in the mechanisms linking obesity and diabetes, and suggests a role for hypothalamic dysfunction in the pathophysiology of diabetes.

1.12.1 Central insulin action and peripheral glucose metabolism

In addition to the direct effects of insulin on the liver, its role in regulating hepatic glucose production (HGP) via signalling events in the hypothalamus has drawn increasing attention. Insulin has been shown to modify peripheral glucose homeostasis through hypothalamic insulin receptors. NIKKO mice, with targeted disruption of the IR specifically in the brain, do not suppress HGP normally in response to a hyperinsulinaemic clamp (Fisher et al., 2005) In addition, central insulin administration has been shown to result in increased insulin sensitivity of peripheral tissues associated with a reduction in HGP (Obici et al.,
Interestingly, blockade of central ATP-activated potassium channel ($K_{\text{ATP}}$ channels) with the sulfonylurea tolbutamide abolishes this effect (Obici et al., 2002b).

In support for the role of $K_{\text{ATP}}$ channels in mediating the effects of central insulin action on peripheral glucose utilisation, a recent study has shown that insulin acts on $K_{\text{ATP}}$ channels in hypothalamic neurons to control HGP via a method involving a decrease in hepatic glucose-6-phosphatase and phosphoenolpyruvate kinase expression (Pocai et al., 2005). Transection of the vagal nerve to the liver nullified this effect, pointing to a role for the autonomous nervous system in transmitting insulins central actions to the periphery. Despite significant advances in understanding with regards to the signalling events involved in $K_{\text{ATP}}$ channel activation, the exact identity of the hypothalamic neuronal populations involved in the regulation of hepatic glucose output are still unclear. However, two recent studies have implicated the ARC as a key site for regulating peripheral glucose homeostasis.

In STZ-DM rats (with uncontrollable diabetes induced by streptozotocin), the blockade of hypothalamic insulin signalling was achieved via the infusion of a PI3K inhibitor into the ARC, prior to peripheral insulin injection. This was found to attenuate insulin-induced glucose lowering by approximately 35% - 40% (Gelling et al., 2006). Conversely, increased PI3K signalling, induced by hypothalamic overexpression of either IRS2 or AKT, enhanced the glycaemic response to insulin by approximately 2-fold in STZ-DM rats. These results thus indicate a crucial role for intact ARC insulin signalling in the peripheral response to insulin treatment.

In the second study, Morton et al (Morton et al., 2005) also used adenoviral microinjection techniques to express the LepR or a constitutively active mutant of AKT in the mediobasal hypothalamus. In both cases they showed that this resulted in improved peripheral insulin sensitivity and glucose response in rats lacking a functional leptin receptor (Morton et al., 2005).

Although the use of such microinjecting adenoviral technology does not allow the specific targeting of specific cell types, these studies have nonetheless highlighted a key role for the
ARC in the regulation of peripheral glucose homeostasis. Although the exact identity of the hypothalamic neurons that control hepatic glucose output remain unknown, the melanocortin pathway has been implicated (Farooqi et al., 2003; Marks and Cone, 2001; Obici et al., 2001; Schwartz, 2001). Melanocortin signalling acutely affects insulin levels and glucose uptake, and humans with MC-R4 mutations are extremely insulin resistant, more so than would be predicted by their degree of obesity (Fan et al., 1997; Farooqi et al., 2003; Obici et al., 2001).

1.13 Obesity, diabetes and insulin resistance

1.13.1 A triad of metabolic disorders

Obesity, insulin resistance and type 2 diabetes mellitus (T2DM) are a tightly linked common triad of metabolic disorders (Kahn and Flier, 2000). Despite major advances in unravelling the complex networks regulating food intake and energy balance, our understanding of the precise pathophysiological relationships between obesity, insulin resistance and T2DM is still rudimentary.

1.13.2 Diabetes Mellitus

Diabetes mellitus is currently the most common metabolic disease in the world with more than 180 million individuals affected worldwide. Global estimates for the year 2030 predict a further growth of more than 50%, to reach a total of 366 million affected individuals. Most of this increase will occur as a result of a 150% rise in the developing countries of Africa, Asia, and South America (WHO, 2007).

Of all of those with diabetes worldwide, 90% of individuals have T2DM, the non-insulin-dependent form of diabetes (Cummings and Schwartz, 2003).
1.13.3 Diabetes- a life threatening condition

Cardiovascular morbidity in patients with T2DM is two to four times greater than that of non-diabetic people (Zimmet et al., 2001). It is also important to note that the life expectancy of affected individuals is also reduced (Damsgaard et al., 1997; Gu et al., 1998; Sasaki et al., 1997). Indeed, each year 3.2 million deaths are attributable to diabetes worldwide (WHO 2007).

1.13.4 The pathophysiology of T2DM

T2DM is a polygenic disease and may involve polymorphisms in multiple genes encoding proteins involved in insulin signalling, insulin secretion and intermediary metabolism. Identifying the genetic variants that increase risk of T2DM has been a significant challenge. Recent studies have added considerably to our knowledge of the molecular mechanisms involved in the development of this disease.

The latest genome wide association studies of T2DM have revealed diabetes susceptibility loci in and around the genes IGF2BP2, CDKAL1, CDKN2A/CDKN2B, TCF7L2, SLC30A8, HHEX, FTO, PPARG, and KCNJ1 (Saxena et al., 2007; Scott et al., 2007; Sladek et al., 2007; Zeggini et al., 2007). These studies confirm the existence of at least ten T2DM loci, and emphasise the contribution of multiple variants of modest effect. The regions identified underscore the importance of pathways influencing pancreatic β-cell development and function in the etiology of T2DM.

Environmental factors especially diet, physical activity and age, can also interact with genetic predisposition to affect disease prevalence and severity (Stern, 2000). T2DM is preceded by the development of insulin resistance in target tissues, but the molecular causes of its initiation and progression are still unclear.
1.13.5 Insulin resistance

In the initial stages of insulin resistance, islets initially compensate for the loss of sensitivity by expanding β-cell mass and insulin secretory capacity. Eventually this heightened demand cannot be met resulting in loss of β-cells and reduction in circulating insulin levels. This is despite continuing peripheral insulin resistance, and the consequence is chronic hyperglycaemia and overt T2DM (Stumvoll et al., 2005).

Insulin resistance is not only a fundamental aspect of the etiology of type 2 diabetes, but is also linked to a wide array of other conditions including hypertension, hyperlipidemia, atherosclerosis and polycystic ovarian disease (Kahn and Flier, 2000). It is characterised by defects at many levels including decreases in receptor concentration and kinase activity, concentration and phosphorylation of insulin receptor substrate proteins, phosphatidylinositol 3-kinase (PI3K) activity, glucose transporter translocation and the activity of intracellular enzymes (Saltiel and Kahn, 2001).

A combination of both genetic and environmental factors can profoundly influence insulin sensitivity. A strong genetic component for insulin resistance is suggested by the concordance between monozygotic twins and increased prevalence in first-degree relatives of affected individuals (Kahn et al., 1996). With regards to environmental factors, although obesity is the predominant risk factor for the onset of insulin resistance, other environmental factors such as physical inactivity also contribute. The precise mechanisms whereby obesity causes insulin resistance are complex and not completely understood.

1.14 The role of insulin in peripheral glucose homeostasis

To understand the cellular and molecular mechanisms responsible for T2DM, it is necessary to conceptualise the framework within which glycaemia is controlled (Figure 1.5).
1.14.1 Pancreatic islet function and glucose uptake

Plasma glucose levels are tightly regulated between 4 and 7 mM in normal individuals (DeFronzo, 2004). This narrow range is governed by a balance between glucose gut absorption, hepatic glucose production and uptake of glucose by peripheral tissues and organs. The balance between the utilisation and production of glucose is maintained at equilibrium by the opposing actions of insulin and glucagon. Increasing blood glucose levels result in the release of insulin from β-cells, which stimulates glucose transport into muscle and fat via GLUT4 glucose transporter mechanisms, whilst inhibiting hepatic glucose production (Kahn and Flier, 2000). Decreasing glucose levels result in the release of glucagon from the α-cells within the islets of Langerhans. This in turn results in the release of glucose stores and newly synthesised glucose into the bloodstream. Thus, the co-ordinated action of insulin and glucagon ensure that glucose homeostasis is maintained throughout a wide variety of physiological states (DeFronzo, 2004).

Figure 1.5: Regulation of plasma blood glucose by opposing actions of insulin and glucagon.
1.15 Insulin receptor signalling pathways

The binding of insulin to its receptor induces a cascade of intracellular signalling events that regulate key cellular functions, including glucose transport and oxidation, synthesis of glycogen, triglyceride and protein, and also regulation of gene expression (Nandi et al., 2004).

1.15.1 The insulin receptor family

The insulin receptor (IR) is present in virtually all vertebrate tissues although the level of expression varies. It belongs to a subfamily of receptor tyrosine kinases (RTKs) that includes the insulin like growth factor 1 (IGF-1) receptor and the insulin receptor-related receptor (IRR). These receptors are tetrameric proteins consisting of two alpha (α) and two beta (β) subunits, that function as an allosteric enzyme in which the α-subunit inhibits the tyrosine kinase activity of the β-subunit. Insulin binding to the α-subunit leads to de-repression of the kinase activity in the β-subunit followed by transphosphorylation of the β-subunits and a conformational change that further increases the kinase activity (Kido et al., 2001). In addition to binding insulin, the IR can bind IGF-1 and IGF-2.

1.15.2 Insulin receptor substrate proteins

Following binding of insulin, subsequent conformational changes expose the catalytic domain of the intrinsic receptor kinase that results in tyrosine phosphorylation of cytosolic insulin receptor substrate (IRS) proteins.

At present the family of insulin/IGF-1 receptor kinase substrates contains at least thirteen members. This includes four insulin receptor substrate (IRS) proteins, three Src-Homology Collagen (SHC) proteins, growth factor receptor bound-2 (Grb-2) associated binder-I (Gab-1), and five members of the p62dok family (DOK1, DOK2, DOK3, DOK4 and DOK5) (Van Obberghen et al., 2001).
11.5.2.1 *Irs proteins*

The insulin receptor substrate (IRS) proteins represent key elements in insulin and IGF action. The IRS1 and IRS2 proteins are 185 kDa and 190 kDa in size respectively. Both are widely expressed in humans and mice. IRS3, a 60 kDa protein is largely restricted to adipose tissue. Expression of IRS4, a 160 kDa protein, is restricted to the thymus, brain, kidney and possibly β-cells (Van Obberghen et al., 2001).

IRS proteins lack intrinsic catalytic activity but are composed of multiple interaction domains and phosphorylation motifs (Van Obberghen et al., 2001) The four IRS proteins share in common an NH2 terminal pleckstrin homology (PH) domain followed by a phosphotyrosine binding (PTB) domain. Both the PH and PTB domains are highly conserved in each IRS protein. In addition, each IRS protein contains a poorly conserved domain of variable length containing several potential tyrosine phosphorylation sites in the COOH terminus (Figure 1.6).

The PH domains are believed to bind to acidic motifs in various proteins or to phospholipids in cell membranes. The PTB domains recognise N-P-x-Y motifs in proteins. Concerning the COOH terminus domain, insulin, IGF-1 and certain cytokine receptors phosphorylate IRS proteins at specific Y-x-x-M motifs within this domain. These motifs serve as docking sites for proteins that contain SH2 (Src-homology-2) domains. Phosphorylation of the tyrosine residues of IRS proteins increases the affinity with which they bind other signalling molecules. Each tyrosine phosphorylated motif of the IRS proteins is able to bind to a specific signalling molecule, and thus different protein complexes can be formed resulting in the engagement of various signalling pathways (White, 2002).
Figure 1.6: Structure and interacting partners of the insulin-receptor substrates. The four insulin-receptor substrate (IRS) isoforms, IRS1, IRS2, IRS3 and IRS4, share a pleckstrin-homology (PH) domain (magenta), a phosphotyrosine-binding (PTB) domain (dark green) and several sites of phosphorylation on tyrosine and serine residues. The positions of the tyrosine residues (Y) that are phosphorylated by the IR and the downstream-signalling proteins that bind to these sites are shown. The positions of the serine residues (S) and the kinases responsible for their phosphorylation are also shown. Blue circles represent sites of positive regulation, whereas red circles represent sites of negative regulation. Circles with both colours depict sites in which the regulation has been reported to be either positive or negative under various conditions. White circles represent sites in which the effect of phosphorylation is currently unknown. phosphatidylinositol 3-kinase (PI3K), growth-factor-receptor-bound protein-2 (Grb2), Src-homology-2 (SH2) domain-containing tyrosine phosphatase-2 (SHP2), AKT, IkB kinase (IKK), c-Jun-N-terminal kinase (JNK), protein kinase C0 (PKC0), p70 ribosomal protein S6 kinase (p70S6K), glycogen synthase kinase-3 β (GSK3β). Adapted from Taniguchi et al., 2006.
1.15.3 SH2 domain-containing proteins

The phosphorylated IRS molecules bind and activate several SH2 containing proteins, including the regulatory subunits of phosphatidylinositol 3-kinase (PI3K), the growth factor receptor-bound protein 2 (Grb2), the SH2 domain phosphatase 2 (SHP2), and phospholipase C. The latter generates 'second messenger' molecules important for insulin signal transduction. SHP2 regulates the phosphotyrosine status of intracellular proteins and Grb2 is a small adaptor molecule linking the insulin signal to the Ras pathway. Functionally most important for the insulin signalling cascade, however, is the activation of phosphatidylinositol 3-kinase (PI3K).

1.15.4 Phosphatidylinositol 3-kinase (PI3K)

These enzymes are lipid kinases that phosphorylate the 3' hydroxyl group of phosphatidylinositol and phosphoinositides leading to the activation of numerous intracellular signalling pathways. These pathways are involved in the regulation of functions as diverse as cell metabolism, vesicle trafficking, survival and polarity. Numerous PI3Ks have been identified using both biochemical approaches and PCR-based cloning strategies. They are divided into three classes (I-III) based on both their substrate preference and sequence homology (Figure 1.7).

Class I PI3Ks are the main type involved in insulin signalling and consist of two groups: class IA and class IB. This classification depends on the receptor types to which they couple. Class IA PI3Ks are activated by RTKs whereas class IB PI3Ks are activated by GPCRs (Katso et al., 2001)
Figure 1.7: There are three classes (I–III) of PI3K which show distinct substrate preferences. In vivo, class I PI3Ks generate phosphatidylinositol-3,4,5-trisphosphate (PIP3) from phosphatidylinositol-4,5-bisphosphate (PIP2). Class III PI3Ks generate phosphatidylinositol-3-phosphate (PI-3-P) from phosphatidylinositol (PI). Class II PI3Ks preferentially generate PI-3-P and phosphatidylinositol-3,4-bisphosphate (PI-3,4-P2) in vitro.

1.15.5 Class IA PI3Ks

Class IA PI3K is a heterodimer that consists of a p85 regulatory subunit and a p110 catalytic subunit. In mammals there are numerous isoforms of each subunit. It is not currently known if specific catalytic and regulatory subunit interactions mediate distinct physiologic outputs. The main role of class IA PI3Ks seems to be the direction of energy into cell growth and proliferations.

1.15.5.1 P85 regulatory subunit isoforms

Three genes: PI3KR1, PI3KR2 and PI3KR3 encode the p85α, p85β and p55γ isoforms of the p85 regulatory subunit respectively. The PI3KR1 gene has also been shown to give rise to two shorter isoforms p55α and p50α by alternative transcription-initiation sites. The structures of the class IA p85 regulatory isoforms are shown in Figure 1.8. The p85 regulatory subunit is vital in mediating the activation of class IA PI3K by RTKs, as the SH2 domains bind to phospho-tyrosine residues in the sequence context pY-x-x-M on activated RTKs or adaptor molecules such as IRS1. This binding results in the removal of the basal inhibition of p110 by p85 and the subsequent recruitment of the p85-p110 heterodimer to its substrate (PIP2) at the plasma membrane.
1.15.5.2 *PI110* catalytic subunit isoforms

Three genes encode the p110 catalytic subunit isoforms. These are *PIK3Cα*, *PIK3Cβ* and *PIK3Cδ*, which encode p110α, p110β and p110δ respectively. All these isoforms are highly homologous (Figure 1.8).

![Diagram of PI3K isoforms](image)

Figure 1.8: Schema depicting the domain structures of various PI3K isoforms. Class IA PI3K is a heterodimer that consists of a p85 regulatory subunit and a p110 catalytic subunit. The class IA p85 regulatory isoforms have a common core structure consisting of a p110-binding domain (also called the inter-SH2 domain) flanked by two Src-homology 2 (SH2) domains. Class IB PI3K is a heterodimer consisting of a p101 regulatory subunit and a p110γ catalytic subunit. Although p110γ shares extensive homology with the class IA p110 proteins, p101 is distinct from p85 proteins. Class II PI3Ks consist of only a p110-like catalytic subunit. The three isoforms of class II PI3Ks share significant sequence homology with the class I p110 subunits. Class II PI3Ks have an extended divergent N terminus, and additional domains at the C terminus. Class III PI3Ks consist of a single member, Vps34. Adapted from Engelman et al., 2006.
1.15.6 Class IB PI3Ks

This class of PI3Ks are not regulated by RTKs, as they do not possess a p85 family regulatory subunit. However, similarly to the class IA kinases they are also heterodimers consisting of a p101 regulatory subunit and a p110γ catalytic subunit. The p101 regulatory subunit facilitates the activation of the p110γ subunit of the heterodimer complex by direct interaction with Gβγ complexes of heterotrimeric G proteins. Although p110γ shares extensive homology with the class IA p110 proteins, p101 is distinct from p85 proteins (Figure 1.8).

1.15.7 Role of PI3K in the insulin signalling cascade

The phosphatidylinositol-3-phosphates products of PI3K such as phosphatidylinositol-3,4-biphosphate (PI-3,4-P₂) and phosphatidylinositol-3,4,5-triphosphate (PIP₃) recruit serine kinases to the plasma membrane including the AGC superfamily of serine/threonine kinases (which include p70S6k, AKT, and SgK isoforms) through binding to their PH domain (Cantrell, 2001). These play a central role in the insulin signal diversification to downstream pathways relating to carbohydrate metabolism and lipolysis, as well as to gene expression, transcription and protein turnover (Shepherd et al., 1998).

1.15.8 AKT and PDK-1

The binding of PIP₃ to the PH domain of the cytosolic serine/threonine kinase AKT (a 57 kDa protein also known as protein kinase B, PKB) and to the 63 kDa phosphoinositide-dependent kinase-1 (PDK-1) results in the co-localisation of these two proteins at the membrane (Cantrell, 2001). During this co-localisation, PDK-1 phosphorylates and activates AKT which in turn phosphorylates a variety of substrates that enable the control of various biological signalling cascades such as glucose transport, protein synthesis, cell proliferation, cell survival, glycogen synthesis and inhibition of lipolysis and gene expression (White, 2002).
1.15.9 Mitogenic insulin effects

In addition to its metabolic effects, insulin plays a major role in regulating cell growth, differentiation, and apoptosis (Shepherd et al., 1998; Vanhaesebroeck and Alessi, 2000). The signal for these responses is initially transmitted through phosphorylated IRS proteins but then diversifies.

One main branch of the cascade follows the described PI3K pathway, with AKT exerting an anti-apoptotic effect when constitutively over-expressed (Sabbatini and McCormick, 1999). AKT phosphorylates the anti-apoptotic protein BAD, activates the IkB kinases (IKKs) that liberate the cell survival transcription factor NF-κB, and also promotes the nuclear exclusion of forkhead transcription factors which subsequently reduce transcription of the apoptosis factor Fas ligand (Vanhaesebroeck and Alessi, 2000). AKT1 appears to be responsible for these processes, as a targeted deletion of this isoform leads to dwarfism and increased apoptosis in mice (Chen et al., 2001; Cho et al., 2001).

A second major branch of mitogenic insulin signalling is the Grb2/SOS/Ras and Raf-1/MEK/ERK serine kinase cascade (Tomlinson, 1999). Although largely distinct from the PI3K pathway, there is cellular communication between the two. Following association with an IRS protein, a complex of Grb2 with the Son of Sevenless (SOS) exchange protein activates the small, membrane-bound G-protein Ras by exchanging GDP for GTP. Once activated Ras operates as a molecular switch stimulating a serine kinase cascade through the stepwise activation of Raf, MAP kinase kinase (MEK) and MAP kinase (ERK) (Roberts, 1992). Activated ERK can translocate into the nucleus where it catalyses the phosphorylation of transcription factors (e.g. elk1, c-fos), initiating a transcriptional programme that leads to cellular proliferation or differentiation.

Emerging consensus is that the acute metabolic effects of insulin require activation of the IRS to PI3K pathway, where as the Ras-MAPK pathway plays a role in certain tissues to stimulate the actions of insulin on growth proliferation. Figure 1.9 is a summary of insulin signalling transduction pathways.
1.15.8.1 AKT mediated effects on glucose homeostasis and protein synthesis

Activated AKT plays an important and diverse role in glucose homeostasis. Three separately encoded proteins are currently known: AKT1, AKT2, and AKT3. The AKT2 isoform mediates the insulin-dependent translocation of GLUT-4 molecules from intracellular storage vesicles into the plasma membrane, thus facilitating glucose influx into the cell and lowering plasma glucose levels (Bae et al., 2003; Cho et al., 2001). Conversely, its absence leads to insulin resistance and hyperglycaemia (Cho et al., 2001). AKT inactivates glycogen synthase kinase 3 (GSK3) by phosphorylation, which in turn activates glycogen synthase and promotes glucose storage in liver and skeletal muscle (Summers et al., 1999). AKT also downregulates phosphoeno/pyruvate carboxykinase (PEPCK), a key enzyme of gluconeogenesis, thus reducing HGP (Gabbay et al., 1996; Summers et al., 1999).

The FOXO forkhead transcription factors are also direct targets for AKT. Their phosphorylation leads to exclusion from the nucleus and thus inhibition of the transcription of the genes involved in gluconeogenesis, ketogenesis, and fatty acid oxidation (Wolfrum et al., 2004). Several studies have shown a role for FOXO1 in mediating the metabolic actions of insulin, including hepatic gluconeogenesis, skeletal muscle glucose disposal, adipocyte differentiation and pancreatic β-cell growth (Kitamura et al., 2002; Furuyama et al., 2003; Nakae et al., 2003; Puigserver et al., 2003; Matsumoto et al., 2006; Naimi et al., 2007).

In addition, AKT can also regulate protein synthesis by inhibiting the activities of Tuberous Sclerosis-1 and 2 (TSC1-TSC2) enzymes (Garami et al., 2003). TSC-1 and TSC-2 have an inhibitory role on the mammalian Target of Rapamycin (mTOR), which mediates protein synthesis by its stimulatory effects upon elongation factor-4E binding protein (4E-BP1) and the p70 ribosomal s6 protein kinase (p70s6k) (Garami et al., 2003). The p70s6k protein is responsible for phosphorylation of the 40S ribosomal protein S6, and thereby regulates the translation of several hundred mRNAs.
Figure 1.9: Insulin signalling cascade. Following ligand binding, the activated insulin receptor recruits insulin receptor substrate (IRS) and other molecules (Grb2, Gab1, Shc). These scaffold proteins initiate a signalling cascade via Sos-Ras-Raf-MEK to activate p44/42 MAPK, which regulates gene transcription related to cell proliferation and apoptosis. p110 recruitment to Irsbound p85 activates PI3K and generates PIP₃ molecules. This leads to Pdk1-mediated AKT activation, which diversifies the insulin signal. Glucose transport is enhanced by AS160-mediated Glut4 translocation, and glycogen synthesis is stimulated by Gsk3 inhibition. AKT increases protein synthesis directly by activating p70⁷⁰S6K and indirectly through TSC1:TSC2 complex phosphorylation, the mTOR-4EBP1 pathway, and the Gsk3-associated de-repression of eIF2B. AKT phosphorylates nuclear Fox transcription factors, leading to their exclusion from the nucleus. Fox proteins control gene expression, elements of the cell cycle and influence apoptosis. AKT also regulates apoptosis through Bcl2-related proteins.
1.16 Murine models of obesity: insights into the role and function of the insulin signalling pathway

The recent generation of transgenic mouse models bearing both global and tissue-specific mutations in various components of the insulin signalling pathway has considerably advanced our knowledge of the role and function of this pathway and its key components.

1.16.1 Role of insulin receptor signalling pathways in hypothalamic function: evidence from animal models

1.16.1.1 Global deletion of the Insulin Receptor (IR)

The generation of mice bearing null IR mutations has been instrumental in dissecting the pathogenesis of insulin resistance, diabetes and growth retardation. Mice with global deletion of IR are born with slight growth retardation, at 90% of normal weight (Louvi et al., 1997). After birth, there is a progressive increase in glucose levels accompanied by a transient robust increase in insulin levels up to 1,000 fold above normal. However, β-cell failure occurs within a few days characterised by extensive degranulation of β-cells (Nakae et al., 2001). Mice eventually die of diabetic ketoacidosis. Thus the insulin receptor is necessary for postnatal fuel homeostasis but not for pre-natal growth and metabolic control (Nandi et al., 2004).

1.16.1.2 Neuronal deletion of the Insulin Receptor

In the CNS, insulin receptors are expressed at high levels in many areas of the brain including the olfactory bulb, the hypothalamus and the pituitary. Although neurons are known to metabolise glucose in an insulin independent manner, the generation of mice with global neuronal deletion of the IR has helped address the physiological role of insulin signalling in the brain.
Mice with global neuronal-specific ablation of the IR (NIRKO mice) were generated using the Cre/LoxP system (Bruning et al., 2000). Inactivation of the IR had no effect on brain development or neuronal survival. However, female NIRKO mice were hyperphagic, with both male and female NIRKO mice demonstrating DIO. Consistent with this, both sexes exhibited about a two-fold increase in perigonadal WAT, with concordant increases in plasma leptin levels. In addition, NIRKO mice developed mild insulin resistance, had elevated plasma insulin levels and hypertriglyceridemia. Surprisingly, both male and female mice had subfertility. Taken together, these results implicate CNS insulin receptor signalling in the regulation of energy disposal, fuel metabolism and reproduction.

The use of anti-sense technology has also highlighted the role of insulin signalling in the hypothalamus (Obici et al., 2002a). In this study, anti-sense oligodeoxynucleotides directed against the IR were injected directly into the third cerebral ventricle of rats. Following a seven day infusion, hypothalamic IR levels decreased by 80% in the ARC region of the hypothalamus. This selective decrease in hypothalamic IRs was accompanied by rapid-onset hyperphagia and increased fat mass. In addition, during insulin clamp studies, physiological hyperinsulinaemia decreased glucose production by 25% in rats treated with the anti-sense oligodeoxynucleotides in contrast to 55% in those treated with control oligodeoxynucleotides.

Taken together, these studies indicate that insulin receptors in discrete areas of the hypothalamus have a physiological role in the control of food intake, fat mass, and hepatic action of insulin.

The use of conditional gene targeting has allowed further dissection of the role of insulin receptor signalling in various insulin target tissues including muscle and liver. Table 1.4 below summarises the various phenotypes of the tissue-specific insulin receptor knockouts that have been generated.
<table>
<thead>
<tr>
<th>Tissue specific insulin receptor knockout</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global</td>
<td>Diabetic ketoacidosis and subsequent death</td>
<td>Louvi et al, 1997</td>
</tr>
<tr>
<td>Muscle</td>
<td>Dyslipidaemia</td>
<td>Bruning et al., 1998</td>
</tr>
<tr>
<td>Cardiac Muscle</td>
<td>Reduced heart size and performance</td>
<td>Belke et al., 2002</td>
</tr>
<tr>
<td>Muscle/adipose tissue</td>
<td>Impaired glucose tolerance</td>
<td>Lauro et al., 1998</td>
</tr>
<tr>
<td>Adipocyte</td>
<td>Protection against obesity and increased longevity</td>
<td>Bluher et al., 2002; Bluher et al., 2003</td>
</tr>
<tr>
<td>Liver</td>
<td>Moderate insulin resistance and hyperglycaemia</td>
<td>Michael et al., 2000</td>
</tr>
<tr>
<td>β-cell</td>
<td>Impaired glucose tolerance</td>
<td>Kulkarni et al., 1999</td>
</tr>
<tr>
<td>Vascular endothelium</td>
<td>Protection from hypoxia-Induced neovascularisation</td>
<td>Vicent et al., 2003</td>
</tr>
</tbody>
</table>

Table 1.4: Summary of the phenotypic effect of the deletion of the insulin receptor in various tissues. Adapted from Nandi et al., 2004.

1.16.2 Different roles of the IRS proteins: evidence from animal models

Gene deletion studies in mice have shown that despite their close homology, the four IRS proteins serve different though complementary roles in insulin/IGF-1 signalling (Table 1.5 and Figure 1.10).
1.16.2.1 *Irs1 null mice*

Disruption of *Irs1* in mice results in both pre- and post natal growth retardation (Araki et al., 1994). In addition, mice exhibit insulin resistance in peripheral tissues and impaired glucose tolerance. However, diabetes does not develop in these mice as insulin secretion increases to compensate for the mild resistance to insulin. Altogether, this suggests an important role of IRS1 in both insulin and IGF-1 actions.

1.16.2.2 *Irs2 null mice*

*Irs2* null mice exhibit mild peripheral insulin resistance and β-cell deficiency at birth (Withers et al., 1998). Subsequent β-cell failure in the face of continued peripheral insulin resistance causes overt fasting hyperglycaemia without ketoacidosis, a common characteristic of human T2DM. The early β-cell failure was found to be a result of reduced IRS2-mediated IGF-1 signalling causing defects in β-cell development and survival (Withers et al., 1999). The metabolic phenotype showed marked sexual dimorphism, whereby male animals developed overt diabetes from 8-10 weeks of age and females displayed compensatory hyperinsulinaemia for more than one year.

In addition to the role of IRS2 signalling in suppression of hepatic gluconeogenesis and the regulation of lipolysis and glycogen metabolism in skeletal muscle, IRS2 also plays complex roles in neuroendocrine function (Burks et al., 2000c). Independent of the diabetic phenotype and despite having elevated leptin levels, *Irs2* null females mice are hyperphagic and develop obesity (Burks et al., 2000c).

These findings not only suggest the presence of leptin resistance but also that CNS insulin action or components of the insulin signalling pathways are also necessary for intact neuronal responsiveness to leptin. Indeed, STAT3 phosphorylation in response to leptin is defective in *Irs2* null mice, suggesting some signalling convergence between leptin and insulin (Burks et al., 2000c). These findings support the hypothesis of potential cross talk
between insulin and leptin signalling in the CNS, with IRS2 implicated as a convergence point between the two pathways. In contrast to Irs1 KO mice, Irs2 KO mice have defective growth in only some tissues, including certain regions of the brain, islets and retina. Finally, Irs2 null females are infertile, and have small anovulatory ovaries with reduced number of follicles (Burks et al., 2000c).

1.16.2.3 Irs3 and Irs4 null mice

Irs3 null and Irs4 null mice have normal or near normal growth and metabolism. Ablation of Irs3 is devoid of a clear phenotype (Bjornholm et al., 2002). Ablation of Irs4 is associated with modest growth retardation and insulin resistance (Bjornholm et al., 2002; Fantin et al., 2000).

Figure 1.10: Isoform-specific functions of IRS proteins. Summary of the relative contribution of each insulin-receptor substrate (IRS) isoform (purple boxes) to the biological actions that are regulated by insulin (black arrows), as determined by knockdown and knockout studies. Adapted from Taniguchi et al., 2006.
<table>
<thead>
<tr>
<th>IRS protein</th>
<th>Development and growth</th>
<th>Metabolic phenotype</th>
<th>Neuroendocrine phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRS1</td>
<td>Intrauterine and post-partum growth retardation (~40%).</td>
<td>Insulin resistance in skeletal muscle <em>in vivo</em>. Impaired glucose uptake in muscle and fat <em>in vitro</em>.</td>
<td>None reported.</td>
<td>Araki et al., 1994</td>
</tr>
<tr>
<td>IRS3</td>
<td>None reported.</td>
<td>None reported.</td>
<td>None reported.</td>
<td>Bjornholm et al., 2002</td>
</tr>
</tbody>
</table>

Table 1.5: The roles of the Irs proteins: summary of knockout mouse phenotypes. Adapted from Withers, 2001.

1.16.3 Signalling by PI3K isoforms: insights from gene-targeted mice

A number of biochemical and cell biological studies have provided significant insights into the distinct and cell specific roles of the class I isoforms. However such studies have been limited by the fact that all class I PI3Ks exhibit a similar level of sensitivity to the classical PI3K inhibitors wortmannin and LY294002. Other approaches have therefore been taken, such as the microinjection of isoform-specific neutralising antibodies. These studies have also had limited success, as not all cells are amenable to microinjection, and so do not allow
the investigation of specific isoform functions in different organs or other physiological settings involving live animals.

Nonetheless, our understanding of the *in vivo* function of the various isoforms of class I PI3Ks has been expanded considerably in recent years with the use of gene deletion studies in mice. These studies have highlighted the key role of class IA PI3K signalling in the context of the regulation of growth and metabolism. The dysregulation of this pathway is crucial for the pathophysiology of a number of human diseases, most notably in T2DM and cancer.

1.16.3.1 Targeted disruption of the catalytic subunits

All class I catalytic isoforms have been inactivated by gene targeting. A description of studies focusing on p110δ and p110γ isoforms, predominantly expressed in cells of the immune system, are beyond the scope of this work. However, the results of studies targeting the p110α and p110β isoforms, both of which have broad tissue distribution, are summarised in Table 1.6 below. These studies indicate an important role for PI3Ks during embryonic development as both the *p110α* null (Bi et al., 1999) and *p110β* null animals (Bi et al., 2002) were embryonically lethal. Interestingly, despite the embryonic lethality of *p110α* null and *p110β* null mice, animals that are heterozygous for deletion of both the *p110α* and *p110β* alleles (*p110α*+/−,*p110β*+/−) are normal compared to controls (Brachmann et al., 2005). These studies indicate non-redundant roles for *p110α* and *p110β*, since lacking one copy of each gene did not replicate the phenotypes seen when both copies of either gene is targeted.

With regards to the function of the catalytic subunits in normal metabolic function, the study of Foukas and colleagues demonstrated a critical role for *p110α* in growth factor and metabolic signalling (Foukas et al., 2006). Mice heterozygous for a *p110α* kinase dead knockin exhibited reduced somatic growth, hyperinsulinaemia, glucose intolerance, hyperphagia and increased adiposity. The explanation for the metabolic phenotype
displayed by these mice stems from the highly selective recruitment and activation of p110α to IRS signalling complexes. To date, no groups have replicated the studies of Foukas et al with regards to the p110β catalytic subunit, and so the role of this isoform in the regulation of metabolism is still unknown.

<table>
<thead>
<tr>
<th>Targeted subunit</th>
<th>Viability</th>
<th>Metabolic Phenotype</th>
<th>Biochemical Features</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>p110α KO (homozygous)</td>
<td>Embryonic lethal. [E10.5]</td>
<td>Not applicable.</td>
<td>Increased expression of p85.</td>
<td>Bi et al., 1999</td>
</tr>
<tr>
<td>p110β KO (homozygous)</td>
<td>Embryonic lethal. [E3.5]</td>
<td>Not applicable.</td>
<td>Not applicable.</td>
<td>Bi et al., 2002</td>
</tr>
<tr>
<td>p110α KI (heterozygous)</td>
<td>Viable.</td>
<td>Reduced somatic growth.</td>
<td>Severely blunted signalling via IRS proteins.</td>
<td>Foukas et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hyperinsulinaemia.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucose tolerance.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hyperphagia.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased adiposity.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p110α&lt;sup&gt;−/−&lt;/sup&gt;p110β&lt;sup&gt;−/−&lt;/sup&gt; KO (heterozygous)</td>
<td>Viable.</td>
<td>Normal.</td>
<td>Normal.</td>
<td>Brachmann et al., 2005</td>
</tr>
</tbody>
</table>

Table 1.6: Metabolic phenotypes of mice with targeted PI3K catalytic subunits. KO, Knockout, KI, knockin. Adapted from Vanhaesebroeck et al., 2005.

### 1.16.3.2 Targeted disruption of the regulatory subunits

To date, four mouse lines with targeted deletion of the class IA PI3K regulatory subunits have been generated. The resulting phenotypes of these four models are summarised in the below Table 1.7. It should be taken into account that deletion of class IA regulatory subunits often alters the PI3K subunit profile in cells, and as consequence it is very difficult to ascertain the roles of the various regulatory subunit isoforms.
<table>
<thead>
<tr>
<th>Targeted Subunit</th>
<th>Metabolic Phenotype</th>
<th>Biochemical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>p85α</td>
<td></td>
<td>Changes in PI3K subunit expression</td>
</tr>
<tr>
<td>p50α</td>
<td>Hypoinsulinaemia.</td>
<td>Decreased p110α.</td>
</tr>
<tr>
<td>(pan p85α)</td>
<td></td>
<td>Increased p110β.</td>
</tr>
<tr>
<td>homozygous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(perinatal lethality)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p85α</td>
<td>Increased glucose tolerance.</td>
<td>Increased p85β.</td>
</tr>
<tr>
<td>p55α</td>
<td>Increased insulin sensitivity.</td>
<td>Increased p110α and p110β unaffected.</td>
</tr>
<tr>
<td>p50α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pan p85α)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>heterozygous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p85α only</td>
<td>Increased insulin sensitivity.</td>
<td>Increased p55α and p50α in muscle and fat cells.</td>
</tr>
<tr>
<td>(still express p85α)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p85β</td>
<td>Increased insulin sensitivity.</td>
<td>Increased p85α, p55α, p50α, 110α and p110β not affected</td>
</tr>
<tr>
<td></td>
<td>Hypoglycaemia.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hypoinsulinaemia.</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.7: Viability and metabolic phenotypes of mice with targeted regulatory subunits. pY denotes tyrosine phosphorylation. Adapted from Vanhaesebroeck et al., 2005.
1.16.3.3 Effect of targeting of regulatory subunits on p110 catalytic subunits and other non-targeted regulatory subunits

Data suggests that p110 isoforms do not demonstrate a binding specificity for distinct regulatory subunits (Vanhaesebroeck et al., 2005). As a result, deletion of regulatory subunits interferes with the ability of all catalytic subunits to become recruited to pY (phosphorylated tyrosine), with the level of impact being relative to the expression level of the targeted regulatory subunit. This is evident when comparing the effects of deletion of p85α with the effects of p85β deletion, on the level of p110 expression levels. The p85α isoform is widely expressed and is the most abundant class IA regulatory subunit. Upon its deletion (pan-85α null animals), the expression levels of all isoforms is severely reduced (Fruman et al., 2000). In contrast, the deletion of p85β (expressed at considerably lower levels) has no affect on the expression of other Class IA subunits (Ueki et al., 2002). These findings are consistent with the suggested role of the regulatory subunits in the stabilisation of the p110 catalytic subunits (Ueki et al., 2002).

With regards to the effect on regulatory subunits, targeting of the various products of the PIK3R1 gene also changes the expression pattern of non-targeted regulatory subunits. Due to the likelihood of the various regulatory subunits having distinct biological and signalling functions, interpretation of any observed phenotype in transgenic lines targeting regulatory subunits is further complicated. In addition, it is possible that the removal of one regulatory subunit isoform results in the facilitation of recruitment of other isoforms even if not upregulated (Vanhaesebroeck et al., 2005).

1.16.4 Role of signals downstream of AKT in the hypothalamic regulation of energy homeostasis

1.16.4.1 Role for p70S6k

Previous studies have indicated a key role for p70S6k (an effector of mTOR) in the regulation of glucose homeostasis. Mice null for this kinase are hypoinsulinaemic, glucose intolerant and have reduced β-cell mass (Pende et al., 2000). These animals exhibit normal fasting blood glucose levels, indicating an increased sensitivity to insulin. With regards to hypothalamic function, Um and colleagues have since reported that
p70\textsuperscript{56k} mice are protected against age related obesity and DIO due to an enhancement in β-oxidation (Um et al., 2004). These animals remain insulin sensitive due to the apparent loss of a negative feedback loop from p70\textsuperscript{56k} to IRS1. In contrast, on HFD both WT mice and ob/ob mice display significant increases in p70\textsuperscript{56k} activity and increases in IRS1 phosphorylation. This study therefore suggests that under conditions of nutrient satiation, p70\textsuperscript{56k} has the ability to negatively regulate insulin signalling and thus energy homeostasis (Um et al., 2004).

1.16.5 Role for mTOR

A recent study has implicated a key role for the serine-threonine kinase, mTOR, in the regulation of food intake (Cota et al., 2006). Cota and colleagues demonstrated colocalisation of mTOR with NPY and POMC in the ARC, and that changes in expression level of this protein correlate with the energy status of the animal. For example, following a 48-h fast, mTOR levels are considerably lowered in the ARC compared to fed conditions. Administration of L-Leucine (a branched amino acid shown to stimulate mTOR activity) directly into the ARC of mice, resulted in significant reductions in food intake. The use of rapamycin (an mTOR inhibitor) proved that this anorexia was dependent on hypothalamic mTOR signalling. The anorexigenic effects of ICV leptin treatment were also shown to be associated with increased hypothalamic mTOR activity. Taken together, these findings highlight an important role for hypothalamic mTOR signalling in food intake and energy balance regulation, and indicate that the anorexigenic effects of CNS adiposity signalling may be mTOR dependent.

1.17 Role for insulin signalling pathways in the regulation of reproduction

Recent studies have highlighted a role for the insulin signalling pathway in the regulation of reproductive function. Both male and female NIRKO animals display subfertility and impaired breeding performance, as indicated by timed mating studies (Bruning et al., 2000). This reduction in male fertility was found to be due to impaired spermatogenesis caused by reductions in luteinising hormone (LH). Ovaries from female NIRKO mice contained reduced numbers of antral follicles and corpora lutea, also due to reduced
levels of LH. As already described, IRS proteins have been shown to mediate the effects of the insulin receptor upon cellular and whole body physiology including reproduction (Withers, 2001). Mice lacking Irs1 display only mild defects in reproductive function (Burks et al., 2000c; Withers, 2001). However, female Irs2 null mice are infertile due to reduced pituitary LH levels and gonadotroph cell number and reduced gonadotrophin-stimulated ovulation. In addition, females exhibit markedly reduced numbers of ovarian follicles and corpora lutea (Burks et al., 2000c).

Insulin also stimulates GnRH secretion in vivo and ICV administration of insulin restores reproductive behaviour in diabetic rats (Burcelin et al., 2003; Kovacs et al., 2003). In vitro studies using immortalised GnRH neuronal cell lines have suggested that insulin regulates GnRH expression acting through the MAP kinase pathway (Salvi et al., 2006). Insulin signalling has also been shown to have complex roles in ovarian function including the regulation of ovarian steroidogenesis, follicular development and granulosa cell proliferation (Adashi et al., 1997; Poretsky et al., 1999; Willis et al., 1996). The strong association of insulin resistance and ovarian dysfunction in the polycystic ovarian syndrome also suggests a role for insulin signalling in ovarian function (Franks et al., 1999). CNS insulin signalling pathways therefore play a role in regulating female reproductive function in rodents.

1.18 Leptin receptor signalling cascade

The leptin receptor (LepR) belongs to the type 1 cytokine family which consist of a single membrane-spanning domain. Currently six leptin receptor isoforms are known to exist: Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re and Ob-Rf. Each of these isoforms has an identical extracellular ligand-binding domain at the amino terminus but differ at the carboxyl terminus. Only the Ob-Rb (the long receptor isoform) contains intracellular motifs which are necessary for signal transduction (Banks et al., 2000).

Upon leptin binding the receptor recruits and activates a member of the janus kinase (JAK) family, primarily JAK2 (Banks et al., 2000). Once activated, JAK2 phosphorylates the intracellular domain of the leptin receptor, resulting in the creation of a binding site for the signal transducers and activators of transcription (STAT) molecules. In response to leptin stimulation, activated STAT3 translocates to the nucleus where it functions as a
transcription factor to stimulate transcription of target genes that mediate some of leptins cellular effects (Banks et al., 2000). A family of proteins known as suppressor of cytokine signalling (SOCS) molecules provide intracellular negative feedback to this signalling system and are synthesised in response to STAT3 activation following leptin stimulation. Leptin receptor activation is also known to activate ERK-regulated pathways (Banks et al., 2000). Figure 1.11 is a summary of the leptin receptor signalling cascade.

![Leptin signalling pathways](image)

**Figure 1.11:** Leptin signalling pathways. The long form of LepR contains intracellular motifs required for signal transduction. Upon leptin binding the receptor recruits and activates JAK2. Activated, JAK2 phosphorylates the intracellular domain of the leptin receptor, creating a binding site for STAT molecules. In response to leptin stimulation, activated STAT3 translocates to the nucleus where it activates leptin-dependent gene transcription. Adapted from Banks et al., 2000.
1.19 Murine models of obesity and T2DM: insights into role and function of the leptin signalling pathway

Much of our current understanding of both the cellular and molecular activities of leptin has been derived from studies of murine models of obesity and diabetes. These models include the spontaneously occurring mutants \(ob/ob\) and \(db/db\), as well as a number of models generated by gene-targeting approaches. A selection of these, which have contributed to our understanding of the role of leptin in the CNS, are described in further detail below.

1.19.1 Role of the leptin receptor (LepR)

1.19.1.1 Mice with neuronal deletion of the LepR

McMinn and colleagues (McMinn et al., 2005) generated animals with neuron-specific deletion of the \(LepR\) in approximately 50% (C F/F mice) and approximately 75% (C Delta17/F mice) of hypothalamic neurons. Both models were significantly heavier than controls with increased percentage fat mass and hyperleptinaemia. However, both mutant strains remained fertile and cold tolerant. Animals with 75% deletion of \(LepR\) (C Delta17/F) animals also displayed hyperinsulinaemia and glucose intolerance.

These findings support the hypothesis that body weight, adiposity, plasma leptin concentrations, and glucose intolerance are proportional to hypothalamic LepR deficiency. However, fertility and cold tolerance remain intact unless hypothalamic LepR deficiency is complete. These findings are consistent with the findings of Cohen et al, in which animals were generated with neuron specific deletion of LepR. The extent of obesity in these animals was negatively correlated with the level of LepR expression in hypothalamus (Cohen et al., 2001). In support of this, de Luca and colleagues found that expression of LepR specifically in the brain of \(db/db\) animals results in reversal of the obesity, diabetes and infertility phenotype (de Luca et al., 2005).
1.19.1.2 Animals with disrupted LepR-Stat3 signalling

The activation of the transcription factor STAT3 is known to be mediated by Tyr 1138. Bates and colleagues generated a model with specific disruption of the LepR-STAT3 signal, by replacing the gene encoding the LepR with one in which the Tyr 1138 is replaced with a serine residue (Bates et al., 2004). They found that similarly to db/db animals, mice homozygous for this mutation (s/s mice) were hyperphagic and obese. However, unlike db/db mice, these animals are fertile, long and less hyperglycaemic. In addition, unlike db/db mice which display elevated hypothalamic NPY expression and suppressed expression of the hypothalamic melanocortin system, s/s mice display only the latter (Bates et al., 2003). These studies indicate that LepR-STAT3 signalling mediates the effects of leptin on melanocortin production and body energy homeostasis, whereas distinct LepR signals regulate NPY and the control of fertility, growth and glucose homeostasis.

1.19.2 Role of STAT3

Mice with global deletion of Stat3 die early in embryogenesis, prior to gastrulation (Takeda et al., 1997). However the use of conditional alleles of this gene have allowed analysis of its role in a tissue specific manner. The models that are of particular relevance to this thesis are described below.

1.19.2.1 Mice with mice with neural-specific disruption of Stat3

Gao and colleagues generated animals with neuronal specific disruption of Stat3 by crossing Stat3lox animals with mice expressing a neuron specific cre recombinase (Gao et al., 2004). These Stat3(N-/-) mice had no apparent developmental abnormalities but were found to be susceptible to neonatal lethality. Stat3(N-/-) animals also exhibited a hyperphagic, obese, diabetic, hyperleptinaemic and infertile phenotype. Treatment with a melanocortin-3/4 receptor agonist was found to abrogate the hyperphagia of these animals. Stat3(N-/-) animals also had reduced energy expenditure and displayed hypothermia following fasting or cold stress. With regards to neuropeptide expression, Stat3(N-/-) animals also exhibited a marked reduction in Pomc expression, with an
increase in $Npy$ and $Agrp$ expression. These studies thus indicate an essential role for STAT3 in the CNS regulation of energy homeostasis and reproduction.

1.20 Potential convergence of insulin and leptin signalling pathways

Several lines of evidence indicate potential cross-talk between insulin and leptin signalling pathways in the control of glucose homeostasis, body adiposity and reproductive function.

1.20.1 Cell culture studies

Soon after the discovery of leptin, it was shown to modify the actions of insulin in various model systems. A potential site of intracellular interaction for both insulin and leptin action involves the enzyme PI3K, although generally considered a downstream effector of IRS1 and IRS2 signalling.

Studies in hepatocyte cell lines have demonstrated that leptin enhanced insulin-induced association of IRS1 with PI3K and increased PI3K activity (Cohen et al., 1996; Kelleher et al., 1997; Zhao et al., 2000). The assessment of IRS-mediated PI3K signalling in the liver and muscle of $ob/ob$ mice indicated that these mice exhibited reduced $Irs1$ and $Irs2$ expression, leading to a differential decrease in IRS-PI3K signalling in these tissues (Kerouz et al., 1997). Subsequent studies in hepatocytes have demonstrated that leptin activates PI3K via tyrosine phosphorylation of IRS1 and IRS2, leading to activation of downstream targets such as AKT and phosphodiesterase 3B (PDE3B) (Zhao et al., 2000). Thus, leptin action in hepatocytes appears to involve signalling through IRS-PI3K pathways. Similar findings have been made in both muscle precursor cell lines (Kelleher et al., 1997) and in cultured pancreatic β-cells (Harvey et al., 2000).

1.20.2 In vivo studies

The first in vivo studies to support a hypothesis for the potential overlapping actions of insulin and leptin (Figure 1.12), found that the administration of leptin improves glucose homeostasis in both normal rodents (Sivitz et al., 1997) and models of leptin deficiency
(Halaas et al., 1995; Schwartz et al., 1996a). More importantly, these improvements were via mechanisms independent of effects on food intake. In further support for potential convergence between insulin and leptin signalling, Kim and colleagues (Kim et al., 2000) found that both hormones, when infused intravenously in rodents, were able to activate STAT molecules to differing degrees in several tissues. Furthermore, they observed that leptin, like insulin, is capable of activating IRS-PI3K pathways in adipose tissue and liver (albeit to a lesser extent than insulin).

![Diagram of Leptin and Insulin Receptors](image)

Regulation of Energy Homeostasis

Figure 1.12: Potential cross-talk between insulin and leptin intracellular signalling via PI3K. The insulin receptor tyrosine kinase catalyses the tyrosine phosphorylation of IRS2, which associates with PI3K, resulting in activation of PI3K activity and downstream signal transduction. It is hypothesised that JAK2 can act through a similar, IRS dependent mechanism to activate PI3K. Both the leptin and insulin receptors interact with numerous other signal transduction pathways (not shown) but could be involved in the regulation of energy homeostasis.
1.20.3 Direct evidence for cross-talk between insulin and leptin signalling in the CNS

Convergence between insulin and leptin signalling pathways in the hypothalamic regulation of energy homeostasis is implicated by the concentration of insulin and leptin receptors in the ARC. In addition, these hormones exert similar effects on neuropeptide gene expression and are generated in proportion to body fat stores, thus linking changes in body fat mass to adaptive adjustments of feeding behaviour.

The intracellular mechanisms that mediate the effects of insulin and leptin in hypothalamic neurons are not well understood. However, the electrophysiological studies of Spanswick and colleagues have provided direct experimental support for the existence of cross-talk between insulin and leptin signalling systems in the CNS regulation of appetite and body weight. They investigated the effects of leptin on hypothalamic neurons in rat brain slice cultures and found that leptin caused ‘glucose responsive’ neurons in the ARC and VMH to hyperpolarise through the activation of K<sub>ATP</sub> channels, resulting in a reduced firing rate (Spanswick et al., 2000). The same population of neurons were also hyperpolarised by insulin via the same K<sub>ATP</sub> channel-dependent mechanism, with individual neurons shown to respond to insulin and leptin in an identical manner (Spanswick et al., 2000). This ability of leptin and insulin to activate neuronal K<sub>ATP</sub> channels has been shown to be blocked by inhibitors of PI3K (Mirshamsi et al., 2004; Niswender et al., 2001; Spanswick et al., 2000).

Moreover, in vivo, the pre-treatment of rats with an ICV infusion of PI3K inhibitors (LY294002 or wortmannin) was found to completely block the ability of both ICV leptin and insulin to reduce food intake. Recent studies have also implicated a role for PI3K in mediating the reported effects of leptin on glucose homeostasis in the hypothalamus, and suggest that hypothalamic leptin signalling is an important determinant of glucose metabolism and that the underlying neuronal mechanism involves PI3K (Morton et al., 2005).

These studies therefore highlight the role of PI3K activation, a signalling molecule that classically mediates insulin action in peripheral tissues, in mediating the anorexigenic
effects of both insulin and leptin in the hypothalamic regulation of energy homeostasis. It should be noted that even though insulin and leptin signalling converge at the level of PI3K, the two hormones appear to elicit distinct signalling events downstream of PI3K. Unlike insulin, the ICV injection of leptin results in only a transitory phosphorylation of IRS proteins and association of IRS with PI3K, but does not result in activation of AKT in whole hypothalamic extracts (Carvalheira et al., 2005). In contrast to this transitory effect of leptin on IRS phosphorylation, leptin has been shown to robustly activate the MAPK pathway under the same experimental conditions.

1.20.4 Evidence for the modulation $K_{ATP}$ channel activity by PI3K generated PIP$_3$ in hypothalamic neurons

The above mentioned studies have highlighted the role of activated PI3K in the regulation of cell excitability via stimulation of $K_{ATP}$ channels channels in the brain. It is postulated that this might provide a potential molecular mechanism for mediating the central effects of insulin and leptin on energy and glucose homeostasis.

Three mechanisms for the modulation of $K_{ATP}$ channel activity by PI3K generated PIP$_3$ have been proposed (summarised in Figure 1.13): (1) PIP$_3$ increases the probability that $K_{ATP}$ channels are open, which indirectly lowers the ability of ATP to inhibit the channels (Baukrowitz et al., 1998; Shyng and Nichols, 1998), (2) PIP$_3$ directly decreases ATP binding to the channel (MacGregor et al., 2002) and (3) PIP$_3$ activates degradation of the local actin filaments around $K_{ATP}$ channels (Mirshamsi et al., 2004).

However, the hypothesis that the hypothalamic regulation of energy homeostasis of both insulin and leptin is via the alteration of the electrical activity of their target neurons should be viewed with caution. More detailed investigations have demonstrated that only 45% of unidentified ARC neurons follow this pattern (Mirshamsi et al., 2004), and that activation of hypothalamic $K_{ATP}$ channels by insulin is abolished in obese rats. In addition, it has been shown that in identified POMC neurons, leptin stimulates while insulin inhibits electrical activity (Choudhury et al., 2005). This is despite the findings of a recent study, discussed in further detail below, that both insulin and leptin stimulate PI3K in POMC neurons.
Figure 1.13: Generation of PIP₃ leads to K_{ATP} channel opening and consecutive cell hyperpolarisation. Insulin activates PI3K, which phosphorylates PIP₂ on position 3' in the inositol ring, generating PIP₃. The lipid phosphatase PTEN antagonises this by dephosphorylating PIP₃ to generate PIP₂. PIP₃ accumulation leads to activation of K_{ATP} channels and, thus, to potassium outflow. This leads to membrane hyperpolarisation and silencing of the neuron. Three different mechanisms for channel opening have been suggested: (i) PIP₃ binding to the KIR6.2 subunit of the potassium channel increases the probability that the channel is open, which indirectly lowers inhibition by ATP; (ii) PIP₃ competes with ATP for binding to the KIR6.2 subunit, thereby lowering ATP’s ability to close the channel; and (iii) PIP₃ activates degradation of the local actin cytoskeleton. Adapted from Plum et al., 2006.
1.20.5 Direct evidence for cross-talk between insulin and leptin signalling in distinct hypothalamic neuronal populations

Recent work has also indicated a role for PI3K in integrating the action of insulin and leptin on hypothalamic neurons, namely the POMC and AgRP populations of the ARC. Although both populations are implicated as mediating the effects of insulin and leptin, it is still unclear how changes in the levels of these adiposity signals are perceived or integrated by them.

Xu and colleagues developed mice in which a fluorescent reporter for PI3K activity is targeted to either AgRP or POMC neurons, and used 2-photon microscopy to measure dynamic regulation of PI3K by insulin and leptin in brain slices (Xu et al., 2005b). PI3K was chosen as a marker as it is the primary target of action by insulin, with leptin thought to act similarly.

They found that the addition of leptin triggered membrane accumulation of the PI3K reporter protein in POMC neurons. Conversely, the withdrawal of leptin triggered membrane accumulation of the PI3K reporter protein in AgRP neurons, consistent with the neuropeptide phenotype of these neurons, and the finding that low leptin levels result in their activation. With regards to insulin, the addition of this compound resulted in membrane accumulation of the PI3K reporter protein in POMC neurons.

Surprisingly, leptin and insulin had opposite effects on AgRP neurons, with membrane accumulation of the reporter protein inhibited by leptin but stimulated by insulin. This activation of PI3K by insulin in AgRP neurons is the opposite of what would be predicted for a physiologic adiposity signal. The use of inhibitors of synaptic transmission showed that leptin directly activates PI3K in POMC neurons, but the effect of leptin withdrawal in activating PI3K in AgRP neurons requires synaptic transmission and therefore must be mediated indirectly. In the case of insulin, synaptic transmission was not required to mediate the activation of PI3K in either AgRP or POMC neurons. The authors also demonstrate that the leptin-induced PI3K activation in POMC neurons is independent of STAT3 function.
Taken together, these results suggest a fundamental difference in the way POMC and AgRP neurons modulate PI3K activity in response to leptin. This is not only in direction of modulation but also in the underlying mechanism, with leptin having a primary effect on POMC neurons but a secondary effect on AgRP neurons (Figure 1.14). This study indicates that the functions of both insulin and leptin on energy balance do not overlap completely.

Figure 1.14: Unifying mechanism for leptin modulation of key arcuate nucleus neurons in which PI3K activity is a mediator and/or marker of neuronal activation and neuropeptide release in both AgRP (pink) and POMC (green) neurons. The effects of insulin on PI3K activity are direct in both neuronal subtypes, but the effects of leptin on PI3K activity in AgRP neurons require synaptic transmission from POMC or other inhibitory presynaptic neurons. IR, insulin receptor; LepR, leptin receptor. Adapted from Xu et al., 2005b.

1.20.6 Role for FOXO1 as an alternative mechanism for the interaction of insulin and leptin signalling at the level of hypothalamic neuropeptide expression

Although the above highlighted studies do indicate a role for PI3K in mediating the anorexigenic effects of insulin and leptin on energy homeostasis, little is known of the signals downstream of PI3K. Leptin has recently been shown to inhibit the hypothalamic expression of NPY and AgRP in a mechanism that requires intact PI3K signalling (Morrison et al., 2005). The anorexigenic effects of leptin are known to be mediated by
STAT3, but little is known of the potential interaction of the PI3K and STAT3 dependent actions of leptin. FOXO1 is known to control metabolism and cellular differentiation in a PI3K-dependent manner (Accili and Arden, 2004). Two groups have recently investigated whether this control is via the regulation of neuropeptide-dependent food intake (Kim et al., 2006; Kitamura et al., 2006).

The results of these studies suggest a model in which leptin signalling through JAK2-STAT3 inhibits Agrp expression by squelching FOXO1-dependent transcription of Agrp, and that this inhibition of FOXO1 function is independent of PI3K. In other words, activation of the PI3K-AKT pathway could decrease Agrp transcription by inhibiting FOXO1 activity, while promoting STAT3-mediated Pome transcription by reducing FOXO1 antagonism of STAT3. Thus, despite many studies implicating PI3K as a direct point of convergence between insulin and leptin pathways in the regulation of energy homeostasis, these findings suggest an additional mechanism of interaction, namely by transcriptional squelching of distal effectors; FOXO1 (for insulin) and STAT3 (leptin) (Figure 1.15).

Figure 1.15: Model for the reciprocal regulation of Pomc and Agrp gene transcription via FOXO1 and STAT3. Adapted from Morton et al., 2006.
1.21 Summary

The use of animal models has provided significant evidence for the role of leptin and insulin action in the CNS regulation of food intake, energy expenditure, reproductive function and peripheral glucose homeostasis. Both hormones circulate in proportion to body fat stores and are proposed to regulate energy homeostasis via their ability to elicit key effector pathways in the CNS resulting in a response where by feeding behaviour, autonomic outflow and substrate metabolism are adjusted in ways that promote homeostasis of both energy stores and fuel metabolism.

Recent evidence has suggested a key role for the ARC in the regulation of energy homeostasis, and specifically the POMC/CART and NPY/AgRP neurons within this region. Upon entering the ARC, insulin and leptin are proposed to bind to their corresponding receptors present on the first-order NPY/AgRP and POMC/CART neurons. These first order neurons subsequently control food intake and energy homeostasis via interactions with second order neurons located in other hypothalamic regions involved in food intake and energy homeostasis. Thus, a mechanism which allows transduction of peripheral signals into behavioural and metabolic responses to regulate energy homeostasis is achieved (Schwartz et al., 2000).

Although little is known about the about the role of insulin and leptin in distinct neuronal populations such as the POMC/CART and NPY/AgRP neurons, due to their similarities as anorexigenic compounds, both insulin and leptin have been widely considered to exert their anorexigenic effects via a similar mechanism. The enzyme, PI3K, has been shown to be act downstream of both the insulin and leptin signalling cascades in the hypothalamus and has been implicated as mediating the anorexigenic effects of both hormones.
1.22 Aims

In addition to the important role for insulin signalling in the classical peripheral target tissues such as liver, muscle and fat, it is now evident that the brain is also a key target for insulin action. Recent evidence has indicated that intact CNS insulin receptor signalling is required for both energy homeostasis and peripheral glucose homeostasis (Bruning et al., 2000; Obici et al., 2002a; Obici et al., 2002b; Plum et al., 2006). More specifically, a key role for IRS2 mediated signalling pathways in the CNS has been implicated by the phenotype of the Irs2 null mouse. In addition to the insulin resistance, β-cell failure and subsequent diabetic phenotype of Irs2 null mice (Withers et al., 1998), these animals also display a phenotype of hypothalamic dysfunction (obesity, hyperphagia, subfertility), and resistance to the anorexigenic effects of peripherally administered leptin (Burks et al., 2000c). However, these studies have raised a number of key points:

- The identity of the neuronal populations mediating the effects of insulin on food intake and body weight regulation is unknown.

- Although the severe impaired glucose homeostasis of Irs2 null animals is thought to be due to deletion of Irs2 in the β-cell, the relevant contribution of CNS IRS2 mediated insulin signalling to this phenotype, if any, is unclear.

- It remains unclear whether deletion of Irs2 in the ovary is solely responsible for the infertility of female Irs2 null animals, or if the deletion of Irs2 in the CNS also contributes to this phenotype.

- The resistance of Irs2 null animals to the anorexigenic effects of leptin indicate that IRS2 may be a point of convergence between insulin and leptin signalling pathways in the CNS.
The enzyme PI3K is known to be a major downstream effector of insulin signalling, and has been implicated as a potential point of convergence between insulin and leptin signalling cascades in the hypothalamic regulation of energy and glucose homeostasis (Mirshamsi et al., 2004; Morton et al., 2005; Niswender et al., 2001; Spanswick et al., 2000). Although several classes of PI3K exist, it is the class IA isoforms that are considered to be the main class involved in mediating the effects of insulin (Katso et al., 2001). In support of this, a recent in vivo study has highlighted the key role for the p110α catalytic subunit (a member of class IA) signalling in the context of the regulation of growth and metabolism (Foukas et al., 2006).

The aim of this thesis was to investigate the role of insulin signalling pathways in the hypothalamic regulation of energy homeostasis. More specifically, the roles of IRS2 and p110β pathways in neurons of the ARC (namely the AgRP and POMC populations) implicated as key targets of insulin in the hypothalamic regulation of energy homeostasis were studied. To do so, the following mouse lines were generated:

1. Mice with Irsp2 deletion in all neurons
2. Mice with Irsp2 deletion in POMC neurons
3. Mice with Irsp2 deletion in AgRP neurons
4. Mice with p110β deletion in POMC neurons
5. Mice with p110β deletion in AgRP neurons

It was predicted that the phenotypic characterisation of these transgenic lines would address the outstanding queries regarding the role of hypothalamic IRS2 mediated insulin signalling pathways in the regulation of energy homeostasis, glucose homeostasis and reproductive function. In addition, these studies were predicted to assess the contribution of the p110β isoform in the PI3K effects on metabolic function and enable the validation of a role for PI3K as a potential mechanism of mediating the anorexigenic effects of both insulin and leptin.
Chapter 2
Methods and materials
2 Methods and Materials

2.1 Conditional gene targeting using the Cre-loxP system

The animal models described in this thesis were generated with the use of conditional gene targeting technology. Conditional gene targeting refers to a gene modification in the mouse that is restricted to either certain cell types (tissue-specific), to a stage within development (temporally specific), or both (Lewandoski, 2001). To date, the cre-loxP system is the best characterised system for the achievement of conditional gene inactivation in mice (Wilson and Kola, 2001). Cre-loxP technology was introduced in the 1980's (Sauer and Henderson, 1988; Sternberg and Hamilton, 1981) and patented by DuPont Pharmaceuticals. It has been successfully applied in yeasts, plants, mammalian cell cultures, and mice (Araki et al., 1987).

The cre-loxP system is compromised of two components: the cre recombinase enzyme and a small stretch of DNA recognised by the recombinase (loxP site) (Stark et al., 1992; Van Duyne, 2001). The cre recombinase is produced by the bacteriophage P1 and is a member of the λ integrase superfamily of site-specific recombinases that cleave DNA at a distinct target sequence and ligate it to the cleaved DNA of a second identical site to generate a contiguous strand. This recombination is carried out with absolute fidelity such that not a single nucleotide is gained or lost overall. The loxP site is a 34 bp, as size that is unlikely to occur at random in even the largest vertebrate genome and yet small enough to be effectively neutral towards gene expression when positioned in chromosomal DNA for genetic manipulations.

Two separate mouse strains are typically generated and intercrossed for a conditional gene targeting experiment (Figure 2.1). One mouse strain expressed the cre recombinase in selected tissues, depending on which promoter has been selected to drive recombinase expression. This strain is mated with a strain that carries the gene of interest flanked (floxed) by the loxP sites. The location of the loxP sites has to be appropriately chosen such as that the function of the gene is not affected and that deletion of the floxed gene segment will lead to inhibition of transcription and/or translation of the gene of interest, or to the synthesis of a non-functional protein. It is important to note, that in offspring, cells expressing the recombinase delete the target gene segment whereas the target gene
remains functional in cells of all other tissues where cre is not expressed. However, a potential limitation of this cre-loxP system is that if the gene promoter chosen to drive the cre is expressed in cells/tissues other than the cells/tissue of interest, the targeted gene will also be inactivated in these populations. This should thus be taken into consideration upon interpretation of any resultant phenotypes generated.

Figure 2.1: Gene targeting using the cre-loxP recombination system to inactivate a gene in a desired cell type. (A) Schema of a standard homologous recombination method using mouse ES cells, in which three loxP sites are introduced along with a marker M at a target locus A (typically a small gene or an internal exon which if deleted would cause a frameshift mutation). Subsequent transfection of a cre recombinase gene and transient expression of this gene results in recombination between the introduced loxP sites to give different products. Type I recombinants are used to generate mice in which the target locus is flanked by loxP sites. Such mice can be mated with previously constructed transgenic mice (B) that carry an integrated construct consisting of the cre recombinase gene linked to a tissue-specific promoter. Offspring that contain both the loxP-flanked target locus plus the cre gene will express the cre gene in the desired tissue type, and the resulting recombination between the loxP sites in these cells results in tissue-specific inactivation of the target locus A. Adapted from Strachan and Read, 1999.
2.2 Animals

Animals were housed in specific-pathogen free barrier facilities and maintained under a controlled environment (temperature 21-23 °C, 12-h light-dark cycle, lights on at 07:00) with *ad libitum* access to food (RM1 diet SDS UK Ltd) and water. All animal procedures were approved by the British Home Office Animals Scientific Procedures Act 1986 and the UCL Animal Users Ethics Committee.

2.2.1 *Irs2lox* mice

*Irs2lox* mice on a C57/Bl6 background were generated in the Withers laboratory using conditional gene targeting (Figure 2.2) (Choudhury et al., 2005). These animals contain a floxed allele of *Irs2* (i.e. the *Irs2* locus is flanked by two *loxp* sites that are target sites for Cre recombinase). These transgenic animals showed no phenotypical differences compared to wild type animals.

2.2.2 *P110βlox* mice

*P110βlox* mice on a C57/Bl6 background were generated in the laboratory of Professor Vanhaesebroeck (Ludwig Institute, UCL) using conditional gene targeting (Figure 2.3). These animals contain a floxed allele of *p110β*, and showed no phenotypical differences compared to wild type animals.

It is important to note that exons 19 and 20 of the *p110β* kinase domain were floxed. Thus, removal of these exons following Cre-mediated recombination results in the splicing of exon 18 and exon 21, creating an out-of-frame fusion protein in which the normal C-terminus is not retained. However, mutant p110β protein still retains its ability to associate with regulatory subunits, thus maintaining the stoichiometry within the tissue/cell of interest.
Figure 2.2: Generation of Irs2lox mice. (A) Schema of targeting construct design, simplified restriction map of the Irs2 locus, the locus after homologous recombination and the deletion of neomycin cassette (Neo), and Southern blotting and PCR genotyping strategies used to identify these events. External probe A was used to identify homologous recombination (HR), probe B to detect the selection cassette, and probe C to detect the coding region of IRS2. HSV-tk, herpes simplex virus thymidine kinase. (B) Southern blot analysis with probe A demonstrating homologous recombination after targeting. (C and D) Southern blots using probe B after Cre-mediated recombination demonstrating deletion (Del) of the neomycin cassette and using probe C to demonstrate retention of IRS2 coding region confirming type 2 recombination. (E) PCR analysis with primers P1 and P2 of HR clone that has lost the neomycin cassette, but retained the loxP site downstream of the IRS2 coding region.
Figure 2.3: The targeting construct, showing a simplified restriction map of the p110β locus, the locus after homologous recombination, and the deletion of the selectable marker following in vitro Cre-mediated recombination.

2.2.3 *NesCre* mice

*NesCre* mice on a C57/Bl6 background (Tronche et al., 1999) were obtained from the Jackson laboratory.

2.2.4 *POMCCre* and *AgRPCre* mice

*POMCCre* and *AgRPCre* animals (Xu et al., 2005a; Xu et al., 2005b), both on a FVB/N-C57BL/6J mixed background, were obtained from Dr. G. Barsh, Stanford University.
2.2.5  \textit{Z/eg mice}

The mice designated \textit{Z/eg} (lacZ/EGFP), express lacZ throughout embryonic development and adult stages (Novak et al., 2000). Cre excision, removes the lacZ gene, which activates expression of the second reporter, enhanced green fluorescent protein (eGFP). Crossing these animals with those expressing the Cre-recombinase enzyme allows the monitoring of the recombination event of \textit{loxP} sites. These animals were obtained from the Jackson laboratory.

2.2.6  \textit{RosaYFP mice}

These animals contain enhanced yellow fluorescent protein (eYFP) cDNA inserted into the ubiquitously expressed \textit{ROSA26} locus, flanked by \textit{loxP} sites (Srinivas et al., 2001). The crossing of these animals with those expressing Cre-recombinase, results in eYFP expression in the cell/tissue in which recombination of the \textit{loxP} sites is taking place. These animals were obtained from the Jackson laboratory.

2.2.7  \textit{\textit{R26R} mice}

\textit{Gt(Rosa)26Sortm1Sor} allele (\textit{R26R}) mice contain a \textit{LacZ} gene inserted into the ubiquitously expressed \textit{ROSA26} locus, flanked by \textit{loxP} sites (Soriano, 1999). The crossing of these animals with those expressing Cre-recombinase, results in expression of LacZ in the cell/tissue in which recombination of the \textit{loxP} sites is taking place. These animals were obtained from the Jackson laboratory.

2.3  \textit{Generation of NesCreIrs2lox mice}

\textit{Irs2lox} mice were crossed with \textit{NesCre} mice, to obtain mice heterozygote for \textit{Irs2lox} and positive for the \textit{NesCre} transgene. These were then crossed with \textit{Irs2lox} heterozygote mice to obtain \textit{NesCreIrs2KO} animals and all other genetic combinations.
2.4 Generation of POMCCreIrs2lox mice

Irs2lox mice were crossed with POMCCre mice, to obtain mice heterozygote for Irs2lox and positive for the POMCCre transgene. These were then crossed with Irs2lox heterozygote mice to obtain POMCCreIrs2KO and all other genetic combinations.

2.5 Generation of AgRPCreR26R Irs2lox mice

Ectopic lacZ expression has been found in approximately 30% of AgrpCreR26R mice (Kaelin et al., 2004). To ensure that no mice in which germline deletion of Irs2 were studied, AgRPCre and R26R mice were intercrossed to produce AgrpCreR26R animals. These were crossed with Irs2lox homozygote mice to generate AgrpCreR26R Irs2lox\(^{+/-}\) mice. These were crossed with Irs2lox heterozygote mice to obtain AgRPCreR26R Irs2KO mice (hereby referred to as AgRPCreIrs2KO) and all other possible combinations.

2.6 Generation of POMCCreR26R Irs2lox mice

POMCCreIrs2KO mice were crossed with animals carrying the R26R allele. These were then intercrossed, to obtain POMCCreR26R Irs2KO mice and other mice of intermediary genotypes.

2.7 Generation of POMCCrep110\(\beta\)lox mice

P110\(\beta\)lox mice were crossed with POMCCre mice, to obtain mice heterozygote for p110\(\beta\)lox and positive for the POMCCre transgene. These were then crossed with p110\(\beta\)lox heterozygote mice, to obtain POMCCrep110\(\beta\)KO and all other genetic combinations.
2.8 Generation of AgRPCre\(^{110}\beta\text{lox}\) mice

\(P^{110}\beta\text{lox}\) mice were crossed with AgRPCre mice to obtain mice that were heterozygote for \(p^{110}\beta\text{lox}\) and positive for the AgRPCre transgene. These animals were crossed with \(p^{110}\beta\text{lox}\) heterozygote mice, to obtain AgRPCre\(^{110}\betaKO\) and all other genetic combinations.

2.9 Generation of POMCCre\(^{110}\beta\text{lox}\)Z/eg mice

POMCCre\(^{110}\betaKO\) mice were crossed with animals carrying the Z/eg transgene to give POMCCre\(^{110}\beta\text{lox}\)\(^{+/-}\)Z/eg mice. These were intercrossed to obtain POMCCre\(^{110}\betaKOZ/eg\) mice and other animals of intermediary genotypes.

2.10 Generation of AgRPCre\(^{110}\beta\text{KOYFP}\) mice

AgRPCre\(^{110}\betaKO\) mice were crossed with mice positive for the Rosa26YFP transgene to obtain AgRPCre\(^{110}\beta\text{lox}\)\(^{+/-}\)YFP animals. These were then intercrossed to obtain AgRPCre\(^{110}\betaKO\) YFP mice and other mice of intermediary genotypes.

2.11 Genotyping strategies

Mice were tail tipped (~ 2 mm) at around 14 days of age, using ethyl chloride spray as a local anaesthetic. Polymerase chain reaction (PCR) genotyping was then carried out upon extracted DNA, in order to determine the genotype of each mouse.

2.11.1 DNA extraction of tail tips

Each tail tip was placed in a 1.5 ml eppendorf tube and 600\(\mu\)l 0.1M NaOH added. Tubes were heated at >100°C for 10 minutes and then left to stand at room temperature (RT) for 10 minutes. 100\(\mu\)l 1M Tris (pH 6.8) was then added and 1\(\mu\)l of the resulting mixture was used for PCR genotyping. The PCR strategies for detecting the genotypic status of the various genes of interest differed. The details of these strategies are outlined below.
2.11.2 NesCre genotyping

NesCre genotyping was performed using the following primers: Cre 1084 5' GCG GTC TGG CAG TAA AAA CTA TC 3', Cre 1085 5' GTG AAA CAG CAT TGC TGT CAC TT 3' together with internal positive control primers for the IL2 gene: IL2 forward and IL2 reverse (5' TAGGCCACAGAATTGAAGATCT 3' and 5' GTAGTTGAAATTCTAGCATCATTCC 3' respectively).

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<th>PCR reaction mixture</th>
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<tbody>
<tr>
<td>Reddymix</td>
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<tr>
<td>Cre1084</td>
<td>0.75 µl</td>
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<tr>
<td>Cre1085</td>
<td>0.75 µl</td>
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<tr>
<td>IL2F</td>
<td>0.25 µl</td>
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<tr>
<td>IL2R</td>
<td>0.25 µl</td>
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10 µl of this mixture was added to 1 µl of DNA.

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<th>PCR profile</th>
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<tr>
<td>94 °C for 3 mins</td>
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<tr>
<td>94 °C for 30 secs</td>
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<tr>
<td>60 °C for 30 secs</td>
</tr>
<tr>
<td>72 °C for 1 min</td>
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<tr>
<td>72 °C for 10 mins</td>
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<td>Samples stored at 4 °C until analysis.</td>
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PCR product analysis

This resulted in a 324 bp IL2 internal control product in all samples. For samples that were NesCre positive, a 100 bp product was also generated (Figure 2.4).

![Image of gel electrophoresis](image)

**Figure 2.4**: NesCre genotyping. Samples A are present and samples B are negative for the Cre allele.
2.11.3 *POMCCre* genotyping

*POMCCre* genotyping was performed using the following primers: PC1 5' AGTGTGGCTCAATGTCCTTCTCG 3' and PAC2 5' CGGCATAACCAGTGAACAGCATG 3' together with internal positive control primers for the *IL2* gene: IL2 forward 5' CTaggccacagaattgaaaagatct 3' and IL2 reverse 5' GTAGGTGAAATTCTAGCATCCATCC 3'.

<table>
<thead>
<tr>
<th>PCR reaction mixture</th>
<th>PCR profile</th>
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<tbody>
<tr>
<td>Reddymix</td>
<td>9 µl</td>
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<td>PC1</td>
<td>0.75 µl</td>
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<tr>
<td>PAC2</td>
<td>0.75 µl</td>
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<tr>
<td>IL2F</td>
<td>0.25 µl</td>
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<tr>
<td>IL2R</td>
<td>0.25 µl</td>
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</tbody>
</table>

10 µl of this mixture was added to 1 µl of DNA.

94 ºC for 4 mins
94 ºC for 30 secs
55 ºC for 30 secs
72 ºC for 1 min
72 ºC for 7 mins
x 30

Samples stored at 4 ºC until analysis.

**PCR product analysis**

This resulted in a 324 bp *IL2* internal control product in all samples. For samples that were *POMCCre* positive, a 500 bp product was also generated (Figure 2.5).

![PCR gel image](image)

Figure 2.5: *POMCre* genotyping. Samples A are positive and samples B are negative for the *Cre* allele.
2.11.4 AgRPCre genotyping

AgRPCre genotyping was performed using the following primers: AC1 5' GTACCTAAGGATGGAGGAGAC 3' and PAC2 5' CCGCATACAGTGAAACACCATG 3' together with internal positive control primers for the IL2 gene: IL2 forward 5' CTagGCCACAGAATTGAAAGATCT 3' and IL2 reverse 5' GTAGGTGAAATTCTAGCATCATCC 3'.

<table>
<thead>
<tr>
<th>PCR reaction mixture</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Reddymix</td>
<td>9 µl</td>
</tr>
<tr>
<td>AC1</td>
<td>0.75 µl</td>
</tr>
<tr>
<td>PAC2</td>
<td>0.75 µl</td>
</tr>
<tr>
<td>IL2F</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>IL2R</td>
<td>0.25 µl</td>
</tr>
</tbody>
</table>

10 µl of this mixture was added to 1 µl of DNA.

<table>
<thead>
<tr>
<th>PCR profile</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>94 °C for 4 mins</td>
<td></td>
</tr>
<tr>
<td>94 °C for 30 secs</td>
<td>→</td>
</tr>
<tr>
<td>55 °C for 30 secs</td>
<td></td>
</tr>
<tr>
<td>72 °C for 1 min</td>
<td></td>
</tr>
<tr>
<td>72 °C for 7 mins</td>
<td></td>
</tr>
</tbody>
</table>

Samples stored at 4 °C until analysis.

PCR product analysis

This resulted in a 324 bp IL2 internal control product in all samples. For samples that were AgRPCre positive, a 500 bp product was also generated (Figure 2.6).

![AgRPCre genotyping](image)

Figure 2.6: AgRPCre genotyping. Samples A are present and samples B are negative for the Cre allele.
2.11.5 *Irs2lox* genotyping

*Irs2lox* genotyping was performed using primers that flank the 3' loxP site: loxP forward 5' ACTTGAAGGAAGCCACAGTCG 3' and loxP reverse 5' AGTCCACTTTCTGACAAGC 3'.

<table>
<thead>
<tr>
<th>PCR reaction mixture</th>
<th>PCR profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reddymix</td>
<td>94 °C for 1 min</td>
</tr>
<tr>
<td>PF</td>
<td>94 °C for 30 secs</td>
</tr>
<tr>
<td>PR</td>
<td>53 °C for 30 secs x 30</td>
</tr>
<tr>
<td>0.5 µl</td>
<td>72 °C for 1 min</td>
</tr>
<tr>
<td>0.5 µl</td>
<td>72 °C for 10 mins</td>
</tr>
<tr>
<td></td>
<td>Samples stored at 4 °C until analysis.</td>
</tr>
<tr>
<td>10 µl of mixture</td>
<td></td>
</tr>
<tr>
<td>of DNA</td>
<td></td>
</tr>
</tbody>
</table>

**PCR product analysis**

This resulted in a 200 bp product for the wild-type allele and/or a 250 bp product for the *Irs2lox* allele (Figure 2.7).

![Image of gel electrophoresis with bands labeled A, B, C, indicating 200 bp and 250 bp products](image)

Figure 2.7: *Irs2lox* genotyping. Samples A are homozygote for the *Irs2lox* allele. Samples B are heterozygote for the *Irs2lox* allele. Samples C are wild-type for the *Irs2lox* allele.
2.11.6 *R26R* genotyping (LacZ and YFP)

*R26R* genotyping was performed using the following three primers: Rosa A 5’-AAAAGTCGCTCTGAGTTGTAT-3’, Rosa B 5’GCGAAGAGTTTGTCCTCAACC-3’ and Rosa C 5’-GGAGCGGGAGAAATGGATATG-3’.

<table>
<thead>
<tr>
<th>PCR reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reddymix</td>
</tr>
<tr>
<td>Rosa A</td>
</tr>
<tr>
<td>Rosa B</td>
</tr>
<tr>
<td>Rosa C</td>
</tr>
</tbody>
</table>

24 μl of this mixture was added to 1 μl of DNA.

<table>
<thead>
<tr>
<th>PCR profile</th>
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<tbody>
<tr>
<td>94 °C for 2 mins</td>
</tr>
<tr>
<td>93 °C for 30 secs</td>
</tr>
<tr>
<td>58 °C for 30 secs</td>
</tr>
<tr>
<td>65 °C for 1 min</td>
</tr>
<tr>
<td>72 °C for 10 mins</td>
</tr>
</tbody>
</table>

Samples stored at 4 °C until analysis.

**PCR product analysis**

This resulted in a 550 bp wildtype band and/or a 250 bp transgene band (Figure 2.8).

Figure 2.8: *R26R* genotyping. Samples 1-4 are homozygote for the *R26R* allele. Samples 5 and 6 are heterozygote for the *R26R* allele. Samples 7-9 are wild-type for the *R26R* allele.
2.11.7 *Irs2* deletion

*Irs2* deletion was analysed using primers located ~1.1 kb upstream of the 5' *loxP* site (DF 5' GGGAACCTGACAAAGTGAATG 3') and ~0.2 kb downstream of the 3' *loxP* site (loxp 5' AGTCACCTTTCTGACAAGC 3').

<table>
<thead>
<tr>
<th>PCR reaction mixture</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Reddymix</td>
<td>44 µl</td>
</tr>
<tr>
<td>DF</td>
<td>2 µl</td>
</tr>
<tr>
<td>PR</td>
<td>2 µl</td>
</tr>
</tbody>
</table>

48 µl of this mixture was added to 2 µl of DNA.

<table>
<thead>
<tr>
<th>PCR profile</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>95 °C for 3 mins</td>
<td></td>
</tr>
<tr>
<td>95 °C for 1 min</td>
<td></td>
</tr>
<tr>
<td>52 °C for 1 min</td>
<td><img src="image" alt="x 30" /></td>
</tr>
<tr>
<td>72 °C for 1 min</td>
<td></td>
</tr>
<tr>
<td>72 °C for 7 mins</td>
<td></td>
</tr>
</tbody>
</table>

Samples stored at 4 °C until analysis.

**PCR product analysis**

The presence of a 1.3kb product indicated that recombination had occurred between the *loxP* sites (Figure 2.9).

![Figure 2.9: PCR for Irs2 deletion. Sample A is positive and sample B is negative for deletion of Irs2.](image)
2.11.8 *P110floox* genotyping

*P110floox* genotyping was performed using the following two primers: B3: 5’ AGTGAACGCTATGACATCACACCAGC 3’ and B98: 5’ AAGTACAAACATCCAAGCAA 3’

<table>
<thead>
<tr>
<th>PCR reaction mixture</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>17.875 µl</td>
</tr>
<tr>
<td>Titanium Buffer</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>dNTPs (2.5 mM)</td>
<td>2 µl</td>
</tr>
<tr>
<td>B3</td>
<td>1 µl</td>
</tr>
<tr>
<td>B98</td>
<td>1 µl</td>
</tr>
<tr>
<td>Titanium Taq</td>
<td>0.125 µl</td>
</tr>
<tr>
<td>25 µl of this mixture was added to 3 µl of DNA.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PCR profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>94 °C for 3 mins</td>
</tr>
<tr>
<td>94 °C for 30 secs</td>
</tr>
<tr>
<td>65 °C for 30 secs</td>
</tr>
<tr>
<td>72 °C for 1 min 30 secs</td>
</tr>
<tr>
<td>72 °C for 7 mins</td>
</tr>
<tr>
<td>Samples stored at 4 °C until analysis.</td>
</tr>
</tbody>
</table>

PCR product analysis

This resulted in a 304 bp product for the wild-type allele and/or a 372 bp product for the *p110floox allele* (Figure 2.10).

![Image](123.png)

**Figure 2.10:** *P110floox* genotyping. Samples 1, 2, 4 and 6 are heterozygote for the *p110floox* allele. Samples 3, 5 and 7 homozygote for the *p110floox* allele. Sample 8 is WT for the *p110floox* allele.
2.11.9 Z/eg genotyping

Z/eg genotyping was performed using the following two primers: Z/eg forward 5' CCTCTGCAAAAAATTATGGGG 3' and Z/eg reverse 5' ACTATGGTGTGCTGACTAATTG 3'.

<table>
<thead>
<tr>
<th>PCR reaction mixture</th>
<th>PCR profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reddymix</td>
<td>94 °C for 1 min</td>
</tr>
<tr>
<td>ZF</td>
<td>94 °C for 30 secs</td>
</tr>
<tr>
<td>ZR</td>
<td>55 °C for 30 secs</td>
</tr>
<tr>
<td>22 μl</td>
<td>72 °C for 1 min 30 secs</td>
</tr>
<tr>
<td>2 μl</td>
<td>x 29</td>
</tr>
<tr>
<td>24 μl of this mixture was added to 1 μl of DNA.</td>
<td>72 °C for 10 mins</td>
</tr>
</tbody>
</table>

Samples stored at 4 °C until analysis.

PCR product analysis

This resulted in a 750 bp product if samples were positive for the Z/eg allele (Figure 2.10).

Figure 2.11: Z/eg genotyping. Samples 1, 4 and 5 are negative for presence of the Z/eg allele. Samples 2, 3, 6, 7 and 8 are positive for the Z/eg allele.
2.12 LacZ staining on earpunches

Tissue from ears was taken from mice using standard ear punchers. The tissue was incubated in the dark overnight at 37\°C in β-galactosidase stain solution (1 x PBS, 2mM MgCl$_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 5mM potassium ferricyanide and 5mM potassium ferrocyanide) containing a 1:40 dilution of X-gal (40mg/ml). The presence of blue staining of the tissue indicated β-galactosidase activity and therefore, ectopic Cre expression.

2.13 In vivo physiological studies

2.13.1 Investigation of body weights

All body weights were recorded during the early light phase between 9am and 12pm using a Sartorius BP60 balance (Sartoruis, Goettingen, Germany) recording to the nearest 0.01g.

2.13.2 Body length

Body length (naso-anal distance) was measured in cm using a standard ruler, either post-mortem or in anaesthetised mice with the observer blinded to the genotype.

2.13.3 Feeding studies

2.13.3.1 Analysis of food intake

For analysis of food intake mice, were singly housed and allowed to acclimatise for a week. Each individual was then given a pre-weighed amount of food (~100g) and allowed ad libitum feeding. Food intake and body weight were measured for seven days at the ages indicated. Food was weighed daily in the early light phase (8am-10am) unless otherwise stated.
2.13.3.2  Response to fasting

Singly housed animals were exposed to two 16 hour (h) overnight fasts (at least 4 days apart) in a two week period preceding the study to acclimatise them. Two evenings before the study, the animals were removed into a clean cage to ensure that no small food pieces were present among the bedding. The evening before the study (at approximately 5pm), food was removed from the cages and the mice fasted for approximately 16-h overnight. In the early light phase (8am-9am) approximately 75g of food was weighed, recorded and given to each animal. The food was then weighed after 1-h, 2-h, 4-h, 8-h and 24-h post-refeeding. The body weight of the mice was also recorded. All measurements were made with a Sartorius BP60 balance (Sartorius, Goettingen, Germany) recording to the nearest 0.01g.

2.14  High fat diet (HFD) feeding

Animals were weighed at 6 weeks of age. Controls and knockouts (KOs) were each divided into two body-weight matched groups. A control and KO group were maintained on a standard chow diet (4.3% fat, RM1, Special Diet Services). The remaining control and KO groups were placed on HFD consisting of 45% fat, 35% carbohydrate and 20% protein (Research Diets, New Brunswick, NJ, USA). All groups were weighed (between 9am and 11am) once a week, for the number of weeks indicated.

2.15  Response to peripheral MTII treatment

Mice were singly housed at least two weeks before the study. The week preceeding the study, animals were handled and sham injected with saline (100 µl) each morning (at approximately 9 am). Two evenings prior to the study, animals were removed into a clean cage to ensure that no small pieces of food remained within the bedding. The evening before the study (at approximately 5 pm), food was removed from the cages and the mice were fasted for approximately 16-h overnight. The MTII (5 mg) (Bachem) was reconstituted in 10ml of sterile PBS resulting in a final concentration of 0.5 µg/µl, and made the morning of the study.
In the early light phase (8am-9am), animals were injected intraperitoneally (i.p.) with 100 µl of saline or 50 µg of MTII (in a volume of 100 µl). Immediately after injections, animals were returned to their home cage containing a known amount of chow (~75g). Food was then reweighed at 1-h, 2-h, 4-h, 8-h and 24-h post-injection using a Sartorius BP60 balance (Sartorius, Goettingen, Germany) recording to the nearest 0.01g. The study was performed in a cross-over design with each animal acting as its own control. Thus, following a week washout period, the study was repeated with animals that had received MTII in the first phase now receiving saline and vice versa.

2.16 Response to peripheral leptin treatment

Animals were singly housed for at least two weeks prior to the study. For 10 days prior to the study, animals were handled and sham injected with 100 µl saline on a daily basis (at approximately 6pm). The daily food intake and body weight of each mouse was also measured each evening to ensure that animals were not stressed and were eating normally. Two days prior to the study, animals were removed into a clean cage to ensure no small pieces of food remained within the bedding.

On the first day of the study (day 0), the animals were weighed in the afternoon (at approximately 4pm) and the food was removed from each cage. The control animals were then divided into two body-weight matched groups: one to receive leptin and the other to receive the vehicle control. KO animals were also randomly divided into two body-weight matched groups, each to receive the different treatment. The protocol that was used is summarised below:

Day 0- each mouse was injected i.p. with either 100 µl of vehicle or leptin (R&D Systems Inc., MN, USA), at a dose equivalent to 5 µg/g) at approximately 6pm. To each cage, a pre-weighed amount of food was then added. The next day (day 1), food was measured at 9am and again at approximately 4pm, at which point it was removed from the cage into a sealed bag. Body weights were also measured at approximately 4pm in order to calculate the injection dose for the evening. Mice were injected with either leptin or vehicle (as before) at around 6pm. Following the injections, the food from the sealed
bag was returned to each cage. On day 2, the steps of day 1 were repeated. On day 3, the food and body weight of each animal was recorded at approximately 9am and 6pm.

This study was designed as a cross-over experiment, so that each animal served as its own control. Thus, following a one week washout period, the study was carried out on the same animals. All the animals that received vehicle control in the first week of the study received leptin, and vice versa.

2.17 Analysis of body composition

Three methods were used to determine the body composition of the various animal models studied: magnetic resonance imaging (MRI), dual-energy x-ray absorptiometry (DEXA) scanning and weighing of fat pads.

2.17.1 Magnetic resonance imaging (MRI)

Mice were scanned using a 4.7T Varian system (post-mortem). Whole body images (between 50-60 slices; 2 mm thick) were obtained for each mouse using a spin-echo sequence (TR4500/TE20). Semi-automatic image segmentation software (sliceOmatic v4.2, Tomovision) was used to separate and quantify respective tissue volumes (Ross et al., 1991). For adipose tissue, the volume was multiplied by a correction factor of 0.9 in order to account for hydration (Tang et al., 2002). This work was carried out by Dr. Po Wah in the laboratory of Professor Jimmy Bell, Imperial College London.

2.17.2 Dual-energy X-ray absorptiometry (DEXA) scanning

Mice were terminally anaesthetised and their body composition analysed using a DEXA scanner. This study employed the PIXImus DEXA scanner (Lunar, GE Healthcare, USA) specifically designed to study the skeleton of small rodents. DEXA scanning works on the principle that X-rays reflect from bone in a density dependent manner. The PIXImus system exposes the entire animal to a cone shaped beam of high energy (80 kV)
and low energy (55 kV) x-rays. A CCD (charged couple device) camera detects the radiation hitting a luminescent panel below the test subject. The varied compositions of bone mineral, fat and lean tissue means they differentially absorb or reflect the dual energy X-rays. Thus, by digitally processing the detected radiation, the PIXImus can calculate the relative quantities of bone, fat and lean tissue along the x-ray path. During each scan, the dual x-ray exposure is performed four times and on completion, the data is collated to produce a ‘summary’ image, the density of which is compared to a standard object (mouse phantom) used to calibrate the instrument. All data was analysed using the PIXImus software (version 1.8, Lunar, GE Healthcare, USA).

2.17.3 Analysis of adiposity by fat pad weights

Animals were sacrificed by either cervical dislocation or terminal anaesthesia (see below). As an indicator of total body white adipose tissue (WAT) mass, the gonadal fat pad and mesenteric fat depots were carefully dissected out, pooled and weighed. With regards to brown adipose tissue (BAT), the two depots on the dorsal aspect of the thorax (between the scapulae) were dissected out. All weights were measured to the closest 0.0001g.

2.18 Metabolic studies

2.18.1 Determination of fasting blood glucose (FBG) levels

Mice were fasted for approximately 16-h overnight, and blood glucose levels were determined during the early light phase (8am-10am) via tail vein bleeds using a Glucometer Elite glucometer (Bayer). Ethyl chloride was used as a local anaesthetic.
2.18.2 Determination of glucose homeostasis by intra-peritoneal glucose- tolerance tests

Mice were fasted for 16-h and blood glucose levels determined as described above. Mice were then injected i.p. with 1.5 g kg\(^{-1}\) of D-glucose, and blood glucose measured at 15, 30, 60 and 120 minutes post-injection.

2.18.3 Determination of fasting insulin and leptin levels

Blood samples taken from animals following a 16-h overnight fast were centrifuged at 10,000 rpm for 15 minutes at 4 °C. The plasma was then immediately removed and frozen at -80 °C until assayed. Plasma insulin levels were measured using an ultrasensitive rat insulin ELISA (CrystalChem Inc.) with mouse insulin standards. Plasma leptin levels were measured using a mouse leptin ELISA (CrystalChem Inc.). All samples were assayed in duplicate and in one assay to avoid inter-assay variation.

2.18.4 Determination of resting metabolic rate and activity

Resting metabolic rate (RMR) was studied in male mice (ages indicated in result sections) at 30 ± 0.1°C (thermoneutral), 22 ± 0.1°C (housing temperature) or both temperatures by openflow respirometry using a paramagnetic oxygen analyser (Series 1100, Servomex Group Ltd, Crowborough, Sussex, U.K.) as previously described (Selman et al., 2001). Free access to food and water was permitted prior to metabolic measurements.

In brief, mice were weighed (0.01g, Sartorius) and rectal body temperature measured (2751-K, Digitron Instrumentation Ltd, U.K.) in advance of being placed individually within an airtight Perspex chamber inside a temperature-controlled incubator (INL-401N-010, Gallenkamp, U.K.). Silica gel dried air was pumped through the system (Charles Austin Pump Ltd, U.K) at 600-800mls min\(^{-1}\) (DM3A, Alexander Wright Flow Meter, U.K.), re-dried and a sub-sample (~150mls min\(^{-1}\)) passed through the oxygen analyser. Energy expenditure was calculated using the Weir equation (Weir, 1949) with all samples corrected for standard temperature and pressure (McLean and Speakman,
2000) and downloaded directly on to a microcomputer. The lowest 10 consecutive readings in oxygen concentration (equivalent to 5 minutes in the chamber) were taken as an estimate of the RMR (mls O2 min\(^{-1}\)). A general-linear model was used to control for observed body mass differences between groups. This work was carried out by Dr. Colin Selman (UCL), in collaboration with the laboratory of Professor John Speakman, University of Aberdeen.

2.19 Harvesting of tissues

2.19.1 Blood collection

Two differing methods were used for the collection of plasma. Samples were collected from live mice via tail bleed using a capillary blood collection system (Sarstedt, Nümbrecht, Germany). An alternative terminal approach involved the decapitation of dead animals (killed by cervical dislocation) and collection of blood. All samples were allowed to clot, and then placed on ice until centrifugation at 10,000 rpm for 15 minutes at 4 °C. Plasma was immediately separated and stored at −80 °C until analysis. If samples were required in a fasted state, animals were fasted for 16-h overnight, prior to blood collection.

2.19.2 Harvesting of brain and hypothalamus

Mice were either terminally anaesthetised (as previously described) or killed by cervical dislocation. In the case of terminal anaesthesia, animals were subjected to an i.p. injection with an overdose of Sodium pentobarbitone (Euthatal; Rhône Mérieux, Harlow, UK) at a dose of 500 mg kg\(^{-1}\) body weight. The head was then removed, the brain dissected out of the skull, and snap frozen in liquid nitrogen. If required, following removal of the brain from the skull, the hypothalamus was rapidly dissected and snap frozen in liquid nitrogen. As before, if samples were required in a fasted state, animals were fasted for 16-h overnight prior to dissection.

If fixed brains were required, mice were terminally anaesthetised and brains harvested following transcardiac perfusion with phosphate-buffered saline (PBS) and 4%
paraformaldehyde (PFA), using a Marlow Watson peristaltic pump. Brains were post-fixed overnight in 4% PFA, and then transferred to 30% sucrose and subsequently frozen after 48-h.

2.19.3 Harvesting of other tissues

Mice were either terminally anaesthetised (as described above) or killed by cervical dislocation. Peripheral tissues (including samples of liver, quadriceps muscles, fat and ovaries were rapidly dissected and snap frozen in liquid nitrogen for subsequent analysis. If required, pituitaries were harvested, and either snap frozen or homogenised in 1M urea in PBS and stored at -80 °C prior to analysis. The pancreas was removed and fixed in 4% PFA. If tissue samples were required in a fasted state, animals were fasted for 16-h overnight prior to dissection.

2.20 DNA, RNA and protein extraction from tissues

2.20.1 DNA extraction from tissue

Tissue was placed in 525 μl lysis buffer (50 mM Tris pH8, 100 mM EDTA, 100 mM NaCl, 1% SDS) with 35 μl Proteinase K (10 mg/ml) and incubated overnight at 55 °C. Following this, 2 μl of RNase A (0.2mg/ml) was added and the mixture incubated at 37 °C for 1-h. 500 μl 5M NaCl was added, and the solution mixed gently and then 700 μl chloroform:isoamylalcohol (24:1) added. Samples were mixed gently for 2-h at room temperature, and centrifuged at 10,000 rpm for 10 minutes to and the upper aqueous phase removed to a new tube. 700 μl isopropanol was added to precipitate the DNA, which was pelleted by centrifuging at 10,000 rpm for 10 minutes. The DNA pellet was then washed with 70% ethanol and resuspended in 200 μl TE buffer.

2.20.2 mRNA extraction and quantification

Throughout handling of tissue and the extraction process, standard precautions were taken to reduce the risk of RNAsase contamination. Bench surfaces, tools, and pipettes
were treated with RNase inhibitor spray (RNaseZap; Ambion, Huntingdon, UK), filtered pipette tips used, and gloves frequently changed. 100 mg of tissue was homogenised on ice in a 5 ml flat-bottom universal container at 12,000 rpm with a Turrax T25 dispersing tool in 1 ml of a phenol/guanidine isothiocyanate solution according to the manufacturer’s instructions (TRIZOL; Invitrogen, Paisley, UK). The precipitated RNA was resuspended in 60 μl of nuclease-free H₂O (Promega, Southampton, UK).

RNA quantification and analysis of purity were determined by measuring the absorbances at 260 nm and 280 nm in a CE 2041 spectrometer (Cecil Instruments, Cambridge, UK). RNA concentration was adjusted to 100 ng/μl with nuclease-free H₂O.

2.20.3 **Protein extraction and quantification**

Protein lysis buffer (10 mM Tris, 5 mM EDTA, 50 mM NaCl, 30 mM Na₂H₂PO₄, 1% Triton X-100, 50 mM NaF, 0.1 mM Na₃VO₄, adjusted to pH 7.6) was freshly prepared by dissolving a protease inhibitor mixture tablet (MiniComplete; Roche Diagnostics, Lewes, UK) per 10 ml of buffer, and by adding PMSF to a final concentration of 1 mM immediately prior to use. Tissue (300-500 mg) was homogenised on ice in 1 ml of lysis buffer in a 5 ml flat-bottom universal container at 12,000 rpm with a Turrax T25 rotors-tator dispersing tool (IKA, Staufen, Germany). The lysate was incubated on ice for 30 minutes to increase protein solubilisation, and transferred to a 1.5 ml tube. Following centrifugation at 10,000 rpm for 20 minutes at 4 °C, the clear phase between the lipid-containing upper phase and the sedimentsed insoluble tissue components was transferred to a new reaction tube. Protein concentration was determined by a Bradford reaction assay (BioRad, Hemel Hempstead, UK).

2.21 **Immunoprecipitation and western blotting**

Protein was extracted as described above (2.19.3) from snap frozen samples.
2.21.1 Immunoprecipitation of IRS2

For the immunoprecipitation of IRS2, 10 μl of anti-IRS2 sheep polyclonal antibody (generated by the Withers laboratory) was incubated with 2 mgs of lysate at 4 °C, on a rotary mixer overnight (total volume of 1 ml). Following overnight incubation with antibody, samples had 100 μl total bead volume of pre-washed protein-A agarose (Roche Diagnostics) added to them using a wide-orifice tip. The samples were incubated for a further 120 minutes at 4 °C. Immunoprecipitations were then clarified by gentle centrifugation and washed twice with 750 μl of 1 x lysis buffer. Samples were then denatured in 20 μl of 2x SDS-sample buffer (125mM Tris pH 6.8, 20% glycerol, 4% SDS, 1% bromophenol blue, 200mM β-mercaptoethanol) at 100 °C for 5 mins. Following centrifugation at 10,000 rpm for 2 minutes, the supernatant was used for SDS-polyacrylamide gel electrophoresis and subsequent immunoblotting.

2.21.2 Immunoblotting

Polyacrylamide gel electrophoresis was performed using the Mini-Protean electrophoresis system (BioRad). Lysates were resolved on 7.5% SDS-PAGE gels, immersed in running buffer (25mM Tris, 192mM glycine, 0.1% SDS). 20 μl of recovered immunoprecipitate was loaded per lane and 10 μl of a coloured molecular weight marker was also loaded to indicate protein size (Rainbow; Amersham, Little Chalfont, UK). Electrophoresis was performed at 100V for 60-90 minutes. Proteins were transferred from the gel to a polyvinylidene-difluoride membrane (Amersham) in transfer buffer [25mM Tris, 192mM glycine, 20% methanol (vol/vol)] at 100V for 90-120 minutes.

The membrane was incubated in blocking solution consisting of 5% non-fat dried milk in TNT buffer (50mM Tris pH 8.0, 150mM NaCl, 0.1% Tween 20) at room temperature for 60 minutes to reduce non-specific binding of antibody. Primary antibody (anti-IRS2 sheep polyclonal antibody generated by the Withers laboratory) was diluted to a concentration of 1:1,000 with blocking solution, in which the blot was incubated at 4 °C for approximately 16-h. The membrane was washed three times in TNT buffer for 5 minutes and incubated at room temperature for 1-h in HPO-conjugated polyclonal sheep anti-mouse antibody (Amersham, NA 931) (1:10,000 in blocking solution).
Following a further three 5 minute washes in TNT buffer, the blot was analysed by application of a chemiluminescence detection kit (ECL Plus; Amersham) and autoradiography (Hyperfilm ECL; Amersham). Exposed films were quantified with self-calibrating GS 800 densitometer and proprietary software (Quantity One 4.5; BioRad).

2.22 Gene expression studies

RNA was extracted as described in section 2.19.2 from hypothalamic samples snap frozen at the time of dissection, and realtime PCR (RT-PCR) was performed to analyse the gene expression of various feeding neuropeptides.

2.22.1 Reverse transcription

Once extracted, mRNA was reverse transcribed in order to generate complementary DNA (cDNA) template for RT-PCR quantification. The TaqMan Retrotranscription Kit (Applied Biosystems) was used. Briefly, each sample contained 2.5 µl of RT Buffer (10X), 5.5 µl of MgCl₂, 5 µl of dNTPs, 1.25 µl of oligo-DT, 0.5 µl of RNAse inhibitor, 0.5 µl of reverse transcriptase with 1 µg of RNA used as a template. Each reaction was set-up in 0.2 ml reaction tubes (BioRad) and the the total volume in each tube made up to 25 µl with nuclease free water. Samples were incubated at 37 °C for 60 minutes, and the reverse transcriptase subsequently inactivated by heating the samples to 95 °C for 5 minutes. The resulting cDNA was then stored at –20 °C until PCR analysis.

2.22.2 Real-time polymerase chain reaction

Quantitative PCR was performed with the TaqMan system (Applied Biosystems). Proprietary sequence Taqman Gene Expression assay FAM/TAMRA primers (Applied Biosystems, Foster City, CA, USA) were used: AgRP (Mm00475829_g1), CART (Mm0048986_m1) HPRT (hypoxanthine guanine phosphoribosyl transferase, Mm99999915_g1), NPY (Mm00445771_m1), and POMC (Mm00435874_m1).
The reactions were carried out in a 25 μl reaction volume in an ABI Prism 7900 HT thermocycler (Applied Biosystems). The manufacturer’s recommended cycling conditions were employed, which consisted of 50 °C for 2 min and subsequent 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min, with all temperature changes occurring at maximum ramp speed. For the melting curve, two subsequent steps of 95 °C for 15 sec and 60 °C for 15 sec also occurred at maximum ramp speed and were followed by a slow 2% temperature ramp step to 95 °C. The integrated 488 nm Argon laser excitation/detection system used SDS 2.1 software (Applied Biosystems).

2.23 Immunocytochemistry on hypothalamic sections

Thirty μm sections were cut from frozen perfused brains using a sliding microtome and samples and stored at −20 °C in cryoprotectant before use. Samples were washed 3 times in potassium phosphate buffered saline (KPBS) pH 7.4 and then blocked with 2% chicken serum in KPBS/0.4% Triton X-100 for 1-h at room temperature. Samples were then incubated with primary antibodies in KPBS/0.4% Triton X-100 for 48-h at 4 °C. The primary antibodies used were rabbit anti-IRS2 antibody (Upstate) and mouse monoclonal anti-lacZ (Abcam). Slices were then washed 4 x 10 min at room temperature with KPBS, and incubated with appropriate secondary antibodies (fluorescently labelled) for 1-h in KPBS/0.4% Triton X-100 at room temperature before being washed 4 x 10 min in KPBS at room temperature. The fluorescently labelled secondary antibodies used were chicken anti-rabbit IgG-AlexaFluor 594 (red) conjugate and chicken anti-mouse IgG-AlexaFluor 488 conjugate (green) (Molecular Probes). Slices were mounted on polylysine-coated microscope slides and cover-slipped with buffered glycerol.

2.24 Pancreatic immunocytochemistry (ICC) and measurement of islet mass and number

Animals were killed by terminal anaesthesia or cervical dislocation. The pancreas was then removed, cleared of fat and lymph nodes and fixed in 4% PFA, embedded in paraffin and cut into 5 μm sections. Sections were treated with 0.01M citrate buffer for 10 min at 95 °C and incubated with blocking solution consisting of PBS buffer with 5%
normal chicken serum and 2% BSA for 30 min. A cocktail of primary antibodies in blocking buffer containing mouse anti-insulin antibody (clone K36aC10, Sigma-Aldrich) and rabbit anti-glucagon (Abcam Ltd) was applied for 2-h at RT or overnight at 4 °C. The sections were subsequently incubated chicken anti-rabbit IgG-AlexaFluor 594 (red) conjugate and chicken anti-mouse IgG-AlexaFluor 488 conjugate (green) (Molecular Probes) for 2-h at RT. Transmitted light and fluorescent images were captured with Metamorph software using a Zeiss Axiophot 2 microscope or with SimplePCI software using an Olympus BX51 microscope equipped with Hamamatsu 95 black and white camera.

For quantification of β-cell area and islet number, five pancreases were analysed per genotype. For each pancreas four sections, at least 150 μm apart, were analysed. For each section, the total area occupied by insulin-positive cells was scored using Simple PCI software, together with the number of islets on the section. Results are expressed as the percentage of the total islet area and the mean islet density for each pancreatic section. This work was carried out by Dr. Marc Claret (UCL).

2.25 Reproductive studies

2.25.1 Superovulation studies

Female mice, at approximately 8-10 weeks of age, were given an i.p. injection of 10 IU pregnant mare's serum (PMSG, Folligon, Intervet Inc). This was followed 46 hours later by 8 IU of human chorionic gonadotropin (Chorulon, Intervet Inc). Oocytes were flushed from the oviduct 14-16 hours after the second injection. This work was carried out with Dr. Irina Neganova (UCL).

2.25.2 Oestrous cycle progression

To monitor the oestrous cycles of NesCreIrs2KO and control animals, mice were vaginally smeared on a daily basis for complete cycles. The smears were spread onto
glass slides, which were then stained with Giemsa stain. This work was carried out with Dr. Irina Neganova (UCL).

2.25.3 Analysis of reproductive hormone levels

2.25.3.1 Serum levels of oestrogen and progesterone at dioestrous

Blood samples were collected as previously described (2.18.1). Samples were left to coagulate at 4 °C overnight, centrifuged with the serum collected and frozen at -20 °C. Serum oestrogen and progesterone levels were measured using immunofluorometric assays (Delfia, Perkin Elmer, Turku, Finland).

2.25.3.2 Pituitary hormone levels

Pituitaries were harvested during dioestrous. They were homogenised in 1ml of 1M urea in PBS and stored at -80 °C prior to analysis. Radioimmunoassays (RIAs) for pituitary luteinizing hormone (LH), follicle stimulating hormone (FSH) and prolactin were performed as previously described. This work was carried out in collaboration with the laboratory of Professor Stephen Bloom, Imperial College London.

2.25.4 Ovarian histomorphometric analyses

Ovaries were fixed in 10% buffered formalin, embedded in paraffin and cut into 5 μm sections. For histomorphometric analysis sections were stained with haematoxylin-eosin. Follicles, classified according to their diameter and number of granulosa cell layers, were counted in every tenth section, starting from the fifth section, through the entire ovary. Only follicles with a clearly visible oocyte nucleus were counted to avoid double counting. Atretic follicles were defined on the following morphological criteria: follicle and oocyte shape, localisation of the oocyte in the follicle, presence of a degenerated oocyte, altered zone pellucida, the presence of granulosa cells inside zone pellucida, presence of more than 3 pynknotic nuclei in granulosa cells and macrophage invasion. Follicle growth, follicle and oocyte size (diameter) and total follicular and antral cavity
area were measured using LUCIA software (Nikon UK, Kingston-upon-Thames, UK). This work was carried out by Dr. Irina Neganova (UCL).

2.25.5 Timed matings

At 6 weeks of age, six female NesCreIrs2KO animals and six controls were pair mated with wildtype C57/Bl6 males for the duration of 12 weeks. Fertility was assessed by daily monitoring of pregnancies, litter and pup number.

2.26 Suppliers

General laboratory chemicals were purchased from Sigma-Aldrich, Gillingham, UK, except where stated otherwise.

2.27 Data handling and statistics

All data was expressed as an average ± S.E.M. for all data points. Standard error of the means, and all other statistical tests were performed using GraphPad Prism Version 4. Paired and unpaired t-tests and 2-way ANOVA with Bonferroni post-tests were performed as appropriate.
Chapter 3

Phenotypic characterisation of
*NesCreIrs2KO* animals
3 Characterisation of NesCreIrs2KO animals

An important role for IRS2 in energy homeostasis, glucose homeostasis and reproduction has been implicated by studies of animals with global deletion of this gene. Irs2 null mice are insulin resistant, display β-cell failure and as a result develop diabetes (Withers et al., 1998). A key role for IRS2 in the CNS regulation of energy homeostasis is also suggested by the obesity and hyperphagia of these animals (Burks et al., 2000c). These findings implicate a significant role for CNS insulin signalling in the regulation of energy homeostasis and are supported by the hypothalamic dysfunction and impaired glucose homeostasis displayed by NIRKO animals (Bruning et al., 2000).

To investigate the role of IRS2 in the CNS regulation of energy homeostasis and glucose homeostasis in a tissue-specific manner, mice with specific deletion of Irs2 in the CNS were generated. To do so, NesCre animals (which expresses Cre recombinase under the control of a rat nestin promoter) were crossed with Irs2lox animals (animals with a conditional allele of Irs2).

3.1 Proof of Irs2 deletion

Nestin, an intermediate filament protein, is expressed in the central and peripheral nervous system by embryonic day 11. NesCre transgenic animals have been used extensively in recent years (Bruning et al., 2000; Lendahl et al., 1990; Tronche et al., 1999). Despite this, before the commencement of investigations, studies aimed at confirming the lack of Irs2 expression in the CNS of NesCreIrs2KO animals were performed.

Indeed, the use of various methods demonstrated the deletion of Irs2 at the DNA, mRNA and protein levels in the hypothalami of NesCreIrs2KO animals but not controls (Figure 3.1). Thus, the use of NesCreIrs2KO animals as a valid model for the study of IRS2 mediated signalling pathways in the CNS was confirmed. Therefore, a series of systematic analyses to investigate the hypothalamic function, glucose homeostasis and reproductive function of NesCreIrs2KO animals and controls were undertaken.
Figure 3.1: A) PCR analysis of Irs2 deletion in DNA extracted from: 1) NesCreIrs2KO whole brain, 2) NesCreIrs2KO hypothalami and 3) wild-type brain. A 1.3kb band in samples 1 and 2 confirms deletion of Irs2. B) Gene expression analysis of hypothalamic Irs2 levels in control and NesCreIrs2KO animals. NesCreIrs2KO mice display significantly reduced IRS2 levels compared to WT (** P<0.0001). C) Immunoprecipitation and western blotting analysis of IRS2 in whole brain and hypothalamic lysates. No IRS2 protein was detected in samples from NesCreIrs2KO animals, confirming the deletion of the Irs2 gene in the brain of these animals.
3.2 Hypothalamic function

3.2.1 Analysis of body weight

Both male and female *NesCreIrs2KO* animals, were of normal weight (as compared to control littermates) at 3 weeks of age. However, from the age of eight weeks onwards, both male and female *NesCreIrs2KO* mice were significantly heavier than control littermates (Figure 3.2).

![Graph A: Growth curve for male NesCreIrs2KO and control animals](image)

![Graph B: Growth curve for female NesCreIrs2KO and control animals](image)

Figure 3.2: Growth curve for A) male *NesCreIrs2KO* and control animals and B) female *NesCreIrsKO* and control animals. Data represent the mean ± SEM for 6-12 animals of each genotype. * P < 0.05, ** P < 0.01, *** P < 0.001.
3.2.2 Analysis of body composition by MRI scanning

In order to determine whether the increased body weight of NesCreIrs2KO animals was due to changes in lean tissue mass, fat mass or a combination of both these factors, the body composition of 7-month-old males was analysed using MRI scanning. Male NesCreIrs2KO animals displayed an almost two-fold increase in fat mass, as compared to controls (Figure 3.3). No difference in lean tissue mass was detected in these animals.

![Body fat mass of 28 week old male NesCreIrs2KO and control animals](image)

Figure 3.3: Body fat mass of 28-week-old male NesCreIrs2KO and controls as revealed by MRI scanning. Data represent the mean ± SEM for 4 animals of each genotype. *** P < 0.001.

![Photograph of male NesCreIrs2KO (left) and control (right) at 12 months of age.](image)

Figure 3.4: Photograph of male NesCreIrs2KO (left) and control (right) at 12 months of age.
3.2.3 Plasma leptin levels

Consistent with their increased body weight at this age, at 12-week-old male \textit{NesCreIrs2KO} animals had significantly raised fasting plasma leptin as compared to control animals (Figure 3.5).

\begin{center}
\textbf{Fasting plasma leptin levels of 12 week old \textit{NesCreIrs2KO} and control males}
\end{center}

![Graph showing fasting plasma leptin levels](image)

Figure 3.5: Fasting plasma leptin levels were measured on male \textit{NesCreIrs2KO} and control mice at 12 weeks of age following a 16-h overnight fast. Data represent the mean $\pm$ SEM for 8 animals of each genotype. $^*$ $P < 0.05$.

3.2.4 Analysis of feeding behaviour

The increased adiposity of \textit{NesCreIrs2KO} animals compared to controls could be a result of increased energy intake (i.e. food intake), decreased energy expenditure or indeed a combination of these two defects. In order to ascertain the contribution of feeding to the obese phenotype of \textit{NesCreIrs2KO} animals, both the daily food intake of these animals and their feeding response to fasting were analysed.
3.2.4.1 Analysis of food intake

At 12 weeks of age, male and female NesCreIrs2KO mice were significantly hyperphagic compared to controls (Figure 3.6). This suggests that the increased body adiposity of these animals was partly due to their increased caloric intake.

A) Daily food intake of 12 week old male NesCreIrs2KO and control animals

B) Daily food intake of 12 week old female NesCreIrs2KO and control animals

Figure 3.6: The daily (24-h) food intake was measured in: A) males at 12 weeks of age, B) females at 12 weeks of age. Data represent the mean ± SEM for 8-16 animals of each genotype. ** P < 0.01, *** P < 0.001.
3.2.4.2  Response to fasting

No increases in the food intake of NesCreIrs2KO animals at 5 months of age were detected following an overnight fast at 1, 2, 4 or 8 hours post re-feeding. However, both male and female NesCreIrs2KO animals exhibited an increase in 24-hour food intake (Figure 3.7), indicating an abnormality in feeding regulation during the dark phase. The hyperphagia exhibited by NesCreIrs2KO animals, suggests defects in long-term feeding regulation, governed by factors such as insulin and leptin.

A) Fast-refeed response of 5 month old male
NesCreIrs2KO and control mice

```
0.0  2.5  5.0  7.5  10.0  12.5
   |   |   |   |   |   |
   1  2  4  8 24

Time (h) after re-feed
```

B) Fast-refeed response of 5 month old female
NesCreIrs2KO and control mice

```
0.0  2.5  5.0  7.5  10.0  12.5
   |   |   |   |   |   |
   1  2  4  8 24

Time (h) after re-feed
```

Figure 3.7: Re-feeding response in A) 5 month old male mice and B) 5 month old female mice. Following a 16-h overnight fast, approximately 50 g of food was weighed and given to each animal. The food was then weighed after 1-h, 2-h, 4-h, 8-h and 24-h. Data represent the mean ± SEM for 6-8 animals of each genotype. * P < 0.05, ** P < 0.01.
3.2.5 Metabolic Rate

In order to determine whether the obese phenotype of *NesCreIrs2KO* animals was due solely to the hyperphagia of these animals, or also partly due to impairments in energy expenditure, the metabolic rate of 12-week-old *NesCreIrs2KO* mice and relevant controls was assessed.

No significant difference in metabolic rate was seen between *NesCreIrs2KO* mice compared to controls at 12 weeks of age at either thermoneutral (30 ± 0.1°C) and housing temperature (22 ± 0.1°C) (Table 3.1).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>RMR (ml O₂/min)</th>
<th>30 °C</th>
<th>22 °C</th>
<th>Tb (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.52 ± 0.02</td>
<td>0.90 ± 0.07</td>
<td>37.6 ± 0.3</td>
</tr>
<tr>
<td><em>NesCreIrs2KO</em></td>
<td></td>
<td>0.62 ± 0.07</td>
<td>0.91 ± 0.09</td>
<td>37.6 ± 0.2</td>
</tr>
</tbody>
</table>

Table 3.1: Summary of analysis of metabolic rate in 12-week-old male *NesCreIrs2KO* and control animals. Data represent the mean ± SEM for 8 animals of each genotype. Resting metabolic rate (RMR) and body temperature (Tb).
3.2.6 Analysis of body length

Measurement of naso-anal body length of male animals at 12 weeks of age revealed that *NesCreLrs2KO* were significantly longer (~12.4% increase) than controls (Figure 3.8).

![Naso-anal length of 12 week old male NesCreLrs2KO and control animals](image)

Figure 3.8: Naso-anal length was measured in male *NesCreLrs2KO* and control mice at 12 weeks of age. Data represent the mean ± SEM for 4-6 animals of each genotype. ***P < 0.001.

3.2.7 Response to leptin

In order to further analyse the defects relating to the obesity phenotype of *NesCreLrs2KO* animals, the sensitivity of these animals to the anorexigenic effects of peripherally administered leptin was assessed.

Leptin treatment (5mg/kg) inhibited food intake by 20% in both control and *NesCreLrs2KO* mice (Figure 3.9). In addition, following the treatment, control mice displayed a 1.09g loss in body weight, compared with a 1.3g loss of weight in *NesCreLrs2KO* mice. Taken together, these findings indicate that 12-week-old *NesCreLrs2KO* mice are not resistant to the anorexigenic effects of leptin.
Figure 3.9: The response to 3 days of intraperitoneal leptin administration upon A) body weight and B) food intake was determined in acclimatised male NesCreIrs2KO and control mice at 12 weeks of age. Animals were injected once daily prior to the onset of the dark-phase with either leptin (5 mg/kg) or vehicle for three days in a cross-over design study. Both body weight and cumulative food intake were recorded. Data represent the mean ± SEM for 8 animals of each genotype. ** P < 0.01.
3.2.8 Analysis of hypothalamic neuropeptide expression

It was hypothesised that the deletion of Irs2 in the brain of NesCreIrs2KO animals may have altered the expression levels of various neuropeptides involved in the regulation of food intake.

Analysis of the gene expression of hypothalamic neuropeptides of 12-week-old fasted NesCreIrs2KO and control animals, showed no significant differences in Agrp, Cart or Npy expression levels. However, almost a 50% reduction in hypothalamic Pomp gene expression in NesCreIrs2KO mice was detected (Figure 3.10).

Figure 3.10: Summary of gene expression of hypothalamic Agrp, Cart, Npy and Pomp analysed in using RT-PCR. Data shown represents the % change neuropeptide concentration of NesCreIrs2KO animals as compared to control animals. Data represent the mean ± SEM for 6 animals of each genotype. * P < 0.05.
3.3 Glucose Homeostasis

A role for CNS insulin signalling in the regulation of peripheral glucose homeostasis and insulin action has been implicated by a number of studies. Of particular relevance is the study by Obici and colleagues (2002) in which anti-sense oligodeoxynucleotides were used to block hypothalamic insulin receptors (Obici et al., 2002a) and also the impaired glucose homeostasis of NIRKO mice (Bruning et al., 2000). Taken together, these studies suggest a significant role for CNS insulin signalling in the regulation of glucose homeostasis.

In order to investigate the role of IRS2 mediated insulin signalling pathways in the CNS regulation of glucose homeostasis, various studies were carried out on NesCreIrs2KO mice. A further aim of these studies was to assess the contribution of the CNS deletion of Irs2 to the severe dysregulation of glucose homeostasis displayed by global Irs2 null animals.

3.3.1 Fasting blood glucose (FBG) levels

By 12 weeks of age NesCreIrs2KO male mice were significantly hyperglycaemic, compared to controls, with this still evident at 6 months. However, blood glucose while increased, was still within normal limits.

![Figure 3.11: FBG levels (mmol/L) of mice following a 16-h fast of A) males at 12 weeks of age and B) males at 6 months of age. Data represent the mean ± SEM for 8 animals of each genotype. * P < 0.05, ** P < 0.01.](image)
3.3.2 Glucose tolerance

Glucose tolerance tests were performed on male NesCreIrs2KO and control mice at 12 weeks of age and 6 months of age. At both ages, male NesCreIrs2KO mice exhibited impaired glucose tolerance compared to controls.

Figure 3.12: Glucose tolerance tests performed on male mice at A) 12 weeks of age and B) 6 months of age. Data represent the mean ± SEM for 7-9 animals of each genotype. * P < 0.05, ** P < 0.01, *** P < 0.001.
3.3.3 Fasting plasma insulin levels

In order to further understand the mechanisms of disordered glucose homeostasis in *NesCreIrs2KO* animals, the fasting plasma insulin levels of animals at 12 weeks of age were measured. This analysis revealed that *NesCreIrs2KO* animals were significantly hyperinsulinaemic; with more than a two-fold increase in basal levels, compared to control animals. This suggests the presence of insulin resistance in *NesCreIrs2KO* animals.

![Graph showing fasting plasma insulin levels of 12 week old male *NesCreIrs2KO* and control animals](image)

Figure 3.13: Fasting plasma insulin levels were measured on male *NesCreIrs2KO* and control mice following a 16-h overnight fast at 12 weeks of age. Data represent the mean ± SEM for 8-12 animals of each genotype. * P < 0.05.
3.3.4 Pancreatic islet mass and density

Analysis of islet size and density in male mice at 12 weeks of age showed that *NesCreirs2KO* mice exhibited increased β-cell mass of almost double that of control animals. This provides further evidence of a compensatory response to insulin resistance in *NesCreirs2KO* animals and is consistent with both the hyperinsulinaemia and obesity seen at the same age.

**Pancreatic β-cell area of 12 week old male *NesCreirs2KO* and control animals**

![Graph showing comparison between Control and NesCreirs2KO groups](image)

Figure 3.14: The percentage total pancreatic area occupied by β-cells in male *NesCreirs2KO* and control animals at 12 weeks of age. Five pancreases were analysed per genotype at each time point and for each pancreas four sections were analysed. Data represent the mean ± SEM for 5 animals of each genotype. **P < 0.01.**
3.4 Assessment of reproductive function

Reproductive dysfunction is a feature of both the NIRKO mouse and *Irs2* null animals. *NIRKO* animals exhibited impaired spermatogenesis and ovarian follicle maturation due to the hypothalamic dysregulation of luteinising hormone. Global *Irs2* null females have small, anovulatory ovaries with reduced numbers of follicles. The plasma levels of luteinising hormone, prolactin and sex steroids were also reduced in these animals. Pituitaries were also decreased in size and contain reduced numbers of gonadotrophs. Taken together, these studies highlight a role for intact insulin signalling in the brain not only in the regulation of energy disposal and fuel metabolism, but also in the regulation of reproductive function.

The aim of this section of work was to investigate the reproductive function of *NesCreIrs2KO* animals, and to address the contribution of CNS *Irs2* signalling pathways to the infertility phenotype of the *Irs2* null mouse in a tissue specific manner. Males with global deletion of *Irs2* exhibited no reproductive defects. In addition, male *NesCreIrs2KO* animals were known to be fertile, as they were used as breeders prior to the commencement of studies aimed at assessing the fertility of these animals. Therefore the studies described in the following chapter were carried out solely on female animals.

3.4.1 Validation of *NesCreIrs2KO* females as model for studying the role of CNS IRS2 pathways in the regulation of reproductive function

Prior to the commencement of studies, it was vital to ensure that no *Irs2* deletion was occurring in non-CNS tissues that contribute to fertility (more specifically the pituitary and ovary). At both the DNA and mRNA levels, no *Irs2* deletion was detected in either tissue (Figure 3.15), confirming the use of *NesCreIrs2KO* animals as a valid model for studying the contribution of CNS IRS2 pathways to the regulation of female reproductive function. Thus, a series of studies designed to determine various physiological parameters of reproduction in *NesCreIrs2KO* females were performed.
Figure 3.15: A) DNA was extracted from the hypothalami, pituitaries and ovaries of NesCreIrs2KO and control animals. PCR was performed to detect Irs2 deletion in these samples. No bands were amplified in pituitary and ovarian samples, indicating no recombination of the loxP sites in these tissues. B) Irs2 gene expression analysis by RT-PCR. i) Gene expression levels in the pituitaries of NesCreIrs2KO and controls, ii) gene expression levels in the ovaries of NesCreIrs2KO and controls. These analyses revealed that the pituitary and ovarian Irs2 mRNA expression of NesCreIrs2KO animals were equivalent to that of control animals. Data represent the mean ± SEM for 12-17 animals of each genotype.
3.4.2 Characterisation of oestrous cycle

Female *NesCreIrs2KO* mice were found to have an extended oestrous cycle due to a mildly prolonged dioestrous phase (Table 3.2).

<table>
<thead>
<tr>
<th>Phase</th>
<th>Duration (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Proestrous</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Oestrous</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Metoestrous</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Dioestrous</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Total duration</td>
<td>4.9 ± 0.1</td>
</tr>
</tbody>
</table>

Table 3.2: Summary of the oestrous cycle pattern of *NesCreIrs2KO* and control females. The oestrous cycle characteristics of *NesCreIrs2KO* were determined by daily vaginal smears analysed for cell type and morphology with giemsa staining for 3 completed cycles. Data represent the mean ± SEM for 8 animals of each genotype. * P < 0.05.

3.4.3 Ovarian functional and morphological analyses

3.4.3.1 Ovarian function

No differences in the timing of ovulation and the numbers of oocytes recovered were seen when *NesCreIrs2KO* mice and control mice were super-ovulated (total oocytes recovered per animal: control 23.6 ± 2.3 vs *NesCreIrs2KO* 21.1 ± 2.1, n=5, p = N.S.
3.4.3.2 *Ovarian morphological analysis*

Haematoxylin-eosin staining of ovarian sections demonstrated the presence of follicles of all developmental stages and corpora lutea in *NesCreIrs2KO* and control mice (Figure 3.16), consistent with the normal response of *NesCreIrs2KO* animals to superovulation.

Figure 3.16: Ovarian anatomy in control and *NesCreIrs2KO* mice. Low magnification photomicrograph of: A) control and B) *NesCreIrs2KO* animals. Scale bars are 0.5 mm. It should be noted that the brown staining visible in the photomicrograph is peroxidase staining from independent immunohistochemistry studies.
3.4.3.3 Ovarian weights

Ovarian weights of NesCreIrs2KO animals at 12 weeks of age, were indistinguishable from those of control animals (Figure 3.17).

**Figure 3.17:** Ovarian weights of NesCreIrs2KO and control animals at 12 weeks of age. Data represent the mean ± SEM for 15-25 animals of each genotype.

3.4.4 Analysis of reproductive hormone levels

Pituitaries and blood were collected from NesCreIrs2KO and control animals during the dioestrous phase of the oestrous cycle, for the analysis of the levels of various reproductive hormones.

Analysis of serum oestradiol and progesterone levels demonstrated no differences in the levels of these hormones between NesCreIrs2KO and control animals. With regards to pituitary hormones, the levels of LH and FSH in NesCreIrs2KO animals were comparable to controls. However, the level of prolactin in NesCreIrs2KO animals was significantly reduced compared to controls. These results are summarised in the table below (Table 3.3).
<table>
<thead>
<tr>
<th>Hormone levels</th>
<th>Serum/pituitary levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Oestradiol (nmol/L)</td>
<td>0.016 ± 0.002</td>
</tr>
<tr>
<td>Progesterone (nmol/L)</td>
<td>4.198 ± 0.7</td>
</tr>
<tr>
<td>LH (ng/ml)</td>
<td>300.6 ± 37.3</td>
</tr>
<tr>
<td>Prolactin (ng/ml)</td>
<td>4048 ± 840.7</td>
</tr>
<tr>
<td>FSH (ng/ml)</td>
<td>43.78 ± 3.435</td>
</tr>
</tbody>
</table>

Table 3.3: Summary of the dioeuous levels of serum oestradiol and progesterone, and reproductive pituitary hormones. Data represent the mean ± SEM for 8 animals of each genotype. ** P < 0.01.

### 3.4.5 Analysis of breeding performance by timed mating studies

In continuous mating studies, the average duration between litters and the size and number of litters over the study period were similar in NesCreIrs2KO and control mice (Table 3.4).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NesCreIrs2KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of litters</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>Total number of pups</td>
<td>172</td>
<td>137</td>
</tr>
<tr>
<td>Average no. of litters per female</td>
<td>3.833 ± 0.1667</td>
<td>3.167 ± 0.3073</td>
</tr>
<tr>
<td>Average no. of pups per litter</td>
<td>7.478 ± 0.6969</td>
<td>7.211 ± 0.7318</td>
</tr>
<tr>
<td>Average duration between litters (days)</td>
<td>26.06 ± 1.885</td>
<td>28.69 ± 2.725</td>
</tr>
</tbody>
</table>

Table 3.4: Summary of breeding performance of six female NesCreIrs2KO animals and six controls, pair mated with wildtype C57Bl6 males. Performance was assessed by monitoring of the litter number and size, and also duration between litters. Data represent the mean ± SEM for 6 animals of each genotype.
3.5 Summary of hypothalamic function, glucose homeostasis and reproductive function of *NesCreIrs2KO* animals

3.5.1 Hypothalamic function

Studies into the hypothalamic function of *NesCreIrs2KO* mice showed that these animals had considerable abnormalities. Both sexes exhibited significantly increased body weight compared to controls, with an almost two-fold increase in adiposity. Consistent with this, *NesCreIrs2KO* mice were significantly hyperleptinaemic, yet remained sensitive to the anorexigenic effects of peripherally administered leptin. In addition, *NesCreIrs2KO* animals were long and hyperphagic, but still had normal resting metabolic rate.

3.5.2 Glucose homeostasis

Studies investigating the glucose homeostasis of these animals, showed that *NesCreIrs2KO* mice were significantly hyperglycaemic and demonstrated impaired glucose tolerance compared to control animals. In addition, *NesCreIrs2KO* animals displayed hyperinsulinaemia and increased pancreatic islet mass density.

3.5.3 Reproductive function

Female *NesCreIrs2KO* mice were found to have an extended oestrous cycle due to a mildly prolonged dioestrous phase. The levels of serum oestradiol and progesterone levels during dioestrous were however normal. The analysis of pituitary hormones during dioestrous showed that although the levels of LH and FSH were comparable to controls, *NesCreIrs2KO* animals had significantly reduced prolactin levels. No differences in the timing of ovulation and the numbers of oocytes recovered were seen when *NesCreIrs2KO* mice and control mice were super-ovulated. Finally, timed matings between *NesCreIrs2KO* females and control males showed that these animals had normal breeding performance compared to control females.
Chapter 4

Phenotypic characterisation of $POMCCreIrs2KO$ animals
4 Characterisation of $POMCCreIrs2KO$ mice

The role of POMC/CART neurons in the regulation of energy homeostasis via the interaction with adiposity signals, such as insulin, has been widely considered essential in the regulation of energy homeostasis. POMC neurons have been implicated as mediating the anorexigenic effects of insulin on energy homeostasis. Insulin receptors are known to be present on POMC neurons indicating a role for insulin signalling in this neuronal population (Benoit et al., 2002). Administration of both insulin and leptin directly into the third ventricle of mice has been shown to result in increased hypothalamic expression of $Pomc$ mRNA (Benoit et al., 2002; Brown et al., 2006). However, despite these findings, the physiological role of insulin signalling in POMC neurons is still unknown. Numerous studies have also implicated a role for the hypothalamic melanocortin system in the regulation of peripheral glucose homeostasis (Farooqi et al., 2003; Marks and Cone, 2001; Obici et al., 2001; Schwartz, 2001).

In order to fully address the role of IRS2 mediated insulin signalling in POMC neurons, a number of studies were carried out on $POMCCreIrs2KO$ mice designed to assess the hypothalamic function and various parameters of glucose homeostasis of these animals.

4.1 Proof of $Irs2$ deletion

$POMCCre$ transgenic animals have been used extensively in recent years (Xu et al., 2005a; Xu et al., 2005b; Xu et al., 2007). Despite this, before the commencement of investigations, studies aimed at confirming $POMCCreIrs2KO$ animals were lacking $Irs2$ expression in the hypothalamus were performed. A PCR-based strategy showed that recombination of $Irs2lox$ alleles was occurring in the hypothalami of $POMCCreIrs2KO$ animals, and immunocytochemistry analysis demonstrated the deletion of $Irs2$ specifically in POMC neurons (Figure 4.1). These studies therefore established $POMCCreIrs2KO$ animals as a valid model for investigating the physiological role of IRS2 signalling in POMC neurons in the context of energy balance regulation. A number of studies were therefore carried out on $POMCCreIrs2KO$ mice, designed to assess the hypothalamic function of these animals.
Figure 4.1: Studies proving deletion of *Irs2* in hypothalami of *POMCCReIrs2KO*. A) PCR analysis of *Irs2* deletion in DNA extracted from *POMCCReIrs2KO* and control hypothalami. A 1.3kb band in sample confirms deletion of *Irs2*. B) Proof of *Irs2* deletion in POMC neurons: i) co-localisation of POMCCreLacZ expression and IRS2 in *POMCCRelacZ* mice and ii) no co-localisation of POMCCreLacZ and IRS2 in *POMCCReIrs2KOlacZ* mice. Scale bars: 10 μm.
4.2 Hypothalamic function

4.2.1 Analysis of body weight

POMCCreIrs2KO mice and relevant controls were weighed from 5 weeks of age onwards. Both males and females were of normal weight, as compared to control littermates, at all ages studied (Figure 4.2).

Figure 4.2: Growth curve for A) male POMCCreIrs2KO and control animals and B) female POMCCreIrs2KO and control animals. Data represent the mean ± SEM for 6-12 animals of each genotype.
4.2.2 Analysis of body composition by DEXA scanning

Analysis of the body composition of 6 month old male and female animals, showed no significant differences in fat mass, lean tissue mass and percentage fat mass between POMCCreIrs2KO and control animals of both sexes (Figure 4.3). These findings were consistent with the normal body weight of POMCCreIrs2KO animals at this age.

Figure 4.3: Analysis of body composition of 6 month old male and females by DEXA: A) body weight, B) lean tissue mass (g), C) fat mass (g), D) % fat mass. Data represent the mean ± SEM for 6 animals of each genotype.
4.2.3 Plasma leptin levels

Consistent with the lack of increased adiposity observed at this age, the fasting leptin levels of 12-week-old male POMCCreIrs2KO mice were indistinguishable from that of controls (Figure 4.4).

![Fasting plasma leptin levels of POMCCreIrs2KO and controls at 12 weeks of age](image)

Figure 4.4: Fasting plasma leptin levels were measured on male POMCCreIrs2KO and control mice at 12 weeks of age following a 16-h overnight fast. Data represent the mean ± SEM for 6-8 animals of each genotype.

4.2.4 Analysis of body length

Measurement of naso-anal body length at 12 weeks of age showed no difference between POMCCreIrs2KO and control animals (Figure 4.5).

![Naso-anal length of POMCCreIrs2KO and control animals](image)

Figure 4.5: Naso-anal length (body length) was measured in male and female POMCCreIrs2KO mice at 12 weeks of age. Data represent the mean ± SEM for 8 animals of each genotype.
4.2.5 Analysis of feeding behaviour

Despite the lack of differences found in the body weight, body length and body composition of \textit{POMCCre\textit{lrs}2KO} compared to controls, the feeding behaviour of \textit{POMCCre\textit{lrs}2KO} animals was investigated. Studies undertaken included the analysis of food intake and re-feeding response to an overnight fast.

4.2.5.1 Analysis of food intake

At 8 weeks of age, the daily food intake of both male and female \textit{POMCCre\textit{lrs}2KO} mice were indistinguishable from that of control animals (Figure 4.6).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig46a.png}
\caption{Daily food intake of A) male \textit{POMCCre\textit{lrs}2KO} and control animals and B) female \textit{POMCCre\textit{lrs}2KO} and control animals at 8 weeks of age. Data represent the mean ± SEM for 6-8 animals of each genotype.}
\end{figure}
4.2.5.2  Response to fasting

At 10 weeks of age, the feeding response of both male and female POMCcrelrs2KO mice was comparable to that of controls at all time points (Figure 4.7), indicating no impairment in short term feeding regulation mechanisms. These results were consistent with the normal daily food intake exhibited by these animals.

A) Fast-refeed response of 10 week old male animals

![Graph A](image)

B) Fast-refeed response of 10 week old female animals

![Graph B](image)

Figure 4.7: Re-feeding response following a 16-h overnight fast in 10 week old A) male POMCcrelrs2KO and controls and B) female POMCcrelrs2KO and controls. Data represent the mean ± SEM for 6-8 animals of each genotype.
4.2.6 High fat diet (HFD) trial

As can be seen from the results above, both male and female POMCCreIrs2KO displayed no difference in body weight compared to control animals, even at 6 months of age. Placing mice on a HFD has been shown to enhance any underlying obesity phenotype that may not be displayed on a standard chow diet (Bruning et al., 2000).

Male POMCCreIrs2KO and control mice were placed on a 12 week HFD trial from the age of 6 weeks, with the body weights of all mice measured once weekly throughout the trial. The body weights of POMCCreIrs2KO and control animals on a normal chow diet, were measured simultaneously. As expected both POMCCreIrs2KO and control animals gained more weight on the HFD, compared to mice of the same genotype on the standard diet. However, no differences in body weight were seen between POMCCreIrs2KO and control animals on the HFD (Figure 4.8).

![Body weight curve of male POMCCreIrs2KO and controls placed on HFD](image)

Figure 4.8: Body weight curve of male POMCCreIrs2KO and control mice on HFD and normal chow diet. Data represent the mean ± SEM for 6-9 animals of each genotype.
4.3 Glucose Homeostasis

4.3.1 Fasting blood glucose (FBG) levels

At both 8 and 12 weeks of age, male and female *POMCCreIrs2KO* mice had indistinguishable fasting blood glucose levels from control animals (Figure 4.9).

![Graphs showing fasting blood glucose levels](image)

Figure 4.9: Fasting blood glucose levels (mmol/L) of mice following a 16-h fast of A) males at 8 weeks of age, B) males at 12 weeks of age and C) females at 8 weeks of age and D) females at 12 weeks of age. Data represent the mean ± SEM for 6-8 mice of each genotype.
4.3.2 Glucose tolerance

Glucose tolerance tests were performed on both male and female $POMCCreIrs2KO$ and control mice at 8 and 12 weeks of age. At both ages, male and female $POMCCreIrs2KO$ mice exhibited normal glucose disposal compared to controls (Figure 4.10).

Figure 4.10: Glucose tolerance tests performed on: A) male mice at 8 weeks of age, B) male mice at 12 weeks of age, C) female mice at 8 weeks of age and D) female mice at 12 weeks of age. Data represent the mean ± SEM for 6-8 animals of each genotype.
4.3.3 Fasting plasma insulin levels

At 12 weeks of age male *POMCCreIrs2KO* mice had normal plasma insulin levels, as compared to control animals (Figure 4.11).

![Fasting plasma insulin levels of males at 12 weeks](image)

Figure 4.11: Fasting plasma insulin levels were measured on 12-week-old male *POMCCreIrs2KO* and control mice following a 16-h overnight fast. Data represent the mean ± SEM for 6 animals of each genotype.

4.4 Summary of hypothalamic function and glucose homeostasis of *POMCCreIrs2KO* animals

*POMCCreIrs2KO* animals exhibited normal body weight and body length compared to controls. Consistent with the lack of increased adiposity, *POMCCreIrs2KO* animals had normal fasting leptin levels compared to controls. In addition, the daily food intake, fast-refeed response and sensitivity to DIO of *POMCCreIrs2KO* animals was comparable to controls.

With regards to glucose homeostasis, *POMCCreIrs2KO* animals exhibited normal fasting blood glucose levels, glucose tolerance and fasting insulin levels compared to control animals.
Chapter 5

Phenotypic characterisation of 
\textit{AgRPCreIrs2KO} animals
5 Characterisation of AgRPCreIrs2KO mice

The dominant view in the field at the commencement of this work was that NPY/AgRP are primary first-order neurons that mediate the anorexigenic effects of insulin in the regulation of energy homeostasis. The ICV administration of insulin directly into the brain has been shown to inhibit expression of Npy and Agrp (Schwartz et al., 1992; Sipols et al., 1995). Although IRS2 has been shown to co-localise with NPY in the ARC (Pardini et al., 2006), the physiological role of insulin signalling in this NPY/AgRP neuronal population is still unknown.

In order to fully address the role of IRS2 mediated insulin signalling in AgRP neurons, a number of studies were carried out to assess various parameters of energy and glucose homeostasis in AgRPCreIrs2KO mice and controls.

5.1 Assessment of ectopic Cre expression in AgRPCreIrs2KO animals

Ectopic lacZ expression has been found in AgRPCreR26R mice with approximately 30% of these animals exhibiting widespread X-gal staining, affecting multiple brain regions as well as mesenchymal and epithelial tissues throughout the body (Kaelin et al., 2004).

To ensure that no mice in which germline deletion of Irs2 were studied, ear punches were taken from all AgRPCreIrs2KO animals generated (all homozygous for the R26R transgene) and X-gal staining was performed. Animals positive for staining were excluded from all investigations described below.

5.2 Proof of Irs2 deletion

Prior to commencement of phenotypic characterisation of AgRPCreIrs2KO animals, studies aimed at confirming the lack of Irs2 expression in hypothalami of AgRPCreIrs2KO animals were undertaken. A PCR-based strategy did indeed
demonstrate that recombination of *Irs2lox* alleles was occurring in the hypothalami of *AgRPCreIrs2KO* animals, and not controls (Figure 5.1).

![Image](image_url)

Figure 5.1: PCR analysis of *Irs2* deletion in DNA extracted from: A) *AgRPCreR26Rlrs2KO* hypothalami and B) control hypothalami. A 1.3kb band in sample A confirms deletion of *Irs2* deletion. No band was amplified in sample B.

Despite proving deletion of *Irs2* was occurring in the hypothalami of *AgRPCreIrs2KO* animals, there was difficulty in demonstrating loss of *Irs2* expression in AgRP neurons. Immunocytochemical (ICC) studies proved unsuccessful due to the fact that no reliable antibodies against AgRP are currently available. As a result, the lack of IRS2 expression specifically in AgRP neurons could never be confirmed using this method. Alternative strategies aimed at expressing green fluorescent protein (GFP) in AgRP neurons (upon expression of Cre-recombinase in this cell type) also failed. This was due to the fact that the *AgRPCre, Irs2lox* and *Z/eg* alleles could never be genetically brought together, despite intensive breeding efforts. Although appropriate mating crossed were set-up, no *AgRPCreIrs2KOZ/eg* mice were ever generated (despite these crosses yielding offspring of all other intermediary genotypes). However, the *AgRPCre* transgenic mouse line has been used in a number of studies and shown to be a reliable model (Kaelin et al., 2004; Xu et al., 2005b).
5.3 Hypothalamic function

5.3.1 Analysis of body weight

Both males and female AgRPCreIrs2KO animals were of normal weight, as compared to control littermates, at all ages studied (Figure 5.2).

A) Growth curve for male AgRPCreIrs2KO and control animals

B) Growth curve for female AgRPCreIrs2KO and control animals

Figure 5.2: Growth curve for A) male AgRPCreIrs2KO and control animals and B) female AgRPCreIrs2KO and control animals. Data represent the mean ± SEM for 6-12 animals of each genotype.
5.3.2 Analysis of body composition by DEXA scanning

The body composition of 6-month-old males was assessed by DEXA scanning. Results showed no significant differences in fat mass, lean tissue mass, and percentage fat mass between AgRPCreIrs2KO and control animals (Figure 5.3).

![Bar charts showing body composition](image)

Figure 5.3: Analysis of body composition of 6 month old males by DEXA: A) body weight, B) lean tissue mass (g), C) fat mass (g), D) % fat mass. Data represent the mean ± SEM for 6 animals of each genotype.
5.3.3 Plasma leptin levels

Consistent with the lack of increased adiposity at 12 weeks of age, the fasting leptin levels of 12-week-old male AgRPCreIrs2KO animals were indistinguishable from controls (Figure 5.4).

![Fasting plasma leptin levels of 12 week old males](image)

Figure 5.4: Fasting plasma leptin levels were measured on male AgRPCreIrs2KO and control mice at 12 weeks of age following a 16-h overnight fast. Data represent the mean ± SEM for 9 animals of each genotype.

5.3.4 Analysis of body length

Measurement of naso-anal body length in males at 6 months of age showed no significant difference between AgRPCreR26R1rs2KO and control animals (Figure 5.5).

![Naso-anal body length](image)

Figure 5.5: Naso-anal length (body length) was measured in male AgRPCreIrs2KO and control mice at 6 months of age. Data represent the mean ± SEM for 6 animals of each genotype.
5.3.5 Feeding behaviour

Despite the lack of differences found in the body weight, body length and body composition of AgRPRCreIrs2KO animals compared to controls, the feeding behaviour of these animals was investigated. Studies undertaken included analysis of food intake and re-feeding response to an overnight fast.

5.3.5.1 Analysis of food intake

At 12 weeks of age, the daily food intake of both male and female AgRPRCreIrs2KO mice was indistinguishable from that of control animals (Figure 5.6).

![Bar chart of daily food intake for male AgRPRCreIrs2KO and controls](image)

![Bar chart of daily food intake for female AgRPRCreIrs2KO and controls](image)

Figure 5.6: Daily (24-h) food intake was studied at 12 weeks of age in: A) male AgRPRCreIrs2KO and control animals and B) female AgRPRCreIrs2KO and control animals. Data represent the mean ± SEM for 6-9 animals of each genotype.
5.3.5.2  Response to fasting

At 12 weeks of age, the feeding response of both male and female *AgRPCreIrs2KO* mice was comparable to that of controls at all time points measured, indicating no impairment in short term feeding regulation mechanisms. These results were consistent with the normal daily food intake exhibited by these animals (Figure 5.7).

**A) Fast-refeed response of 12 week old male animals**

![Graph showing cumulative food intake](https://example.com/graph1.png)

**B) Fast-refeed response of 12 week old female animals**

![Graph showing cumulative food intake](https://example.com/graph2.png)

Figure 5.7: Re-feeding response following a 16-h overnight fast in A) 12 week old male mice and B) 12 week old female mice. Data represent the mean ± SEM for 6-9 animals of each genotype.
5.4 Glucose Homeostasis

In order to investigate the effect on glucose homeostasis of the deletion of IRS2 mediated insulin signalling in AgRP neurons, various metabolic studies were carried out on AgRPCreIrs2KO mice.

5.4.1 Fasting blood glucose (FBG) levels

At 12 weeks and 8 months of age, both male and female AgRPCreIrs2KO mice had indistinguishable FBG levels to control animals (Figure 5.8).

![Graphs showing fasting blood glucose levels for different ages and sexes.](image)

Figure 5.8: Fasting blood glucose levels (mmol/L) of A) males at 12 weeks of age, B) females at 12 weeks of age, C) males at 8 months of age and and D) females at 8 months of age. Data represent the mean ± SEM for 6-9 mice of each genotype.
5.4.2 Glucose tolerance

At 12 weeks of age, the glucose disposal of both male and female \textit{AgRPCreIrs2KO} animals was comparable to controls (Figure 5.9).

A) Glucose tolerance of males at 12 weeks of age

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{male_glucose_tolerance.png}
\caption{Glucose tolerance tests performed on: A) male mice at 12 weeks of age, B) female mice at 12 weeks of age. Following a 16 -h overnight fast and an intraperitoneal injection of D-glucose (1.5g/kg body weight), blood glucose levels were measured at the indicated time-points. Data represent the mean ± SEM for 6-9 animals of each genotype.}
\end{figure}
5.4.3 Fasting plasma insulin levels

At 12 weeks of age male AgRPCreIrs2KO mice had normal fasting insulin levels as compared to control animals (Figure 5.10).

Fasting insulin levels of males at 12 weeks of age

![Fasting insulin levels of males at 12 weeks of age](image)

Figure 5.10: Fasting plasma insulin levels were measured on male AgRPCreIrs2KO and control mice following a 16-h overnight fast at 12 weeks of age. Data represent the mean ± SEM for 6-8 animals of each genotype.

5.5 Summary of hypothalamic function and glucose homeostasis of AgRPCreIrs2KO animals

AgRPCreIrs2KO animals exhibited normal body weight and body length compared to controls. Consistent with the lack of increased adiposity, (as assessed by DEXA scanning) AgRPCreIrs2KO animals had normal fasting plasma leptin levels compared to controls. In addition, the daily food intake and fast-refeed response of AgRPCreIrs2KO animals was comparable to controls.

With regards to glucose homeostasis, AgRPCreIrs2KO animals exhibited normal fasting blood glucose levels, glucose tolerance and fasting insulin levels compared to control animals.
Chapter 6

Phenotypic characterisation of $AgRPCrep110\betaKO$ animals
6 Characterisation of AgRP<sub>Crep110βKO</sub> mice

The dominant view in the field is that NPY/AgRP are primary first-order neurons that mediate the anorexigenic effects of insulin in the regulation of energy homeostasis. The ICV administration of insulin directly into the brain has been shown to inhibit expression of NPY and AgRP neuropeptides (Schwartz et al., 1992; Sipols et al., 1995). Leptin inhibits the release of AgRP (Hoggard et al., 2004), the expression of which is up-regulated in leptin reduction due to fasting or mutation (Hahn et al., 1998; Wilson et al., 1999). The inhibition of the hypothalamic expression of AgRP by leptin has been shown to be via a mechanism that requires intact PI3K signalling (Morrison et al., 2005). Indeed, several studies have implicated PI3K as a point of convergence between the insulin and leptin signalling cascades, mediating the anorexigenic effects of both hormones in the regulation of energy and glucose homeostasis (Morrison et al., 2005; Morton et al., 2005; Niswender et al., 2003; Niswender et al., 2001). However, a recent study has shown that leptin and insulin have opposite effects on AgRP neurons, with membrane accumulation of PI3K inhibited by leptin but stimulated by insulin (Xu et al., 2005b).

In order to fully address the role of PI3K mediated signalling pathways in AgRP neurons, a number of studies were carried out on AgRP<sub>Crep110βKO</sub> mice (animals which lack functional expression of the catalytic p110β subunit in AgRP neurons), designed to assess the hypothalamic function and various parameters of glucose homeostasis in these animals.

6.1 Proof of p110β deletion

Prior to commencement of the phenotypic characterisation of AgRP<sub>Crep110βKO</sub> animals, studies aimed at confirming the lack of functional p110β expression in hypothalami of AgRP<sub>Crep110βKO</sub> animals were carried out. However, to date, all studies aimed at proving this have been unsuccessful. These studies have included PCR-based strategies designed to detect recombination of the p110β<sub>lox</sub> alleles, and also immunocytochemical (ICC) studies aimed at proving loxP recombination specifically in AgRP neurons. Possible explanations for this potentially stem from the fact that only a
very small percentage of hypothalamic neurons express AgRP. This neuropeptide is expressed exclusively within the ARC, and thus the use of PCR-based strategies performed on whole hypothalamic lysates may not be sensitive enough. With regards to ICC studies, although viable AgRPCrepi110βKOYFP (animals which express enhanced yellow fluorescent protein in AgRP neurons lacking p110β) were generated, there are no reliable antibodies against the C-terminus of p110β currently on the market. It can be argued that given the important role for PI3K in the regulation of growth and proliferation, inactivation of the p110β subunit in AgRP neurons results in cell death. This could also explain the fact that recombination of the floxed alleles could not be detected in hypothalamic lysates from AgRPCrepi110βKO animals. However, hypothalamic slices from AgRPCrepi110βKOYFP animals clearly show the presence of fluorescence in the ARC indicating that AgRP neurons lacking p110β are viable (Figure 6.1).

![Image](image.png)

**Figure 6.1:** Proof of AgRP neuron viability in AgRPCrepi110βKO animals. Hypothalamic sections from: A) AgRPCreYFP control animals and B) AgRPCrepi110βKOYFP animals. No obvious alterations in AgRP neuron dimensions and number could be detected in AgRPCrepi110βKOYFP animals compared to controls.

### 6.2 Hypothalamic function

In order to fully address the role of p110β mediated signalling pathways in AgRP neurons, studies were carried out on AgRPCrepi110βKO mice designed to assess the hypothalamic function of these animals.
6.2.1 Analysis of body weight

From the ages of 10 and 17 weeks, male and female AgRPCrep110βKO animals respectively weighed significantly less than controls (Figure 6.2).

A) Growth curve for AgRPCrep110βKO and control males

![Growth curve for AgRPCrep110βKO and control males](image)

B) Growth curve for AgRPCrep110βKO and control females

![Growth curve for AgRPCrep110βKO and control females](image)

Figure 6.2: Growth curve for male AgRPCrep110βKO and control animals (A). Data represent the mean ± SEM for 26-52 animals of each genotype. Growth curve for female AgRPCrep110βKO and control animals (B). Data represent the mean ± SEM for 6-26 animals of each genotype. * P < 0.05, ** P < 0.01, *** P < 0.001.
6.2.2 Analysis of body length

Measurement of body length at 10 months of age showed male AgRPCrept110lβKO animals were of normal body length, compared to control littermates (Figure 6.3).

![Naso-anal length of male AgRPCrept110lβKO and controls](image)

Figure 6.3: Naso-anal length (body length) was measured in male AgRPCrept110lβKO and control mice at 10 months of age. Data represent the mean ± SEM for 8 animals of each genotype.

6.2.3 Plasma leptin levels

Measurement of the fasting plasma leptin levels of 10-month-old mice showed that AgRPCrept110lβKO animals had an almost 4-fold reduction in leptin levels compared to controls (Figure 6.4).

![Fasting leptin levels of male AgRPCrept110lβKO and controls](image)

Figure 6.4: Fasting plasma leptin levels were measured on male AgRPCrept110lβKO and control mice at 10 months of age following a 16-h overnight fast. Data represent the mean ± SEM for 6-8 animals of each genotype. ** P < 0.01.
6.2.4 Analysis of feeding behaviour

The reduced body weight of both male and female AgRPCrep110βKO animals suggested that this could be a result of decreased energy intake (i.e. food intake), increased energy expenditure or indeed a combination of these two defects. In order to ascertain the contribution of feeding to the lean phenotype, the daily food intake and the feeding response to fasting of male AgRPCrep110βKO and controls were analysed.

6.2.4.1 Analysis of food intake

At 4 months of age, AgRPCrep110βKO mice exhibited significantly reduced daily food intake compared to control animals (Figure 6.5).

![Daily food intake of male AgRPCrep110βKO and control animals](image)

Figure 6.5: The daily (24-h) food intake of singly housed male AgRPCrep110βKO and controls at 4 months of age. Data represent the mean ± SEM for 13-15 animals of each genotype. * P < 0.05.
6.2.4.2 Response to fasting

At 6 months of age, AgRPCrep110βKO animals displayed normal food intake at 1, 2, 4 and 8-h post re-feeding compared to controls. However, consistent with the hypophagia displayed by AgRPCrep110βKO animals, AgRPCrep110βKO animals had significantly reduced cumulative food intake compared to controls at 24-h post re-feeding (Figure 6.6).

Fast-refeed response of male AgRPCrep110βKO and controls

![Graph showing food intake over time](image)

Figure 6.6: Re-feeding response following a 16-h overnight fast in 6 month old males. Data represent the mean ± SEM for 13-15 animals of each genotype. * P < 0.05.

6.2.5 Analysis of metabolic rate

In order to determine whether the lean phenotype of AgRPCrep110βKO animals was due solely to the hypophagia of these animals or also partly due to impairments in energy expenditure, the metabolic rate of 8-month-old male AgRPCrep110βKO mice and relevant controls was assessed. No significant difference in metabolic rate was seen between AgRPCrep110βKO mice and controls at thermoneutral (30±0.1°C) temperature (Figure 6.7). These results indicate that the reduced food intake of AgRPCrep110βKO animals is the major contributory factor to the lean phenotype of these animals.
6.2.6 Response to peripherally administered leptin

PI3K has previously been implicated as a point of convergence for insulin and leptin signalling. To assess the leptin sensitivity of \textit{AgRPCrep110\textbetaKO} and control animals, leptin was administered peripherally to 10-month-old male mice for 3 consecutive days. Both cumulative food intake and body weight were measured over this period.

Following leptin treatment, control animals displayed a 3.3 \% loss in body weight, compared with a 4.3 \% loss in \textit{AgRPCrep110\textbetaKO} mice (Figure 6.8a). In addition, leptin treatment (5mg/kg) inhibited food intake by 14 \% in control mice and by 16 \% in \textit{AgRPCrep110\textbetaKO} mice, as compared to the food intake of the same animals following vehicle treatment (Figure 6.8b). These findings indicate that \textit{AgRPCrep110\textbetaKO} animals are leptin sensitive. Indeed, it appears that \textit{AgRPCrep110\textbetaKO} animals are more sensitive to the anorexigenic effects of leptin on body weight.
A) % change in body weight following peripheral leptin treatment

<table>
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<tr>
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<th>Control</th>
<th>AgRPp110βKO</th>
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<td>Vehicle</td>
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<td>Leptin</td>
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B) Cumulative food intake following peripheral leptin treatment

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<th>Control</th>
<th>AgRPCrep110βKO</th>
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<td>Leptin</td>
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</table>

Figure 6.8: The response to 3 consecutive days of i.p. leptin administration upon A) body weight and B) food intake was determined in acclimatised male AgRPCrep110βKO and control mice at 10 months of age. Animals were injected once daily prior to the onset of the dark-phase with either leptin (5 mg/kg) or vehicle for three days in a cross-over design study. Data represent the mean ± SEM for 6-12 animals of each genotype. * P < 0.05, ** P < 0.01.
6.2.7 Response to peripherally administered MTII

To test whether the reduced lean phenotype and hypophagia of AgRPCrep110βKO animals was due to impairments in melanocortin signalling, animals were subjected to i.p. treatment with MTII, a melanocortin receptor agonist.

Both control and AgRPCrep110βKO animals, which had received MTII treatment, ate significantly less than the equivalent vehicle treated group at 1, 2 and 4-h post re-feeding (Figure 6.9). No significant differences in food intake could be detected between MTII treated control and AgRPCrep110βKO animals at any time points. These studies thus demonstrate that AgRPCrep110βKO animals are sensitive to the anorexigenic effects of melanocortins.

Figure 6.9: The fast-refeed response of animals following an i.p. injection of vehicle or MTII. Following a 16-h overnight fast, mice were injected with either vehicle or MTII, and approximately 50g of food was weighed and given to each animal. The food was then weighed after 1-h, 2-h, 4-h, 8-h and 24-h. Data represent the mean ± SEM for 13-15 animals of each genotype. * P < 0.05, ** P < 0.01, *** P < 0.001.
6.2.8 HFD trial

The sensitivity of male and female AgRPCrep110βKO and controls to HFD was assessed from the age of 6 weeks onwards, for the duration of 28 weeks.

6.2.8.1 Body weights of animals on HFD

Both male and female AgRPCrep110βKO animals gained significantly less weight than control animals on HFD at the end of the 28-week trial (Figure 6.10). Thus AgRPCrep110βKO animals are resistant to diet induced obesity (DIO).

A) Bodyweight curve for HFD-fed males

![Bodyweight curve for HFD-fed males](image)

B) Bodyweight curve for HFD-fed females

![Bodyweight curve for HFD-fed females](image)

Figure 6.10: Body weight curve of AgRPCrep110βKO animals on HFD: A) male control and AgRPCrep110βKO mice, B) female control and AgRPCrep110βKO mice. Data represent the mean ± SEM for 6-9 animals of each genotype. * P < 0.05, ** P < 0.01.
6.2.8.2 Analysis of body fat content

The fat content of 10-month-old male and female AgRPCrep110βKO and controls on HFD was assessed by measurement of fat pads, namely WAT (gonadal and mesenteric) and BAT (two depots between the scapulae) tissue pads. Consistent with their resistance to DIO, both male and female AgRPCrep110βKO animals had significantly reduced WAT mass compared to controls although no significant difference in BAT levels were detected significant (Figure 6.11).

A) Fat pad weight analysis of male HFD-fed AgRPp110βKO and controls

![Fat pad weight analysis of male HFD-fed AgRPp110βKO and controls](image)

B) Fat pad weight analysis of female HFD-fed AgRPCrep110βKO and controls

![Fat pad weight analysis of female HFD-fed AgRPCrep110βKO and controls](image)

Figure 6.11: Body fat content of AgRPCrep110βKO and controls as assessed by fat pad weight analysis on HFD. Fat pad weights of A) males and B) of females. Data represent the mean ± SEM for 6-9 animals of each genotype. * P < 0.05, ** P < 0.01, *** P < 0.001.
6.2.8.3 Analysis of feeding behaviour

In order to understand the mechanisms underlying the resistance of male and female AgRPCrep110βKO animals to DIO, the daily food intake of these animals on HFD was analysed. Similarly to AgRPCrep110βKO mice on a standard chow diet, HFD-fed AgRPCrep110βKO animals ate significantly less than controls (Figure 6.12).

![Daily food intake of males on HFD](image)

Figure 6.12: The daily (24-h) food intake of singly housed male AgRPCrep110βKO and controls on HFD at 8 months of age. Data represent the mean ± SEM for 13-15 animals of each genotype. * P < 0.05.

6.2.8.4 Analysis of metabolic rate

The metabolic rate of 8-month-old HFD-fed male AgRPCrep110βKO mice and controls as assessed to investigate if the resistance to DIO of these animals was due solely to hypophagia. No significant difference in metabolic rate was seen between AgRPCrep110βKO mice and controls at thermoneutral (30±0.1°C) temperature. This indicates that the reduced food intake of AgRPCrep110βKO animals is the major contributor to the resistance to DIO of these animals (Figure 6.13).
6.3 Glucose Homeostasis

In order to investigate the effect on glucose homeostasis of the deletion of *p110β* in AgRP neurons, various metabolic studies were carried out on *AgRPCrep110βKO* mice.

6.3.1 Fasting blood glucose (FBG) levels

At 5 weeks and 10 months of age, both male and female *AgRPCrep110βKO* animals had indistinguishable FBG levels from controls (Figure 6.14A-D). In addition, at 10 months of age, no difference in fasting blood glucose levels could be detected between control and *AgRPCrep110βKO* animals of both sexes subjected to HFD for a duration of 28 weeks (Figure 6.14E and 6.14F).
Figure 6.14: Fasting blood glucose levels of AgRPCrept110βKO and controls. A) 5 week old males, B) 5 week old females, C) 10 month old males, D) 10 month old females, E) males following 28 weeks on HFD and E) females following 28 weeks on HFD. Data represent the mean ± SEM for 6-8 mice of each genotype.
6.3.2 Glucose tolerance

Glucose tolerance tests were performed on male and female *AgRPCrep110βKO* and control mice at 5 weeks and 10 months of age. At this age, both male and female *AgRPCrep110βKO* mice exhibited normal glucose disposal as compared to controls (Figure 6.15).

![Graphs showing glucose tolerance](image)

**A) Glucose tolerance of males at 5 weeks**

![Blood glucose levels over time](image)

**B) Glucose tolerance of females at 5 weeks**

![Blood glucose levels over time](image)

**C) Glucose tolerance of males at 10 months**

![Blood glucose levels over time](image)

**D) Glucose tolerance of females at 10 months**

![Blood glucose levels over time](image)

Figure 6.15: Glucose tolerance tests performed on *AgRPCrep110βKO* and controls. A) males at 5 weeks of age, B) females at 5 weeks of age, C) males at 10 months of age, D) females at 10 months of age. Data represent the mean ± SEM for 6-8 animals of each genotype.

Ten-month-old male and female *AgRPCrep110βKO* and control animals that had been subjected to HFD feeding for a duration of 28 weeks, were also subjected to glucose tolerance testing. Male *AgRPCrep110βKO* had improved glucose disposal compared to controls, although this was not statistically significant. On the other hand, female *AgRPCrep110βKO* had significantly improved glucose tolerance compared to control animals on HFD (Figure 6.16).
Figure 6.16: Glucose tolerance tests performed on: A) male *AgRPCrep110βKO* and controls and B) female *AgRPCrep110βKO* and control, following 28 weeks of HFD feeding. Data represent the mean ± SEM for 6-8 animals of each genotype. * P < 0.05, ** P < 0.01.

### 6.3.3 Fasting plasma insulin levels

At 10 months of age male *AgRPCrep110βKO* mice had significantly reduced fasting insulin levels as compared to control animals (Figure 6.17).

Figure 6.17: Fasting plasma insulin levels of 10 month old male *AgRPCrep110βKO* and control mice following a 16-h overnight fast at 10 months of age. Data represent the mean ± SEM for 6-8 animals of each genotype. * P < 0.05.
6.4 Summary of hypothalamic function and glucose homeostasis of \textit{AgRPCrep110\textbetaKO} animals

6.4.1 Phenotype on standard chow diet

Both male and female \textit{AgRPCrep110\textbetaKO} animals exhibited significantly reduced body weight compared to controls. Male \textit{AgRPCrep110\textbetaKO} animals were significantly hypophagic compared to controls. However the metabolic rate of these animals was normal. At 10 months of age, male \textit{AgRPCrep110\textbetaKO} animals were significantly hypoleptinaemic and demonstrated increased sensitivity to the anorexigenic effects of peripheral leptin treatment, compared to controls. In addition, no abnormalities in naso-anal body length could be detected in \textit{AgRPCrep110\textbetaKO} animals indicating no impairments in melanocortin system. Consistently, \textit{AgRPCrep110\textbetaKO} animals exhibited normal sensitivity to the anorexigenic effects of peripheral MTII treatment.

With regards to glucose homeostasis, both male and female \textit{AgRPCrep110\textbetaKO} animals exhibited normal FBG levels and glucose tolerance, as compared to controls. Consistent with their reduced body weight, 10-month-old male \textit{AgRPCrep110\textbetaKO} animals were significantly hypoinsulinaemic compared to controls.

6.4.2 Phenotype on HFD

Both male and female \textit{AgRPCrep110\textbetaKO} animals exhibited resistance to DIO. Consistently, \textit{AgRPCrep110\textbetaKO} animals of both sexes exhibited significantly reduced WAT mass. This reduced adiposity was shown to be due to reduced food intake; \textit{AgRPCrep110\textbetaKO} animals exhibited decreased daily food intake but normal metabolic rate compared to controls.

With regards to glucose homeostasis, both male and female \textit{AgRPCrep110\textbetaKO} animals exhibited normal FBG levels. Although male \textit{AgRPCrep110\textbetaKO} exhibited normal glucose tolerance, female \textit{AgRPCrep110\textbetaKO} animals demonstrated significantly improved glucose tolerance compared to controls.
Chapter 7

Phenotypic characterisation of $POMCC\text{rep}110\beta\text{KO}$ animals
7 Characterisation of POMCCrep110βKO mice

Within the ARC, POMC neurons have been shown to express both insulin and leptin receptors (Benoit et al., 2002; Cheung et al., 1997). Administration of both these hormones directly into the third ventricle of mice has been shown to result in increased hypothalamic expression of Pomc mRNA (Benoit et al., 2002; Brown et al., 2006). In addition, both insulin and leptin have been shown to activate PI3K expression in POMC neurons (Xu et al., 2005b), implicating this enzyme as a potential point of convergence between the two signalling cascades in the hypothalamic regulation of food intake and body weight. Treatment with PI3K inhibitors attenuates the anorexigenic effects of insulin and leptin (Niswender et al., 2003; Niswender et al., 2001). A role for hypothalamic PI3K-mediated pathways in the regulation of peripheral glucose homeostasis has also been suggested by various studies (Morrison et al., 2005; Morton et al., 2005).

In order to fully address the role of PI3K mediated signalling pathways in POMC neurons, a number of studies were carried out on POMCCrep110βKO mice designed to assess the hypothalamic function and various parameters of glucose homeostasis in these animals.

7.1 Proof of p110β deletion

Prior to commencement of the phenotypic characterisation of POMCCrep110βKO animals, studies aimed at confirming the lack of functional p110β expression in the hypothalami of POMCCrep110βKO animals were carried out. A nested-PCR strategy, performed on cDNA transcribed from hypothalamic lysates of POMCCrep110βKO animals, confirmed the recombination of the p110βflox alleles in the hypothalamus of these animals. This work was performed by Dr. Julie Guillermet at the Ludwig Institute, UCL (Figure 7.1A).

However, ICC studies aimed at proving recombination of the p110βflox alleles specifically within POMC neurons have been unsuccessful. Although viable
POMCCrep110βKOGFP (animals which express green fluorescent protein in POMC neurons lacking p110β) were generated, no reliable antibodies against the C-terminus of p110β are currently available. However, similarly to the situation in AgRPCrep110βKO animals, hypothalamic slices from POMCCrep110βKOGFP animals clearly show the presence of fluorescent neurons in the ARC, indicating that POMC neurons lacking p110β are viable.

Figure 7.1: A) Proof of recombination of floxed alleles by a nested-PCR strategy. Samples 1 and 2 are hypothalamic cDNA samples from control animals. Samples 3, 4 and 5 are hypothalamic cDNA samples from POMCCrep110βKO animals. A 474 bp band indicates presence of the p110βflox allele. A 204 bp band indicates recombination of the floxed alleles. B) Proof of POMC neuron viability in POMCCrep110βKO animals. Hypothalamic sections from: i) POMCCreYFP control animals and ii) POMCCrep110βKOYFP animals. 3V, third ventricle.
7.2 Hypothalamic function

In order to fully address the role of p110β mediated signalling pathways in POMC neurons, a number of studies were carried out on POMCCre p110βKO mice designed to assess the hypothalamic function of these animals.

7.2.1 Analysis of body weight

Both males and female POMCCre p110βKO animals were of normal weight (as compared to control littermates) at all ages studied (Figure 7.2).

Figure 7.2: Growth curve for A) male POMCCre p110βKO and control animals and B) female POMCCre p110βKO and control animals. Data represent the mean ± SEM for 11-39 animals of each genotype.
7.2.2 Analysis of body length

Measurement of naso-anal body length at 10 months of age, showed male POMCCrep110βKO animals to be significantly longer than control littermates (Figure 7.3).

![Naso-anal body length of male POMCCrep110βKO and controls](image)

Figure 7.3: Naso-anal length (body length) was measured in male POMCCrep110βKO and controls at 10 months of age. Data represent the mean ± SEM for 6 animals of each genotype. * P < 0.05.

7.2.3 Analysis of body fat content by fat pad weights

The body fat content of 10-month-old male POMCCrep110βKO and controls was assessed by measurement of fat pads, namely WAT (gonadal and mesenteric) and BAT pads (two depots between the scapulae).

Interestingly, despite the normal body weight of POMCCrep110βKO animals compared to controls, POMCCrep110βKO animals had a significant increase in WAT mass (Figure 7.4).
7.2.4 Plasma leptin levels

Measurement of the fasting leptin levels of 10-month-old mice, demonstrated that *POMCCrep110βKO* animals had indistinguishable leptin levels from controls (Figure 7.5).

Figure 7.5: Fasting plasma leptin levels were measured on male *POMCCrep110βKO* and control mice at 10 months of age following a 16-h overnight fast. Data represent the mean ± SEM for 6-8 animals of each genotype.
7.2.5 Analysis of feeding behaviour

Despite the lack of differences found in the body weight of POMCCrep110βKO animals, the increased adiposity suggested that these animals might have impaired energy homeostasis regulation. In addition, the increased body length indicated potential impairments in melanocortin signalling. Thus, the feeding behaviour of these animals was investigated by analysis of the daily food intake and response to fasting of POMCCrep110βKO and control animals.

7.2.5.1 Analysis of food intake

At 4 months of age, male POMCCrep110βKO mice were significantly hyperphagic compared to control animals (Figure 7.6).

![Daily food intake of male POMCCrep110βKO and controls](image)

Figure 7.6: Daily (24-h) food intake was measured in male POMCCrep110βKO and control animals at 4 months of age. Data represent the mean ± SEM for 14-16 animals of each genotype. * P < 0.05.
7.2.5.2  **Response to fasting**

At 6 months of age, male *POMCCrep110βKO* animals displayed normal food intake at 1, 2, 4 and 8-h post re-feeding compared to controls. However, consistent with the hyperphagia of these animals, *POMCCrep110βKO* animals had significantly increased cumulative food intake compared to controls at 24-h post re-feeding (Figure 7.7).

**Fast-refeed response of male *POMCCrep110βKO* and controls**

Figure 7.7: Re-feeding response following a 16-h overnight fast in 6 month old males. Data represent the mean ± SEM for 14-16 animals of each genotype. * P < 0.05.

7.2.6  **Analysis of metabolic rate**

In order to determine whether the increased adiposity of *POMCCrep110βKO* animals was due solely to the hyperphagia of these animals or also partly due to impairments in energy expenditure, the metabolic rate of 10-month-old male *POMCCrep110βKO* mice and relevant controls was assessed. *POMCCrep110βKO* displayed a non-significant trend for lower metabolic rate at thermoneutral (30±0.1°C) temperature, compared to controls (Figure 7.8).
Resting metabolic rate of male POMCCrep110βKO and controls

Figure 7.8: Summary of analysis of RMR in 10 month old male POMCCre p110βKO and control animals (thermoreutral). Data represent the mean ± SEM for 7 animals of each genotype.

7.2.7 Response to peripherally administered leptin

The hyperphagia and increased adiposity of POMCCrep110βKO animals at 10 months of age suggests a possible underlying resistance to leptin. PI3K has previously been implicated as a point of convergence for insulin and leptin signalling. To assess the leptin sensitivity of these animals, leptin was administered peripherally to 10-month-old male POMCCrep110βKO and control mice for 3 consecutive days.

Following leptin treatment, control animals displayed a 3.45 % loss in body weight, compared with a 4.12 % loss in POMCCrep110βKO mice. In addition, leptin treatment (5mg/kg) inhibited food intake by 15% in controls and by 27% in POMCCrep110βKO mice, as compared to the food intake of the same animals following vehicle treatment (Figure 7.9). Taken together, these results demonstrate that POMCCrep110βKO animals are still sensitive to the anorexigenic effects of peripherally administered leptin.
Figure 7.9: The response to 3 consecutive days of i.p. leptin administration upon A) body weight and B) food intake was determined in acclimatised male POMCcrep110βKO and control mice at 10 months of age. Data represent the mean ± SEM for 7-10 animals of each genotype. * P < 0.05, ** P < 0.01, *** P < 0.001.
7.2.8  Response to peripherally administered MTII

The increased body length and impaired feeding regulation of *POMCCrep110βKO* animals suggested that these animals have disrupted melanocortin signalling. Thus, to assess the melanocortin sensitivity of these animals, both *POMCCrep110βKO* and control animals were peripherally treated with MTII.

As expected, control animals ate significantly less during the first 8 hours post-refeed when treated with MTII compared to vehicle. *POMCCrep110βKO* were also sensitive to the food-inhibiting effects of MTII during the first 8 hours post-refeed (Figure 7.10). This demonstrates that *POMCCrep110βKO* animals remain sensitive to the anorexigenic effects of melanocortins, and that melanocortin pathways remain functional regardless of *p110β* deletion in POMC neurons.

![Sensitivity of male *POMCCrep110βKO* and controls to MTII](chart)

Figure 7.10: The fast-refeed response of animals following an i.p. injection of vehicle or MTII. Following a 16-h overnight fast, mice were injected with either vehicle or MTII, and approximately 50g of food was weighed and given to each animal. The food was then weighed after 1-h, 2-h, 4-h, 8-h and 24-h. Data represent the mean ± SEM for 14-16 animals of each genotype. * P < 0.05, ** P < 0.01, *** P < 0.001.
7.3 Glucose Homeostasis

In order to investigate the effect on glucose homeostasis of the deletion of $p110\beta$ in POMC neurons, various metabolic studies were carried out on POMCCrep110$\beta$K0 and control mice.

7.3.1 Fasting blood glucose (FBG) levels

At both 5 weeks and 10 months of age, male POMCCrep110$\beta$K0 mice had indistinguishable FBG levels from control animals (Figure 7.11).

![FBG levels at 5 weeks of age](image1)

![FBG levels at 10 months of age](image2)

Figure 7.11: FBG levels (mmol/L) following a 16-h fast in male POMCCrep110$\beta$K0 and controls at A) 5 weeks of age and B) at 10 months of age. Data represent the mean ± SEM for 7-9 mice of each genotype.

7.3.2 Glucose tolerance

Glucose tolerance tests were performed on male POMCCrep110$\beta$K0 and control mice at 5 weeks and 10 months of age. At both ages, POMCCrep110$\beta$K0 animals exhibited normal glucose disposal as compared to controls (Figure 7.12).
Figure 7.12: Glucose tolerance tests performed on male POMCCrep110βKO and controls at: A) 5 weeks of age and B) 10 months of age. Data represent the mean ± SEM for 7-9 mice of each genotype.

7.3.3 Fasting plasma insulin levels

At 10 months of age male POMCCrep110βKO mice had normal fasting insulin levels, as compared to control animals (Figure 7.13).

Figure 7.13: Fasting plasma insulin levels of 10-month-old male POMCCrep110βKO and control mice following a 16-h overnight fast. Data represent the mean ± SEM for 7-10 mice of each genotype.
7.4 Summary of hypothalamic function and glucose homeostasis of POMCCrep110βKO animals

Both male and female POMCCrep110βKO animals exhibited normal body weight compared to controls. Male POMCCrep110βKO animals were however shown to have significant increases in fat mass compared to controls, although fasting plasma leptin levels were normal. Consistently, POMCCrep110βKO animals were still sensitive to the anorexigenic effects of peripheral leptin treatment. Analysis of the feeding behaviour of male POMCCrep110βKO animals revealed that they were significantly hyperphagic and exhibited increased cumulative 24-h food intake following an overnight fast, compared to controls. The metabolic rate of male POMCCrep110βKO animals was indistinguishable from that of controls. Measurement of the body length of POMCCrep110βKO animals revealed that they were significantly longer than controls indicating impairments in melanocortin signalling. However, POMCCrep110βKO animals exhibited normal sensitivity to the anorexigenic effects of peripheral MTII treatment.

With regards to glucose homeostasis, POMCCrep110βKO animals exhibited normal FBG levels, glucose tolerance and fasting insulin levels compared to control animals.
Chapter 8
Discussion
8 Discussion

Obesity is defined medically as a state of increased body weight, more specifically adipose tissue, of sufficient magnitude to produce adverse health consequences (Kopelman, 2000). Obesity is especially linked with the development of insulin resistance and T2DM, although the mechanisms that underlie the association of these metabolic disorders are still unknown. Despite this, recent evidence has suggested a key role for the brain in the control of both body weight and glucose metabolism.

It is now a widely accepted view that the brain processes information from adiposity signals, and integrates this input with signals from nutrients and other molecules. In response, energy intake and energy expenditure are adjusted accordingly in ways that promote homeostasis of energy stores and fuel metabolism. Currently, only insulin and leptin meet all the criteria required of a putative adiposity signal. At the commencement of this work, these hormones were predicted to regulate energy homeostasis via their actions in the hypothalamus. This model is outlined below.

Insulin and leptin are proposed to enter the ARC region of the hypothalamus, where they bind to their corresponding receptors present on the first-order NPY/AgRP and POMC/CART neurons. NPY/AgRP neurons are proposed to be inhibited by leptin and insulin whereas POMC/CART neurons are stimulated by these hormones (Schwartz et al., 2000). These neurons then integrate information concerning the energy balance of the individual and relay this to second-order neurons located in other hypothalamic regions. This therefore results in the transduction of the insulin/leptin adiposity signal into a response, affecting the overall energy homeostasis status of the individual.

Prior to the commencement of this work, several lines of evidence had suggested a key role for CNS insulin action in the regulation of energy homeostasis. Administration of insulin directly to the brain has been shown to result in an anorexigenic effect, resulting in a reductions in body weight (Woods et al., 1979). In contrast, the selective decrease of hypothalamic insulin receptors by the administration of anti-sense oligodeoxynucleotide directed against the IR (Obici et al., 2002b) results in rapid-onset hyperphagia and
increased fat mass. The use of transgenic animal models has also implicated a role for intact CNS insulin signalling in the central regulation of energy homeostasis and peripheral glucose metabolism. *NIRKO* mice (with neuron specific deletion of the insulin receptor) are obese, hyperphagic and display mildly impaired glucose homeostasis (Bruning et al., 2000). Females with global deletion of *Irs2* (a major downstream effector of insulin receptor) also display a phenotype of hypothalamic dysfunction with the development of hyperphagia, obesity and infertility (Burks et al., 2000c). Male *Irs2* null animals develop insulin resistance, β-cell failure and a subsequent diabetic phenotype (Withers et al., 1998). Taken together, these studies implicate CNS insulin receptor signalling in the regulation of both energy homeostasis and peripheral glucose metabolism, and indicate that IRs in discrete areas of the hypothalamus have a physiological role in these actions.

Various studies have implicated a role for PI3K pathway in the regulation of hypothalamic energy homeostasis (Morrison et al., 2005; Niswender et al., 2003; Niswender et al., 2001; Spanswick et al., 2000). Classically viewed as a downstream effector of the insulin signalling pathway, increasing evidence suggests a role for this lipid kinase in mediating the anorexigenic effects of both insulin and leptin in the hypothalamic regulation of energy homeostasis. In addition, insulin and leptin have been shown to activate PI3K specifically in distinct hypothalamic neurons implicated in energy homeostasis regulation, namely POMC and AgRP neurons, suggesting a potential mechanism for the integration of these signals in discrete neuronal populations (Xu et al., 2005b).

The most intensively studied of the PI3Ks are those that belong to the class IA. The main role of this subgroup seems to be the direction of energy into cell growth and proliferation. PI3K is a heterodimer that consists of a p85 regulatory subunit and a p110 catalytic subunit. Three catalytic subunit isoforms exist (p110α, p110β and p110δ), with p110α and p110β broadly expressed (Engelman et al., 2006). The relative contributions of these isoforms to normal mammalian physiology remain unknown.
However, recent evidence indicates a critical role for p110α in growth factor and metabolic signalling (Foukas et al., 2006). Mice that were heterozygous for a knock-in mutation, that abrogated p110α kinase activity (but without altering the stoichiometry of other subunits), displayed severely blunted signalling via IRS proteins. These animals were hyperinsulinaemic, glucose intolerant, hyperphagic, and also displayed increased adiposity and reduced somatic growth. The authors also demonstrated a minimal contribution from p110β towards IRS associated lipid kinase activity.

Prior to the commencement of these studies, the exact identities of the neuronal populations mediating the effects of insulin on food intake and body weight regulation were still unknown (although POMC/CART and NPY/AgRP neurons were implicated). In addition, although the severe impaired glucose homeostasis of Irs2 null animals was presumed to be a result of Irs2 deletion in the β-cell, the relevant contribution of the CNS deletion of Irs2 was unknown. Similarly, the extent (if any) of the CNS deletion of Irs2 to the infertile phenotype of Irs2 null females was also unclear. Finally, the resistance of Irs2 null animals to the anorexigenic effects of leptin had also implicated IRS2 as a point of convergence between the insulin and leptin signalling pathways in the CNS.

The aim of the work described in this thesis, was to investigate the role of IRS2 mediated signalling pathways in the hypothalamic regulation of energy homeostasis, glucose homeostasis and reproductive function. The generation and phenotypic characterisation of transgenic mice with Irs2 deletion in all neurons (NesCreIrs2KO), mice with Irs2 deletion in POMC neurons (POMCCreIrs2KO) and mice with Irs2 deletion in AgRP neurons (AgRPcreIrs2KO) allowed these points to be addressed in a tissue-specific and cell-specific manner.
8.1 Implications of the phenotypic characterisation of NesCreIrs2KO animals

8.1.1 Key role for CNS IRS2 pathways in the regulation of energy homeostasis

From approximately 8 weeks of age onwards, NesCreIrs2KO mice develop increased body weight and increased fat tissue mass. Consistently, these animals are hyperleptinaemic yet remain sensitive to the anorexigenic effects of peripheral leptin treatment. In addition, NesCreIrs2KO animals are significantly hyperphagic, indicating that the obesity of these animals is due to their increased caloric intake (no defects in resting metabolic rate were detected). Interestingly, NesCreIrs2KO animals are also significantly longer than controls, implicating disruption in CNS melanocortin signalling pathways. Consistently, NesCreIrs2KO animals demonstrated a significant reduction in Pomc mRNA levels compared to controls, although no changes in Npy or Agrp expression levels were detected.

The significant hypothalamic dysfunction of NesCreIrs2KO animals strongly supports growing evidence implicating a role for insulin signalling in the regulation of food intake and body weight. These studies also highlight the non-overlapping roles of the insulin receptor substrate proteins (IRS). No changes in hypothalamic Irs1 mRNA expression in NesCreIrs2KO animals were detected, demonstrating that IRS1 is unable to compensate for IRS2 deficiency in the whole brain. Thus, IRS2 appears to be the major isoform involved in mediating the anorexigenic effects of insulin signalling in the brain.

8.1.2 CNS IRS2 pathways are not mandatory for leptin action

IRS2 has been implicated as a convergence point for both insulin and leptin action. Irs2 null mice have defective leptin-stimulated hypothalamic STAT3 phosphorylation, suggesting potential convergence between leptin and insulin signalling in the hypothalamus (Burks et al., 2000b). However, the finding that NesCreIrs2KO animals remain sensitive to peripherally administered leptin suggests that the elevated leptin levels displayed by these animals results from their greater adiposity rather than primary
central leptin resistance. This is consistent with the observation that central leptin administration to Irs2 null mice causes reduction in fat mass (Suzuki et al., 2004). This is also consistent with the phenotype of RipCreIrs2KO animals, which lack Irs2 in an uncharacterised population of ARC neurons (Choudhury et al., 2005). Although hyperleptinaemic at 12 weeks of age, measurement of fasting leptin levels of these animals at 4 weeks of age (before the onset of obesity) indicates that the hyperleptinaemia is not due to intrinsic leptin resistance. In support of this, RipCreIrs2KO animals remained sensitive to the anorexigenic effects of peripheral leptin treatment (Choudhury et al., 2005). Taken together, these studies indicate that CNS IRS2 pathways are not mandatory for leptin action.

8.1.3 Possible role for CNS IRS2 pathways in the regulation of peripheral glucose homeostasis

A role for IRS2 mediated insulin signalling in the regulation of peripheral glucose homeostasis is also suggested by studies on NesCreIrs2KO animals. NesCreIrs2KO mice display hyperglycaemia, hyperinsulinaemia and impaired glucose tolerance. These findings are consistent with those of previous studies, which have also indicated a role for CNS insulin signalling in the regulation of peripheral glucose metabolism (Bruning et al., 2000; Obici et al., 2002a).

However, these results should be interpreted with caution as the relationship between obesity and impaired glucose homeostasis is complex. It thus remains unclear whether the impaired glucose tolerance of NesCreIrs2KO animals is a primary cause due to neuronal IRS2 deletion, or a secondary effect of the increased adiposity of these animals. The use of euglycaemic clamp studies would be required to assess the true contribution of CNS IRS2 pathways to the regulation of peripheral glucose homeostasis. However, hyperinsulinaemic-hypoglycaemic clamp studies performed on NIRKO animals do suggest that intact insulin signalling in the CNS is critical for the normal sympathoadrenal response to hypoglycaemia (Fisher et al., 2005). Thus, it is likely that altered neural insulin signalling could contribute to defective glucose counter-regulation in diabetes, and this is potentially true in the case of NesCreIrs2KO animals.
The use of NesCreIrs2KO mice has thus allowed disentanglement of the phenotypes displayed by the global Irs2 null mouse with regards to glucose homeostasis (Burks et al., 2000a; Withers et al., 1998). Although NesCreIrs2KO animals display hyperglycaemia and impaired glucose tolerance, these phenotypes are relatively mild compared to the overt diabetes displayed by Irs2 null animals (Burks et al., 2000a; Withers et al., 1998). The preservation of β-cell mass in NesCreIrs2KO mice also dissociates hypothalamic dysfunction from reduced β-cell mass, and indicates that the deletion of Irs2 in the pancreatic β-cell is a major contributory factor to the impaired glucose homeostasis of Irs2 null animals.

8.1.4 CNS IRS2 pathways are not required for normal reproductive function

Minimal reproductive abnormalities were detected in female NesCreIrs2KO animals. Female NesCreIrs2KO mice display an extended oestrous cycle, due to a mildly prolonged dioestrous phase. However, NesCreIrs2KO animals respond normally to superovulation, and display normal ovarian morphology. The dioestrous levels of oestradiol, progesterone, luteinising hormone, and follicle-stimulating hormone are also normal in NesCreIrs2KO animals. However, the level of prolactin in NesCreIrs2KO animals is significantly reduced compared to controls. Finally, the analysis of breeding performance of NesCreIrs2KO animals by timed mating studies revealed no abnormalities.

The use of NesCreIrs2KO mice has thus allowed disentanglement of the phenotypes displayed by female Irs2 null mice with regards to reproductive function (Burks et al., 2000a; Withers et al., 1998). The use of NesCreIrs2KO indicates that the deletion of Irs2 in the ovary and pituitary is the major contributory factor to the infertility of Irs2 null females.
8.1.5 Evidence for a more important role for IRS2 mediated signalling pathways than IR pathways in the CNS regulation of energy and glucose homeostasis

Similarly to NIRKO animals, impaired energy homeostasis is a phenotype of NesCreIrs2KO mice, although the severity of this dysfunction is actually greater in these NesCreIrs2KO animals. The body weights of male NIRKO mice were indistinguishable from those of control littermates during the first 6 months of life, and no differences in the food intake of male NIRKO animals were detectable. However, female NIRKO animals displayed increased body weight and hyperphagia from approximately 16 weeks of age (Bruning et al., 2000). In contrast, NesCreIrs2KO animals demonstrate no sexual dimorphism, with both male and females exhibiting increased body weight and hyperphagia from approximately 8 weeks of ages onwards.

Similarly to NIRKO animals, NesCreIrs2KO animals exhibit impaired glucose homeostasis. However, again, the phenotype of NesCreIrs2KO animals appears to be more severe. Between the ages of 4 and 6 months of age, NIRKO mice showed normal FBG levels, but were significantly hyperinsulinaemic. However, even as young as 12 weeks of age, male and female NesCreIrs2KO demonstrate fasting hyperglycaemia, impaired glucose tolerance and hyperinsulinaemia. With regards to reproductive function, both female and male NIRKO animals demonstrated impaired fertility (Bruning et al., 2000). However, as previously mentioned, no defects in the reproductive function of NesCreIrs2KO females were found.

Taken together, these results indicate a more important role for IR-mediated pathways than IRS2-mediated pathways in the CNS regulation of reproductive function. However, these studies highlight a more important role for IRS2-mediated signalling pathways compared to insulin receptor pathways with regards to the CNS regulation of energy and glucose homeostasis. There are a number of potential explanations for this.

Firstly, the IGF-1 receptor can also bind insulin, although this is at approximately 70 % less affinity than IGF-1 (Smith et al., 1988). Thus, deletion of the IR in the brain of NIRKO mice is unlikely to be ablating insulin signalling completely, with signalling via the IGF-1 receptor potentially able to mildly compensate.
Secondly, in addition to its role in the insulin signalling cascade, IRS2 is also a downstream component of other signalling pathways such as those of BDNF, IGF-1, growth hormone and interleukin-2, -4, and -7 (Yamada et al., 1997). A role for the neurotrophin BDNF in particular, has been implicated in the hypothalamic regulation of energy homeostasis by a number of studies (Fox and Byerly, 2004; Kernie et al., 2000; Lyons et al., 1999; Xu et al., 2003). Thus, the more severe phenotype of NesCreIrs2KO animals compared to NIRKO animals can be potentially explained by the fact that deletion of Irs2 in the CNS affects more than just the insulin signalling cascade. In contrast, deletion of IR signalling pathways in the CNS would directly affect only insulin signalling in this tissue.

With regards to the subfertility of both male and female NIRKO animals, the findings that female NesCreIrs2KO exhibit normal reproductive function suggests the importance of alternative downstream components of the IR signalling cascade. Indeed, it is possible that in the CNS of NesCreIrs2KO animals, IRS1 has the ability to compensate with regards to signalling pathways involved in the regulation of reproductive function.

Although the phenotype of the NesCreIrs2KO animal suggests a more important role for CNS IRS2 pathways than CNS IR pathways in terms of energy and glucose homeostasis, these implications should be interpreted with some caution. The genetic background of transgenic mouse lines, as well as the methods used in the creation of various mutants, has been shown to contribute considerably to the phenotypic outcome (Barthold, 2004).

8.2 Implications of the phenotypic characterisation of POMCCreIrs2KO and AgRPCreIrs2KO animals

No defects in hypothalamic function could be detected in POMCCreIrs2KO or AgRPCreIrs2KO animals. These animals exhibit no defects in body weight, body composition, body length, food intake and leptin levels compared to controls. Similarly, no defects in glucose homeostasis could be detected in POMCCreIrs2KO or AgRPCreIrs2KO animals. These animals exhibit no defects in FBG levels, glucose tolerance or insulin levels compared to control littersmates.
Although the lack of phenotypic abnormality in either mouse line was unexpected, when interpreted in context with the robust phenotype of *NesCreIr2s2KO* animals, these studies have considerable implications.

### 8.2.1 Non-essential role for IRS2 signalling in hypothalamic POMC and AgRP neurons

Hypothalamic POMC and AgRP neurons have long been implicated as having key roles in the regulation of energy homeostasis (Cowley et al., 2001). These neurons contain leptin and insulin receptors on their membrane and it is via these receptors, that insulin and leptin are proposed to exert their anorexigenic effects. However, *POMCCreIr2KO* and *AgRPCreIr2KO* mice did not exhibit impairments in either energy homeostasis or glucose metabolism. This indicates that POMC and AgRP neurons are not important for the effect of IRS2 mediated insulin signalling on energy homeostasis. Consistent with these findings, mice with deletion of the IR in POMC neurons (*POMCCreIRKO*) and with deletion of the IR in AgRP neurons (*AgRPCreIRKO*) also display no phenotypic abnormalities (J.C. Bruning, personal communication). These unpublished studies also question the potential contribution of insulin signalling in these neuronal populations.

Interestingly, deletion of the *LepR* in POMC neurons resulted in mice that were mildly obese, hyperleptinaemic and had altered expression of hypothalamic neuropeptides (Balthasar et al., 2004). Consistently, female *POMCCreSTAT3KO* mice (with *Stat3* deletion specifically within POMC neurons), exhibit a 2-fold increase in fat pad mass but only a slight increase in total body weight (Xu et al., 2007). Thus, it would appear that POMC neurons are important for mediating the anorexigenic effects of leptin but not insulin.

These findings correspond with our observations of the electrophysiological properties of POMC neurons, performed in animals with florescent POMC neurons (generated from crossing POMCCre and GFP animals). Leptin has been demonstrated to depolarise POMC neurons (Cowley et al., 2001). In contrast, although previously postulated to function in a similar way to leptin, insulin results in the hyperpolarisation and reduced action potential frequency in POMC neurons (Choudhury et al., 2005). These opposite
effects on POMC cell excitability perhaps explain the lack of phenotype in POMC<sup>CreIrs2KO</sup> mice. We are currently in the process of investigating the electrophysiological properties of AgRP neurons in collaboration with the laboratory of Professor Ashord, Dundee University.

It should be noted that the lack of phenotypic abnormalities in both POMC<sup>CreIrs2KO</sup> and AgRP<sup>CreIrs2KO</sup> can potentially be attributed to the fact that genetic manipulations within these neuronal populations are simply intervening in one limb of a multi-neuronal highly integrated circuit. Thus, physiological compensation or over-compensation of other limbs in these systems may occur (Morton et al., 2006). It is important to take into account the fact that POMC and AgRP neurons, besides being targets for insulin action, are also targets for other multiple signals regarding energy homeostasis. Thus, although deletion of Irs2 in these populations may create a relative state of ‘insulin resistance’ in these neurons, due to the presence of other intact signalling pathways (e.g. leptin) still in place in these neurons, normal regulation of energy homeostasis may still proceed.

### 8.2.2 Potential role for other IRS proteins in the regulation of energy homeostasis in POMC and AgRP populations

A role for IRS1 mediated insulin signalling in the anorexigenic effects of insulin in POMC and AgRP neurons cannot be excluded. However, findings that no changes in hypothalamic Irs1 mRNA expression in Nes<sup>CreIrs2KO</sup> animals are observed, suggests that IRS2 is the major isoform involved in mediating the anorexigenic effects of insulin signalling in the brain. It is still possible however, that compensation by IRS1 may still be occurring without detectable changes in gene expression levels.

Recent data also suggests a role for IRS4 in the regulation of central and peripheral energy homeostasis, when the IRS2 branch of the insulin signalling cascade is ablated (Dong et al, Abstract ADA 2006). Thus, a potential role for IRS4 in mediating the effects of insulin in POMC and AgRP neurons cannot be dismissed.
8.2.3 Role for IRS2 signalling in neurons distinct from POMC and AgRP populations

These present studies also implicate IRS2 signalling in neurons distinct from POMC and AgRP populations in the regulation of energy homeostasis. This is because in contrast to the lack of phenotypic abnormalities observed in POMCCreIrs2KO and AgRPCreIrs2KO animals, the deletion of Irs2 in all neurons (NesCreIrs2KO mice) results in marked hypothalamic dysfunction and impaired glucose homeostasis.

Indeed, a recent study has identified a previously uncharacterised ARC population of neurons involved in IRS2 mediated insulin regulation of energy homeostasis (Choudhury et al., 2005). RipCreIrs2KO animals lack Irs2 in the β-cell and also an uncharacterised population of ARC neurons. These animals exhibit many of the characteristics of NesCreIrs2KO animals; they are obese, hyperphagic and leptin sensitive despite hyperleptinaemia. Similarly to NesCreIrs2KO animals, the increased body length displayed by RipCreIrs2KO mice suggests altered melanocortin action. In addition, RipCreIrs2KO display impaired glucose tolerance and reduced β-cell mass mice due to Irs2 deletion in the β-cell. These Rip neurons (which express the Rat insulin II promoter) have been shown to be neither POMC nor AgRP neurons (Choudhury et al., 2005) but may exist as a third population of first-order arcuate neurons in the regulation of energy balance.

In addition, the potential role of “non-ARC” neuronal populations in mediating the effects of adiposity signals, and other signals involved in the regulation of energy homeostasis, has been largely ignored. Recent work has shown that POMC neurons receive strong excitatory input from the VMH, highlighting an important role for VMH populations in energy balance (Sternson et al., 2005). Leptin has also been shown to depolarise and increase the firing rate of steroidogenic factor-1 (SF-1) positive neurons in this nucleus (Dhillon et al., 2006). Sf-1CreLepRKO mice, which lack the LepR in SF-1 neurons, develop obesity and hyperphagia. When placed on a HFD, these animals are particularly sensitive to DIO, displaying hyperphagia and marked impairments in the activation of diet-induced thermogenesis. Taken together, these results demonstrate that leptin action on neurons of the VMH plays an important role in controlling body weight and food intake.
Interestingly, the degree of obesity observed in Sf-1CreLepRKO mice is similar to that seen in POMCCreLepRKO mice, suggesting that these two groups of neurons play a quantitatively similar role in regulating body weight. Further, mice lacking LepRs in both SF-1 and POMC neurons have a body weight phenotype that is approximately the sum of that observed with loss of LepRs in either neuronal group alone, but less obese than db/db mice. This indicates that sites, other than these, are also important for leptin action in body weight regulation. Thus, SF1 neurons may also potentially mediate the anorexigenic effects of insulin, and studies involving the deletion of components of this pathway will be key in determining their contribution.

Consistently, a recent study has demonstrated hypothalamic Irs2 expression exists mainly in the ARC, VMH and PVN, indicating a potential role for this isoform in ARC and ‘non-ARC’ neuronal populations (Pardini et al., 2006). The same study also implicates extra-hypothalamic regions as possible sites for insulin action in the regulation of energy homeostasis. In the hindbrain, Irs2 expression is detectable in the area postrema (AP), medial nucleus of the solitary tract (NTS), motor nucleus of the vagus nerve (DMV) and the hypoglossal nucleus (HN). Interestingly, expression is also found in the ventral tegmental area (VTA), an important area for reward perception, and is detectable in dopamine neurons in this brain area. The IRS2-AKT pathway in dopamine neurons of the VTA has been shown to be key to the regulation of behavioural and cellular responses to opiates (Russo et al., 2007).

8.3 Implications of phenotypic characterisation of AgRPCrep110βKO and POMCCrep110βKO animals

Despite the recent findings for a potentially dominant role for p110α in mediating the actions of PI3K on metabolism (Foukas et al., 2006), the relative contributions of other catalytic isoforms in the hypothalamic regulation of energy homeostasis remain unaddressed. As discussed previously, convergence between insulin and leptin signalling in the CNS, and more specifically in the hypothalamus, has been implicated in the regulation of food intake and body weight at the level of PI3K.
To fully address the role of PI3K in the hypothalamic regulation of energy balance and peripheral glucose homeostasis, mice with p110\(\beta\) deletion in AgRP neurons (AgRPCrep110\(\beta\)KO) and mice with p110\(\beta\) deletion in POMC neurons (POMCCrep110\(\beta\)KO) were generated. It should be noted that at the time of submission of this thesis, studies were at an early stage. Due to this, insights into the role of p110\(\beta\) pathways in the regulation of energy balance are incomplete. However, as a result of the interesting preliminary findings of these investigations, they have been included in this work.

### 8.3.1 Key role for hypothalamic p110\(\beta\) pathways in the regulation of energy homeostasis

*AgRPCrep110\(\beta\)KO* and *POMCCrep110\(\beta\)KO* animals exhibit opposing phenotypes of hypothalamic dysfunction, consistent with the neuropeptide phenotype of the neuronal populations involved.

Examination of the hypothalamic function of *AgRPCrep110\(\beta\)KO* animals, revealed that these animals have significantly reduced body weight and adiposity compared to controls. Consistently, *AgRPCrep110\(\beta\)KO* animals are also significantly hypoleptinaemic and hypophagic compared to controls. Interestingly, both male and female *AgRPCrep110\(\beta\)KO* animals exhibit resistance to diet induced obesity (DIO), with the reduced daily food intake of these animals on this diet found to be the main contributory factor. Conversely, examination of the hypothalamic function of *POMCCrep110\(\beta\)KO* animals revealed that these animals exhibit increased adiposity and are hyperphagic compared to controls. However, both *AgRPCrep110\(\beta\)KO* and *POMCCrep110\(\beta\)KO* animals remain sensitive to the anorexigenic effects of peripherally administered leptin and MTII. Interestingly, *AgRPCrep110\(\beta\)KO* animals are more sensitive than controls to the anorexigenic effects of leptin on body weight.

With regards to glucose homeostasis, no abnormalities in FBG levels or glucose tolerance could be detected in *AgRPCrep110\(\beta\)KO* and *POMCCrep110\(\beta\)KO* animals. However, at 10 months of age, male *AgRPCrep110\(\beta\)KO* animals are significantly hypoinsulinaemic compared to controls, consistent with their reduced body weight. At
the same age, \textit{POMCCrep110\textbeta K}\textit{O} animals have indistinguishable basal insulin levels compared to controls.

Our findings are consistent with those of Xu and colleagues, who showed that in the case of POMC neurons, both insulin and leptin resulted in activation of PI3K in this neuronal population (Xu et al., 2005b). With regards to the phenotype of \textit{POMCCrep110\textbeta K}\textit{O} animals, one can postulate that removal of the \textit{p110\textbeta} isoform in POMC neurons results in reduced PI3K signalling. This could thus be mimicking a state of low insulin and leptin levels in this population, resulting in increased activation of downstream anabolic pathways. With regards to AgRP neurons, Xu and colleagues found that insulin and leptin had opposite effects, with PI3K activity inhibited by leptin but stimulated by insulin (Xu et al., 2005b). Our phenotypic characterisation of the \textit{AgRPCrep110\textbeta K}\textit{O} animal also supports this finding. Again, one can postulate that removal of the \textit{p110\textbeta} isoform in AgRP neurons results in reduced PI3K signalling, thus mimicking a state of high leptin levels in this population, resulting in increased activation of downstream catabolic effector pathways.

Taken together, these preliminary studies implicate an important role for the \textit{p110\textbeta} isoform of PI3K in the regulation of hypothalamic energy homeostasis, and specifically within AgRP and POMC neuronal populations of the ARC.

\textbf{8.3.2 Potential role for \textit{p110\textbeta} as a mediator of other non-insulin signalling pathways involved in the regulation of energy homeostasis in AgRP and POMC neurons}

Phosphorylation of the tyrosine residues of IRS proteins results in the recruitment of p85, which in turn results in recruitment of the p110 catalytic subunit. In contrast to the lack of phenotype observed in \textit{AgRPCrepIrs2KO} and \textit{POMCCrepIrs2KO} animals, both \textit{AgRPCrep110\textbeta KO} and \textit{POMCCrep110\textbeta KO} animals exhibit significant defects in hypothalamic function. This suggests the existence of alternative pathways (i.e. non-insulin/IRS), which signal via \textit{p110\textbeta} to influence the regulation of energy balance in AgRP and POMC neurons. In addition to its role in insulin signalling, PI3K plays a pivotal role as a signalling mediator in a wide variety of cellular responses to other
hormones, neurotransmitters, growth factors and cytokines (Vanhaesebroeck and Waterfield, 1999). Stimulation of almost every receptor that induces Tyr kinase activity also leads to class IA PI3K activation (Vanhaesebroeck and Waterfield, 1999).

8.3.3 Non-essential role for p110β-mediated signalling in hypothalamic leptin action in AgRP and POMC neurons

A number of studies support a role for hypothalamic PI3K action in mediating the effects of leptin on energy homeostasis. For example, the use of PI3K inhibitors has recently demonstrated that the inhibition of hypothalamic expression of NPY and AgRP by leptin involves a mechanism requiring intact PI3K signalling (Morrison et al., 2005). However, both AgRPCrep110βKO and POMCCrep110βKO animals remain leptin sensitive, indicating that the anorexigenic effects of leptin action in the hypothalamus are mediated by other PI3K isoforms in these neuronal populations. An alternative explanation is that the deletion of p110β at the embryonic stage in AgRP and POMC neurons, may be resulting in the activation of alternative compensatory mechanisms in these cells, aimed at maintaining the functional response to leptin.

The administration of ICV leptin to AgRPCrep110βKO and POMCCrep110βKO animals, and subsequent assessment of food intake and body weight changes should allow leptin action in these neuronal populations (lacking p110β) to be accurately assessed.

8.3.4 Non-essential role for p110β-mediated insulin signalling in AgRP and POMC neurons in peripheral glucose homeostasis

Despite recent studies supporting a role for PI3K function in mediating the actions of hypothalamic signalling on peripheral glucose homeostasis (Gelling et al., 2006; Morton et al., 2005), both AgRPCrep110βKO and POMCCrep110βKO animals exhibit no impairments in glucose homeostasis. This suggests a non-essential role for p110β-mediated insulin signalling in these neuronal populations with regards peripheral glucose homeostasis. Although AgRPCrep110βKO animals are hypoinsulinaemic compared to controls, this is consistent with their reduced body weight.
8.4 Conclusions

These studies have demonstrated that CNS IRS2 pathways acting in neuronal populations distinct from POMC and AgRP neurons regulate energy homeostasis and growth. A role for CNS IRS2 mediated insulin signalling in the control of peripheral glucose metabolism is also implicated. Furthermore these studies have shown that CNS IRS2 pathways are not required for leptin action or reproductive function.

Although these studies demonstrate an important role for IRS2 in the CNS regulation of food intake and body weight in mice, the relevance of this to human obesity remains unclear. It is currently not known whether low IRS2 levels in the human brain are involved in the pathogenesis of obesity and diabetes. However, there has been an encouraging proof of concept study with respect to the role of IRS2 in the pancreatic β-cell. It has now been shown that in patients with T2DM, there is a marked reduction in IRS2 expression in pancreatic islets (Gunton et al., 2005). This suggests that reduced IRS2 signalling in islets is involved in the pathogenesis of β-cell disease. Taken into context with the findings presented in this thesis, reduced IRS2 signalling in the CNS may also be a potential mechanism in the development of human obesity.

The investigations presented in this work also highlight a key role for hypothalamic p110β-mediated signalling pathways in the regulation of energy homeostasis. Although at a preliminary stage, these studies clearly demonstrate that p110β is an important mediator of signals that regulate energy balance in AgRP and POMC neuronal populations.

At the time of commencement of this work, the dominant view in the field was that the CNS mechanisms regulating energy homeostasis involved a key role for hypothalamic insulin action in distinct cell types. The studies discussed in this thesis have shown this not to be the case, and it is now evident that numerous signals acting in multiple brain regions are integrated to ensure the homeostatic preservation of energy stores. Overall these studies have provided new insights into the role of insulin signalling mechanisms in the hypothalamic regulation of energy homeostasis.
Future work

Studies involving NesCreIrs2KO animals

Although the impaired glucose homeostasis of NesCreIrs2KO animals suggests an important role for IRS2-mediated insulin signalling pathways in the regulation of peripheral glucose homeostasis, the relationship between obesity and impaired glucose homeostasis is complex. Thus, the use of hyperinsulinaemic-hypoglycaemic clamp studies will be required to assess the true contribution of CNS IRS2 pathways to the regulation of peripheral glucose homeostasis.

Studies involving POMCCre110βKO and AgRPCre110βKO animals

As mentioned above, these investigations are at an early stage and a number of additional studies are required to fully complete the phenotypic characterisation of POMCCre110βKO and AgRPCre110βKO animals. These are outlined below:

1. In order to prove deletion of this isoform specifically within POMC and AgRP neurons (of POMCCre110βKO and AgRPCre110βKO animals respectively), in situ hybridisation techniques will be established aimed at doing so.

2. The detailed analysis of the body composition of POMCCre110βKO and AgRPCre110βKO animals will be performed with the aid of MRI scanning. This work will be carried out in collaboration with Professor Jimmy Bell, Imperial College London.

3. The expression levels of various hypothalamic neuropeptides involved in the regulation of energy homeostasis will be determined. Leptin has recently shown to inhibit the hypothalamic expression of NPY and AgRP in a mechanism that requires intact PI3K signalling (Morrison et al., 2005). Thus, ablation of intact p110β signalling in these distinct neuronal populations may result in downstream changes in various neuropeptide systems.
4. A role for PI3K mediated mechanisms in the neuronal firing of hypothalamic neurons has been implicated in various studies. Thus, to address the role of the inhibition of functional p110β to the electrophysiological characteristics of the POMC and AgRP neurons (within POMC<sub>Cre</sub>110β<sub>KO</sub> and AgRP<sub>Cre</sub>110β<sub>KO</sub> animals respectively) mice expressing fluorescent markers in these neuronal populations lacking p110β will be generated and studied. This work will be carried out in collaboration with the laboratory of Professor. Ashford, University of Dundee.

5. Similarly to studies involving AgRP<sub>Cre</sub>110β<sub>KO</sub> animals, male and female POMC<sub>Cre</sub>110β<sub>KO</sub> animals will be challenged with HFD from the age of 6 week onwards for a total duration of 26 weeks to assess their sensitivity to DIO.

6. The food intake of POMC<sub>Cre</sub>110β<sub>KO</sub> and AgRP<sub>Cre</sub>110β<sub>KO</sub> animals at younger ages will also be assessed. The food intake studies described in this thesis were carried out on male mice at approximately 4 months of age. In the case of AgRP<sub>Cre</sub>110β<sub>KO</sub> animals, which exhibit reduced body weight from the age of 10 weeks of age onwards, it is unclear if the hyophagia of these animals is the causative factor for their reduced body weight or a consequence of it.
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Publications

The following publications have arisen as a result of this work:

