**UNRAVELING NOVEL LEPTIN-DEPENDENT SIGNALING PATHWAYS
IN THE BASOMEDIAL HYPOTHALAMUS**

by

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<u>DEDICATION</u>

Dedicated to:

My parents, Luz Eneida Feliciano and Tomás Villanueva

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LIST OF ABBREVIATIONS

CNS, central nervous system
BBB, blood brain barrier
BAT/WAT, brown adipose tissue/ white adipose tissue
LepRb, leptin receptor-b
GnRH, gonadotropin releasing hormone
LH, luteinizing hormone
FSH, follicle stimulating hormone
ME, median eminence
ARC, arcuate nucleus of the hypothalamus
MB-ARC, mediobasal arcuate nucleus
PVN, paraventricular nucleus of the hypothalamus
PMv, ventral premammillary nucleus
LHA, lateral hypothalamic area
DMH, dorsomedial hypothalamic nucleus
VMH, ventromedial hypothalamic nucleus
3V, third ventricle
AgRP, agouti related peptide
POMC, proopiomelanocortin peptide
NPY, neuropeptide-Y
OX, orexin
Jak2, Janus kinase-2
GRB2, growth factor receptor-bound protein-2
SHP2, Src homology domain-containing tyrosine phosphatase-2
ERK, extracellular signal-regulated protein
SOCS3, suppressor of cytokine signaling-3
PI3K, phosphatidyl-inositol-3-kinase
STAT3/5, signal transducer and activator of transcription-3 or -5
SH2B2, Src homology-containing adaptor protein-2
GFP, green fluorescent protein
mTORC1, mammalian target of rapamycin complex-1
pS6, phosphorylated ribosomal protein S6
S6K1/2, S6 kinase-1/2
AMPK, AMP-dependent protein kinase
GM-CSF, granulocyte macrophage colony stimulating factor
IHC, immunohistochemistry
IF, immunofluorescence
IB, immunoblotting
IR, immunoreactivity
ICV, intracerebroventricular
IP, intraperitoneal
ipGTT/ITT, intraperitoneal glucose tolerance test/ insulin tolerance test
NC/HF, normal chow/ high fat diets

ABSTRACT

UNRAVELING NOVEL LEPTIN-DEPENDENT SIGNALING PATHWAYS
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Leptin is secreted by the adipose tissue in proportion to fat stores and acts on the hypothalamus to inhibit appetite and promote energy expenditure. Leptin also regulates glucose homeostasis independently of feeding, and has a permissive action on reproduction. Activation of the leptin receptor (LepRb) initiates a cascade of signaling events, including the phosphorylation of residues on the intracellular domain of LepRb: Tyr_{985}, Tyr_{1077} and Tyr_{1138}. The ongoing analysis of LepRb signaling mutants *in vivo* has revealed a crucial role for Tyr_{1138} in energy homeostasis, while Tyr_{985} appears to be important for the attenuation of LepRb signaling. However, a number of the molecular mechanisms by which LepRb controls other physiological events, including neuronal firing, reproduction and glucose homeostasis, remain poorly defined. Also unclear are the mechanisms that control leptin regulation of a variety of signals including mTORC1. In this study, we examined the regulation of the mTORC1 pathway in the basomedial hypothalamus, and investigated the specific role of LepRb-Tyr_{1077} in leptin action *in vivo*. We found that leptin does not modulate hypothalamic mTORC1 in a uniform manner,

but rather controls mTORC1 in opposite directions, in different cell types and brain regions. Indeed, neuronal activation is the main regulator of mTORC1 activity in the hypothalamus in response to metabolic perturbations. We also examined the phenotype of mice mutant for LepRb-Tyr$_{1077}$ on a normal chow and a high fat diet. LepRb$^{f1077/f1077}$ (*f/f*) mice had modestly increased body weight and food intake on both diets. Both, male and female *f/f* mice had increased adiposity, and mutant females presented larger pancreas and ovaries than controls. Interestingly, we found that LepRb-Tyr$_{1077}$ seems to be mostly important for the regulation of reproduction and glucose homeostasis given that *f/f* female mice have delayed or absent estrous cycling together with improved glucose clearance. These results suggest novel pathways by which leptin regulates neuronal firing, reproduction and glycemic control. This information not only extends our understanding of leptin action, but could be crucial as we seek to understand the basis of conditions linking metabolism to reproduction and glucose homeostasis.

CHAPTER I

PUTTING IT ALL TOGETHER: OBESITY, LEPTIN-DEPENDENT SIGNALING PATHWAYS AND THE REGULATION OF MAMMALIAN PHYSIOLOGY

INTRODUCTION

The evolution of the obesity epidemic

Throughout the years, scientists have proposed different theories in an attempt to explain the physiological systems that regulate body weight and energy balance. Theories ranging from circulating signals that control appetite to specific genetic and environmental factors (1) were proposed, and altogether, these concepts opened a whole new era of research in the energy homeostasis area. Kennedy and colleagues (2) proposed the concept of a hypothalamic signal aimed to influence appetite and body fat stores in a coordinated manner. Overall, understanding the mechanisms of body weight regulation is important because as food becomes widely available in developed countries, excessive energy storage becomes a huge problem as obesity develops.

Obesity is a medical condition where an individual accumulates excess body fat and has increased body weight or a body mass index of 30.0 kg/m^2 or greater, to the extent that it can increase the risk for a variety of diseases (including Type-II diabetes, cardiovascular disease and cancers). Obesity affects more than 30% of the population in the United States, and its prevalence continues to increase despite all efforts to oppose it (3-5). At its most basic level, obesity develops when energy intake exceeds energy

utilization. The aggregate actions of a variety of hormones modulate energy expenditure, resulting in relatively little change in body weight or adiposity over time.

Complex hormonal interactions in the periphery are crucial to maintain homeostasis of diverse physiological functions and feedback from these interactions is ultimately integrated at the level of the central nervous system (CNS) to control energy balance. Of the hormones responsible for orchestrating energy homeostasis, the pancreatic β-cell-derived hormone insulin is a short-term signal secreted in proportion to meal size and is known to bind to its hypothalamic receptor (insulin receptor) to inhibit food intake (6-8). In the periphery, insulin is a potent anabolic hormone essential for maintenance of whole-body glucose homeostasis by reducing hepatic glucose output and increasing glucose uptake, primarily into muscle and adipose tissue (9).

On the other hand, ghrelin is a recently discovered orexigenic hormone secreted from the stomach (10;11). Ghrelin stimulates the release of growth hormone (which among many important functions induces lipolysis) and it is elevated during fasting. This hormone is known to bind to a hypothalamic ghrelin receptor to induce food intake and increase body weight gain (12;13). While insulin and ghrelin play important roles, they are not the most important central regulators of feeding because deletion of insulin receptor in the CNS only causes mild obesity (14-16) and whole body deletion of ghrelin or the ghrelin receptor has a minimal energy homeostasis phenotype in mice (17-19). Thus, there must be another signal(s) responsible for the overall regulation of feeding and energy balance.

The anorectic hormone, leptin

Mice homozygous for a spontaneous mutation in the obese (*ob*) gene (*ob/ob* mice) or the diabetes (*db*) gene (*db/db* mice) rapidly gain weight and reach three times the normal weight of wild-type controls at a young age. These mice exhibit hyperphagia, hyperglycemia, hypercortisolemia, glucose intolerance, and insulin resistance. These mutations also lead to an impaired immune system, infertility and increased metabolic efficiency (20-22). By performing parabiosis studies in which the blood supply of *ob/ob and db/db* mice were joined to a wild-type mouse by vascular anastomosis, Coleman (23) found that *ob/ob* mice lacked the production of a circulating signal that induced anorexia, and that *db/db* mice lacked the response (presumably the receptor) to this signal. Twenty years later, Zhang and colleagues cloned the *ob* gene and discovered its product, leptin (24). Leptin is a 16 kD protein secreted by the adipose tissue in proportion to fat (energy) stores that inhibits appetite and promotes energy utilization. Soon after the discovery of the obese (*ob*) gene, the leptin receptor was cloned and shown to be identical with the diabetes gene (*db*) (21). Transcription of the leptin gene (*ob)* in mice yields an mRNA of ~3.5 kb that is expressed primarily in white adipose tissue (WAT), although is evident that it is produced in other tissues as well (25-27). In humans, leptin is encoded by a gene located in 7q31.3 and it is translated as a 167 amino acid protein containing an amino-terminal secretory signal of 21 amino acids that is later removed at the microsomes, and thus leptin circulates in the blood as a protein of 146 amino acid residues (24). Leptin is an interleukin-type-6 cytokine that is produced in levels roughly proportional to body fat stores. Thus, leptin is well

positioned to communicate the amount of fat stores and consequently promote physiological processes that depend on energy availability.

Physiological actions controlled by leptin

Of the hormones controlling energy balance, leptin plays a central role (22;28-30). Lack of leptin signaling in mice and humans that are genetically null for either leptin (*ob/ob* mice) or for the leptin receptor (LepRb) (*db/db* mice) results in obesity secondary to increased feeding and decreased energy utilization (28;31-33). These mice display a phenotype that resembles the neuroendocrine starvation response (i.e., hypothyroidism, decreased growth, infertility and decreased immune function) and exogenous leptin replacement restores each of these functions while decreasing appetite (34). Leptin signaling similarly regulates the autonomic nervous system and the immune system (34;35) (Figure 1.1). The lack of leptin does not underlie common forms of obesity, however, as leptin levels are generally elevated in proportion to adipose mass; the failure of high circulating leptin levels in obese states to promote weight loss defines a state of so-called "leptin resistance" (36-38). Several potential mechanisms have been proposed to underlie leptin resistance, including defects in leptin access to the brain, leptin receptor signaling pathways or neurons that mediate downstream leptin action (39) but the etiology of this phenomenon still remains poorly defined.

While adiposity greatly influences insulin sensitivity and glycemic control, leptin also regulates glucose homeostasis independently of feeding and energy balance (40) given that *ob/ob* and *db/db* mice have impaired glycemic control that cannot be

reversed by fasting or pair feeding (41). Also, in humans and rodent models of lipodystrophy, leptin treatment improves insulin sensitivity and diabetes prior to changes in energy balance (42-46). These effects on glucose homeostasis appear to be mediated specifically by leptin in the CNS (47-49). Furthermore, incubation of primary islet cultures with leptin results in suppression of insulin secretion (50) which suggests that leptin may also regulate glucose homeostasis via insulin production in the pancreatic β-cells (51).

Leptin, energy stores and reproduction

The "critical weight hypothesis" (52) states that there is a threshold level of body fat required in order for females to achieve menarche and maintain normal ovulatory cycles. In normal women, a 10-15% decrease in body weight interrupts ovulation and menstrual cycle due to decreased secretion of gonadotropin releasing hormone (GnRH) by the hypothalamus (53;54). Leptin has proven to play an important role in reproductive physiology given that leptin-deficient *ob/ob* mice are sterile but leptin administration increases ovary weight and corrects sterility in both male and female mice (55). Prepubertal female mice injected with leptin experienced accelerated reproductive maturation demonstrated by the classical pubertal signs like vaginal opening, estrous and cycling (56;57). Thus, adequate leptin levels enable the onset of reproductive competence and lack of leptin in rodents and humans with genetic leptin deficiency or lipodystrophy results in hypothalamic amenorrhea, which is reversible with leptin replacement (43;44). Moreover, food restriction (which decreases leptin levels) and inadequate body fat levels, disrupts the activity of the reproductive axis, but leptin

treatment reverses this effect by partially restoring testosterone in male mice and luteinizing hormone (LH) levels and cyclicity in female mice (34;58). Additionally, leptin treatment preserves LH pulse frequency, sexual maturation, ovulation and sexual behaviors in rodent models (59;60).

Leptin receptors

Leptin acts through a cell-surface receptor (LepRb) that is a member of the type I cytokine receptor family (61). The mouse and human leptin receptor are highly similar in amino acid sequence for both the extracellular and intracellular domains (61). The *lepr* gene contains 17 common exons and several alternatively spliced exons. Alternative splicing of the transcript from a single *lepr* gene and/or proteolytic processing of the subsequent protein product produces multiple LepR isoforms (62;63) and in mice, the five distinct LepR isoforms that have been identified are designated LepRa-LepRe (64). Of these, LepRa-LepRd contain identical extracellular and transmembrane domains, but diverge in sequence because of splicing at 3' exons, while LepRe only contains an extracellular domain. The LepRb specific exon encodes a longer intracellular domain than other LepRs. The short forms of LepR (a, c, d) are widely found in the periphery (65) and although their function is not clear, their roles may include the transport of leptin, especially across the blood-brain barrier (BBB) (66;67). In all species, LepRb appears to account for most if not all of leptin action (61;68-70) since loss of LepRb in *db/db* mice results in the same phenotype observed in leptin-deficient *ob/ob* mice.

Leptin receptor distribution in the brain

While LepRb may be expressed in other tissues, central nervous system (CNS) LepRb accounts for the majority of its role in energy balance (35;51;71-73). LepRb is expressed in a variety of brain regions, including the hypothalamus and the hindbrain (74-77) (Figure 1.2). The arcuate nucleus of the hypothalamus (ARC) is the most sensitive area for circulating leptin compared to other hypothalamic sites and baseline leptin levels are sufficient to stimulate LepRb neurons that contact the portal circulation of the median eminence (ME), outside the BBB (78). While many hypothalamic nuclei contain LepRb-expressing neurons, two populations of LepRb neurons within the ARC define an important, well-understood and approachable neural circuit (74;79). Leptin promotes the expression and secretion of anorexigenic peptides derived from proopiomelanocortin (POMC) in POMC-expressing ARC neurons, while blocking the synthesis and secretion of orexigenic agouti-related peptide (AgRP) and neuropeptide-Y (NPY) from ARC neurons that express these peptides (Figure 1.3). LepRb-expressing neurons in the ventromedial hypothalamic nucleus and the ventral tegmental area also regulate feeding (80;81) and many other large populations of LepRb-expressing neurons (in regions such as the dorsomedial hypothalamic nucleus, the lateral hypothalamic area, the ventral premammillary nucleus, and others) most likely participate as well (74;79).

Leptin receptor signaling

Leptin binding to LepRb initiates a cascade of signaling events beginning with the activation of the constitutively receptor-associated Janus kinase-2 (Jak2), a tyrosine kinase; this represents a crucial step, as LepRb has no intrinsic enzymatic activity of its

own (61;82). In addition to promoting the autophosphorylation of Jak2 to increase its

activation, Jak2 stimulates the phosphorylation of multiple residues on the intracellular

domain of LepRb: Tyr_{985}, Tyr_{1077} and Tyr_{1138} (83). Each of these phosphorylation sites lies

in a unique amino-acid motif, and each of these residues thus recruits a distinct set of

downstream signaling proteins when phosphorylated (Figure 1.4). In cultured cells,

phosphorylated Tyr_{985} recruits the SH2-containing tyrosine phosphatase-2 (SHP2) to

mediate the first step in the activation of the extracellular signal-regulated kinase (ERK)

cascade (83-85). Phosphorylated Tyr_{985} also binds the suppressor of cytokine signaling-3

(SOCS3), which serves as a negative regulator of LepRb signaling (86). Tyr_{1138} recruits

the signal transducer and activator of transcription-3 (STAT3), a latent transcription

factor that then becomes phosphorylated, translocates to the nucleus, and mediates the

regulation of gene expression (84;87). Tyr_{1138}→STAT3 signaling promotes the expression

of SOCS3, as the afferent arm of a feedback loop that attenuates LepRb signaling

(84;86;88) (Figure 1.4). Also, the detection of leptin-induced pSTAT3 by

immunohistochemistry (IHC) or immunoblotting (IB) techniques mirrors LepRb

activation and function in the CNS (89;90). The phosphorylation of Tyr_{1077} promotes the

recruitment, tyrosine phosphorylation, and transcriptional activation of STAT5, although

Tyr_{1138} may also play a minor role in the regulation of STAT5 phosphorylation (83;91).

Moreover, there are Jak2-dependent signals given that phosphorylated Jak2 (at Tyr_{813})

binds to the SH2 domain of SH2B2 (92), which is an endogenous enhancer of leptin

signaling, and genetic deletion of SH2B2 results in leptin resistance and obesity (93).

Leptin additionally regulates a number of other intracellular signaling pathways by

mechanisms that remain to be clarified, including the activation of phosphatidylinositol kinase-3 (PI3K), the mammalian target of rapamycin (mTOR) and the inhibition of the AMP-dependent protein kinase (94-97). The complexity of the signaling pathways regulated by leptin becomes apparent when we consider the opposite regulation of some signals by leptin in distinct types of LepRb neurons. For instance, while leptin promotes the activation of PI3K in POMC neurons, it inhibits PI3K in AgRP neurons (98;99). Therefore, the analysis of how leptin and LepRb regulate these signaling pathways requires further studies *in vivo*.

The role of individual LepRb signals in leptin action in vivo

In the Myers laboratory, we have probed the function of specific LepRb tyrosine residues/signaling pathways *in vivo* by the generation and study of homologously targeted "knock-in" mice in which sequences encoding substitution mutants of specific LepRb phosphorylation sites replace the endogenous *lepr* allele (22;100). This approach expresses LepRb mutants from the *lepr* locus, ensuring their correct level and site of expression. This ongoing analysis has revealed a crucial role for Tyr_{1138} in the regulation of feeding and overall energy expenditure, but has also revealed that this signaling pathway is not required for the control of fertility, immune function, or several other leptin actions (40;64;101;102). The finding of similarly dysregulated feeding and body adiposity in mice null for STAT3 in the CNS (*NStat3KO*) compared to those mutant for LepRb Tyr_{1138} (*Lepr$^{s1138/s1138}$*) is consistent with the role for the $Tyr_{1138}\rightarrow$STAT3 pathway in energy homeostasis (103). The infertility of *NStat3KO* mice, which contrasts with the reproductive competence of *Lepr$^{s1138/s1138}$* animals, may reflect the importance of

neuronal STAT3 in the response to estrogen (and perhaps other factors), as well as leptin (104). Insulin resistance and glucose intolerance are improved in $Lepr^{s1138/s1138}$ mice compared to *db/db* animals suggesting that LepRb/STAT3-independent signals may contribute to the regulation of glucose homeostasis by leptin. Thus, in addition to LepRb/STAT3-mediated adiposity signals, non-LepRb/STAT3 leptin signals mediate an important adiposity-independent role in promoting glycemic control (40).

In contrast to the hyperphagic and obese phenotype of animals mutant for Tyr_{1138}, mice carrying a mutation of Tyr_{985} ($Lepr^{l985/l985}$) demonstrate a lean phenotype with exaggerated leptin sensitivity. Additionally, $Lepr^{l985/l985}$ mice exhibit normal neuroendocrine function. Thus, *in vivo*, the major role of Tyr_{985} appears to be in the attenuation of LepRb signaling, presumably via the SOCS3-mediated feedback loop (100). The physiologic role for SHP2-mediated signaling in LepRb action thus remains unclear.

Furthermore, mice with the intracellular domain of LepRb containing only the Jak2 binding site (LepRb$^\Delta$/LepRb$^\Delta$ mice) have a phenotype that closely resembles *db/db* mice (105), suggesting that leptin action is primarily mediated by pathways controlled by the phosphorylation of tyrosine residues within LepRb.

While leptin reportedly stimulates the activity of the mammalian target of rapamycin complex 1 (mTORC1) pathway, and leptin's anorectic response is known to be dependent on hypothalamic mTORC1 signaling (94), LepRb poorly regulates mTORC1 signaling in cultured cells, although Tyr_{985} mediates the phosphorylation of ribosomal protein S6 via the Rsk pathway (83).

The knowledge of the biology and the physiological effects of leptin have contributed greatly to our understanding of the processes that control energy intake and expenditure, but we are still at an early stage in the investigation of the molecular mechanisms by which LepRb controls neuronal physiology. It is thus important to determine how leptin controls these other physiological aspects via LepRb. We already know that Tyr_{985} regulates LepRb feedback and that Tyr_{1138} is mostly responsible for the regulation of feeding and adiposity, but numerous aspects of leptin signaling remain unclear, including the mechanism by which leptin controls mTORC1 *in vivo*, and the role for LepRb-Tyr_{1077} in the overall regulation of physiology.

Given our inability to detect the regulation of mTORC1 by LepRb in cultured cells, we postulate that the regulation of mTORC1 *in vivo* may be indirect of LepRb, as via the regulation of neural physiology. We will thus study the activation of neuronal activation (and hypothalamic mTORC1) in response to leptin and other nutritional cues. We also postulate that LepRb-Tyr_{1077} regulates physiological functions not attributable to Jak2-autonomous signals, LepRb-Tyr_{985} or -Tyr_{1138}, such as reproduction and glucose homeostasis.

To test the aforementioned hypotheses and for the fulfillment of this doctoral dissertation, I propose the following specific aims: (1) Determine the regulation of the mTORC1 pathway in the basomedial hypothalamus by examining the regulation of mTORC1 by nutritional/metabolic cues such as leptin, LepRb signals, insulin, ghrelin, and fasting, and by investigating the site (neuronal populations) of mTORC1 activation in the arcuate nucleus (ARC) in response to the above cues; (2) Investigate the role of LepRb-

Tyr$_{1077}$ *in vivo* by examining leptin action in mice with replacement of Tyr$_{1077}$ (*Lepr$^{f1077/f1077}$*).

These studies will be crucial as we seek to elucidate the complete mechanism of leptin action, and these results will hopefully identify pathways that may be targeted for therapy in obesity and Type 2 diabetes.

FIGURES: CHAPTER I
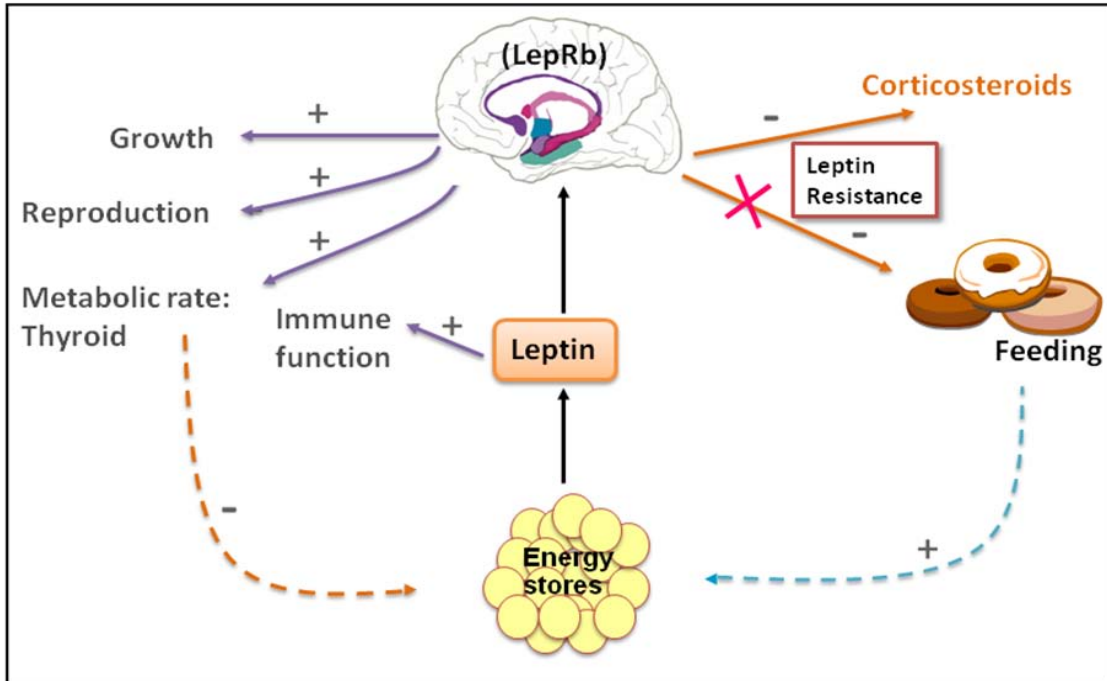
Figure 1.1: **Leptin acts on the central nervous system to control physiology**
Leptin is a hormone secreted by the adipocytes in proportion to fat (energy) stores. It travels in the circulation into the brain where it binds to leptin receptor (LepRb)-expressing cells to increase metabolic rate, promote thyroid function reproduction and growth. Leptin also suppresses feeding and corticosteroids, and increases immune response. Leptin resistance is a poorly understood process in obesity that blocks inhibition of feeding by leptin (Adapted from Bates, S., The role of leptin receptor signaling in feeding and neuroendocrine function, *TRENDS in Endocrinology and Metabolism*, 2003).
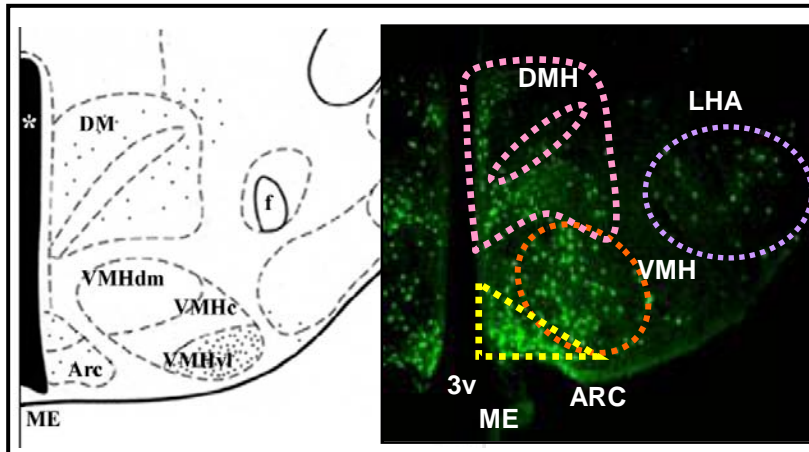
Figure 1.2: Leptin receptor expression in the hypothalamus
Coronal section (30µm) showing the neuroanatomical distribution of leptin receptor-expressing cells (anti- GFP) within the basomedial hypothalamus. 3v-third ventricle; ARC-arcuate nucleus; VMH-ventromedial hypothalamus; DMH-dorsomedial hypothalamus; LHA-lateral hypothalamic area; ME-median eminence. Picture on the left taken from Sica *et al. BMC Neuroscience* 2009.
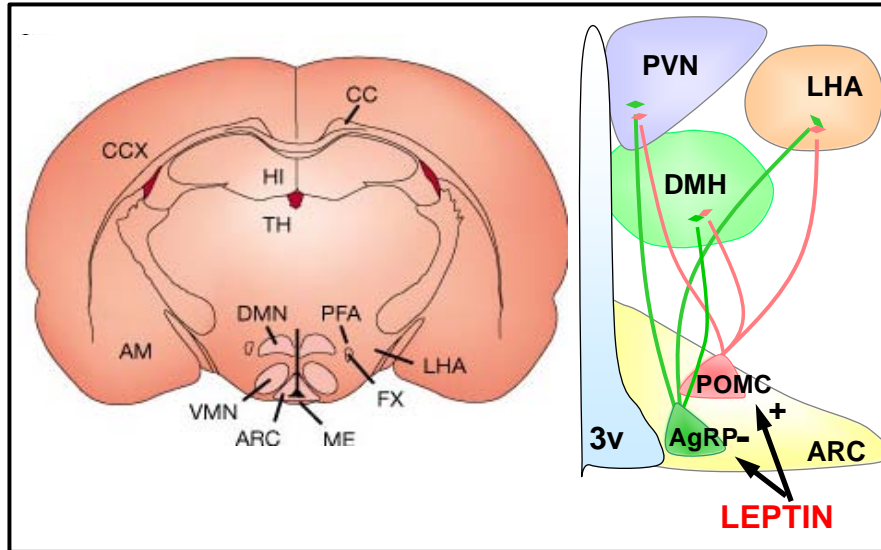
Figure 1.3: Neuronal circuits in the ARC

Diagram of a coronal section of the mouse brain (left, from Schwartz, M.W., *et. al.* Central nervous system control of food intake, *Nature*, 2000) and a model of the effect of leptin in hypothalamic neuropeptides in a zoomed-in diagram of the ARC (right) where leptin activates anorexigenic (POMC) neurons and inhibits orexigenic (AgRP/NPY) neurons to control energy homeostasis. These neurons synapse to other hypothalamic areas that are also known to express the long form of the leptin receptor (LepRb). 3v-third ventricle; ARC-arcuate nucleus; DMH-dorsomedial hypothalamus; PVN-paraventricular nucleus; LHA-lateral hypothalamic area.
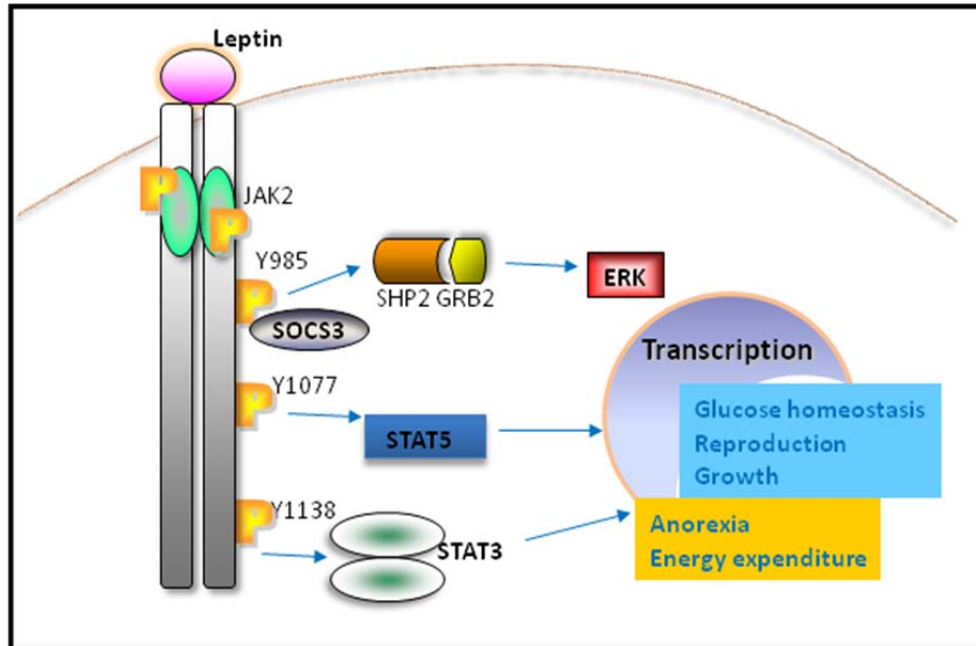
Figure 1.4: Leptin receptor (LepRb) signaling pathways
Leptin binding to the long form of the leptin receptor (LepRb) activates the associated Janus kinase-2 (Jak2), which autophosphorylates and activates downstream tyrosine residues (Tyr$_{985}$, Tyr$_{1077}$, Tyr$_{1138}$). LepRb- Tyr$_{985}$ recruits SHP-2 which recruits GRB2 and activates the ERK signaling cascade. Phosphorylated Tyr$_{1138}$ recruits the signal transducer and activator of transcription-3 (STAT3) and its nuclear translocation mediates transcription of several genes including the suppressor of cytokine signaling-3 (SOCS3) which binds to Tyr$_{985}$ and promotes feedback inhibition of LepRb signaling. LepRb-Tyr$_{1138}$→ STAT3 is a very important pathway on the regulation of anorexia and energy expenditure, whereas LepRb-Tyr$_{1077}$ mediates the recruitment of STAT5. Altogether, these signaling pathways regulate leptin-dependent physiological functions such as energy balance, glucose homeostasis, reproduction, immune function and growth.

CHAPTER II

REGULATION OF THE MAMMALIAN TARGET OF RAPAMYCIN COMPLEX-1 (mTORC1) PATHWAY IN THE BASOMEDIAL HYPOTHALAMUS BY LEPTIN AND NUTRITIONAL CUES

SUMMARY

The medial basal hypothalamus (MBH), including the arcuate nucleus (ARC) and the ventromedial hypothalamic nucleus (VMH), integrates signals of energy status to modulate metabolism and energy balance. Leptin and feeding regulate the mammalian target of rapamycin complex 1 (mTORC1) in the hypothalamus, and hypothalamic mTORC1 contributes to the control of feeding and energy balance. To determine the mechanisms by which leptin modulates mTORC1 in specific hypothalamic neurons, we immunohistochemically assessed the mTORC1-dependent phosphorylation of ribosomal protein S6 (pS6). In addition to confirming the modulation of ARC mTORC1 activity by acute leptin treatment, this analysis revealed the robust activation of mTORC1-dependent ARC pS6 in response to fasting and leptin deficiency in leptin receptor-expressing Agouti-related protein (AgRP) neurons. In contrast, fasting and leptin deficiency suppress VMH mTORC1 signaling. The appropriate regulation of ARC mTORC1 by mutant LepRb isoforms correlated with their ability to suppress the activity of AgRP neurons, suggesting the potential stimulation of mTORC1 by neuronal activity. Indeed, fasting- and leptin deficiency-induced pS6-immunoreactivity (IR) extensively colocalized with cFos-IR in ARC and VMH neurons. Furthermore, ghrelin, which activates orexigenic ARC neurons, increased ARC mTORC1 activity and induced

colocalized pS6- and cFos-IR. Thus, neuronal activity promotes mTORC1/pS6 in response to signals of energy deficit. In contrast, insulin, which activates mTORC1 via the phosphatidylinositol 3-kinase pathway, increased ARC and VMH pS6-IR in the absence of neuronal activation. The regulation of mTORC1 in the basomedial hypothalamus thus varies by cell and stimulus type, as opposed to responding in a uniform manner to nutritional and hormonal perturbations.

INTRODUCTION

The prevalence of obesity and its pathologic sequelae, including type-2 diabetes and cardiovascular disease, has reached epidemic levels and continues to increase in developed countries (3-5;106). Understanding the mechanisms that regulate feeding and energy balance thus represents a crucial first step in identifying potential targets for the therapy of obesity and diabetes. The discovery of leptin and the elucidation of crucial leptin-regulated neurons in the brain have facilitated the study of these mechanisms. Adipocytes secrete leptin in approximate proportion to fat/energy storage, so that circulating leptin levels reflect the status of long-term energy stores (28). Adequate leptin levels signal via a specific long-form leptin receptor (LepRb) on metabolic sensing neurons in the brain to modulate food intake, metabolism, and neuroendocrine energy utilization (107-109).

Numerous specialized neural populations in the brain express LepRb, including those in several basomedial hypothalamic nuclei important for energy balance, such as the arcuate nucleus (ARC) and the ventromedial hypothalamic nucleus (VMH). The ARC

in particular contains a well-studied and -understood circuit composed of two distinct neural populations (29;109;110). Proopiomelanocortin (POMC)-expressing neurons secrete peptides that act via central melanocortin (and perhaps other) receptors to suppress appetite and increase metabolic rate; leptin promotes the activity of these neurons (111). In contrast, leptin suppresses the activity of ARC neurons that express and secrete neuropeptide Y (NPY) and Agouti-related protein (AgRP), both of which stimulate appetite and decrease energy utilization (112). In addition to responding to leptin, many ARC neurons sense and respond to other indicators of energy status, including insulin and a variety of nutrients (113-115).

Among the indicators of cellular energy status implicated in the regulation of ARC neural circuits are the AMP-dependent protein kinase (AMPK, which is activated in response to increased AMP/ATP ratios, among other signals of decreased cellular energy) (95;116), and the mammalian target of rapamycin (mTOR) (94;117). mTOR functions within two distinct multimolecular complexes; of these, mTOR complex 1 (mTORC1) responds to nutritional (especially amino acid) cues of energy repletion to promote macromolecular biosynthesis (118-120). Insulin similarly promotes anabolic mTORC1 action, as does signaling via protein kinase C, Ca^{+2}, and other pathways. Among other downstream signals, stimulation of mTORC1 activates the S6 kinases (S6K1 and S6K2) to promote the phosphorylation of ribosomal protein S6 (pS6), an important regulator of protein translation (118;121). Acute rapamycin treatment specifically inhibits mTORC1 and mTORC1-dependent signals, such as S6K1/2 (119;120). Refeeding-, amino acid-, and leptin-stimulated activation of mTORC1-dependent signals in whole

ARC, and hypothalamic mTORC1 activity is important for the anorectic response to amino acids and leptin (94). In contrast, constitutive mTORC1 signaling in POMC neurons impairs their anorectic action and promotes obesity (122).

These data suggest the potential complexity, and neural cell type-specificity, of mTOR regulation by signals of nutritional status and energy sufficiency. We thus sought to determine the mechanisms by which leptin and LepRb control mTORC1 activity in defined populations of ARC neurons. Consistent with the complexity of hypothalamic mTORC1 action, we report that while leptin modestly increases mTORC1 in the ARC, ARC mTORC1 is also stimulated by the activation of orexigenic AgRP neurons in response to leptin deficiency and other signals of negative energy balance. In contrast, VMH neuronal activity and mTORC1 are conversely attenuated by signals of negative energy balance. Insulin activates mTORC1 more widely and by neural activity-independent mechanisms.

MATERIALS AND METHODS

Reagents

Leptin was from NHPP (Torrance, CA), insulin was from Novo Nordisk (Princeton, NJ), and ghrelin was the gift of Matthias Tschöp, M.D. (University of Cincinnati, Cincinnati, OH). Rabbit anti-phospho S6 (Ser 240/244) was purchased from Cell Signaling (Boston, MA), chicken anti-GFP from Ab-cam (Cambridge, MA), goat anti-cFos was from Oncogene Sciences (Uniondale, NY), goat anti- β -Gal from Biogenesis (Poole, UK), and donkey serum was from Jackson ImmunoResearch Laboratories, Inc. (West

Grove, PA). Donkey anti-chicken (or anti-rabbit) Alexa-488 conjugated and donkey anti-goat Alexa-564 conjugated antibodies were purchased from Molecular Probes, Inc. (Eugene, OR). All other immunohistochemical supplies were purchased from Sigma-Aldrich (St. Louis, MO).

Animals

Animals for immunohistochemical analysis were bred in our colony in the Unit for Laboratory Animal Medicine (ULAM) at the University of Michigan, except for 10-12 weeks old male S6K1$^{-/-}$ animals (123), which were produced by heterozygote intercrosses at the University of Cincinnati Genome Research Institute animal facility. All procedures were in accordance with the guidelines of and with the approval of the respective University Committee on the Care and Use of Animals at the University of Michigan and University of Cincinnati.

Mice containing the coding sequence of Cre recombinase targeted into the 3'-untranslated region of the LepRb-specific exon 18b in the *Lepr* gene (LepRb^{cre} mice) were bred with *Gt-(ROSA)26Sor^{tm2sho}* (Rosa26-EGFP) mice, in which a floxed transcription blocker precedes the EGFP reporter gene sequence. Cre-mediated recombination/removal of the transcription blocker results in EGFP expression in LepRb-expressing cells (LepRb^{EGFP} "reporter" mice) (124). Heterozygote *Lepr^{db}* (*db/+*), *Lepr^{Tm1mgmj}* (*s/+*) (101), and *Lepr^{Tm2mgmj}* (*l/+*) (125) mice were self-crossed to generate animals homozygous for each altered *lepr* allele. Heterozygous mice expressing LacZ from the AgRP locus (*a/+*) were produced as previously described (126).

Mice were housed in groups of 2-4 with *ad libitum* access to food and water. Some

mice were fasted for 24 hours (or as indicated) before sacrifice between 9 a.m. and 12

p.m.; access to water was not restricted. For immunohistochemical analysis, *ad libitum-*

fed animals remained with food in the cage until (9 a.m. and 12 p.m.) the day of sacrifice

between.

Intraperitoneal (IP)/Intracerebroventricular (ICV) injections

10-12 weeks-old male mice received an ip injection of leptin (5mg/kg), insulin

(400mU/mL), or 1X sterile phosphate buffered saline (PBS) for one hour prior to

sacrifice. Stereotaxic implantation of cannulae was performed under isoflurane

anesthesia into the left cerebral ventricle. After 7 days of recovery, 3µl of leptin

(1mg/mL), PBS, ghrelin (2µg/µl), insulin (300mU/ml), DMSO, or rapamycin (2µg/µl) were

injected icv one hour prior to sacrifice.

Perfusion and Immunohistochemistry (IHC)

Handling of all animals was limited to <1 hour prior to anesthesia and perfusion to

minimize stress-related induction of c-Fos levels. Perfusion and IHC procedures were

performed essentially as described previously (90). In brief, mice were deeply

anesthetized with an overdose of pentobarbital (150 mg/kg, IP) and transcardially

perfused with sterile PBS followed by 4% paraformaldehyde. Brains were removed,

post-fixed and cryoprotected before sectioning into 30µm coronal slices, which were

collected into 4 representative series and stored at -20˚C until further use.

For IHC, sections were pretreated in ice-cold methanol, 0.6% glycine and 0.03% SDS,

and then blocked with donkey serum and incubated in the primary antibodies (rabbit-

anti-pS6 [1:100], chicken anti-GFP [1:1000], goat anti-c-Fos [1:1000] and/or goat anti-β-Gal [1:3000]).   Detection of primary antibodies was done by immunofluorescence (donkey anti-chicken (or anti-rabbit)-Alexa 488 and anti-goat-Alexa 564 [all 1:200 dilution]).

Quantification of IHC signals from hypothalamic sections and statistics

Regions of the hypothalamus denoted as ARC and VMH for counting purposes are depicted in Figure 2.1. For quantification of pS6-positive neurons, LepRb (EGFP) neurons, AgRP neurons and pS6/EGFP, pS6/AgRP or pS6/c-Fos double-labeled neurons, pictures of matched brain areas were taken on channels for Alexa 488 and Alexa 564 from at least three sections containing the arcuate nucleus (ARC) of the hypothalamus for each brain between bregma -1.58mm to -1.94mm (according to the Paxinos and Franklin mouse brain atlas). All sections were arranged from rostral to caudal to examine the distribution of single- and double-labeled neurons.   Using Adobe® Photoshop software, both channels (red and green) were combined in a RGB picture and single and double-labeled neurons were counted and recorded digitally to prevent multiple counts. Total number of single- and double-labeled neurons was presented as means +/- SEM and differences were analyzed by two-way ANOVA followed by a Student's T-test to determine significant differences between groups. Differences were accepted for p values < 0.05, with an n value ≥ 3.

RESULTS

Activation of ARC mTORC1 and suppression of VMH mTORC1 by fasting and leptin deficiency

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In order to more closely examine the molecular mechanisms and cellular specificity of the regulation of the mTORC1 pathway by leptin, we employed the immunohistochemical detection of pS6-IR as a marker for mTORC1 activity in the hypothalamus. Reasoning that decreased baseline levels of leptin would facilitate the detection of the leptin response, we examined pS6-IR in the hypothalamus of *ad libitum*-fed and 24-hour fasted wild-type (*+/+*) mice following treatment with leptin (5 mg/kg, ip) or vehicle (Figure 2.2). This analysis revealed an increased number of pS6-IR ARC neurons in fasted *+/+* animals with and without leptin treatment, but only a trend toward increased numbers of pS6-IR neurons with leptin in the *ad libitum*-fed animals. In contrast to the ARC, fasting suppressed pS6-IR in the VMH. (See Figure 2.1 for depictions of the regions counted for this and subsequent analyses). We also compared *ad libitum* fed *+/+* mice to leptin-deficient (*Lep^{ob/ob}*) mice (Figure 2.3), revealing an increase in the number of pS6-IR ARC neurons and a trend toward decreased pS6-IR in VMH neurons (p= 0.06) in *Lep^{ob/ob}* animals relative to *ad libitum*-fed controls. This increase in the number of pS6-IR ARC neurons was especially pronounced in the medial basal ARC (MB-ARC) near the median eminence. These findings suggested that fasting and leptin deficiency mediate the phosphorylation of S6 in MB-ARC neurons, and also that the conditions of leptin repletion or the increased MB-ARC pS6-IR at baseline with 24 hour-fasting impair our ability to examine leptin-mediated stimulation of pS6-IR. We thus re-examined the leptin effect in animals that had been fasted for a shorter period of time (overnight, approximately 16 hours) (Figure 2.4). This analysis revealed an

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increase in pS6-IR neurons with leptin treatment in the MB-ARC similar to that previously described in rats following a similarly modest fast (94).

Since the finding of MB-ARC mTORC1 activation implied by the increased MB-ARC pS6-IR in fasted and *Lep^ob/ob^* animals was unexpected, we examined the mTORC1-dependency of the fasting effect by examining pS6-IR in S6K1^-/-^ animals (123) and in animals treated with intracerebroventricular (ICV) rapamycin (a specific mTORC1 inhibitor, when used acutely) (118-120). S6K1 and the related S6K2 interact with and are activated by mTORC1 to mediate the mTORC1-dependent phosphorylation of S6. Lack of S6K1 significantly decreased pS6-IR in the MB-ARC of knockout mice under *ad libitum* and fasted conditions compared to wild type mice under similar conditions (Figure 2.5). S6K1^-/-^ animals exhibited some residual pS6-IR in the fasted state, however, suggesting that while S6K1 mediates a portion of the basal and fasting-induced pS6-IR in the MB-ARC, other kinases (e.g. S6K2) also contribute. ICV rapamycin treatment abrogated MB-ARC pS6-IR (including during fasting), demonstrating the mTORC1-dependence of pS6-IR in the MB-ARC (Figure 2.6). Thus, fasting promotes increased mTORC1 activity in neurons of the MB-ARC in mice.

Neuronal populations demonstrating pS6-IR in the MB-ARC

In order to determine whether the fasting-mediated activation of pS6 occurred in LepRb-expressing neurons, we examined pS6-IR and its colocalization with EGFP in LepRb^EGFP^ mice, which demonstrate EGFP expression specifically in LepRb-expressing neurons throughout the brain (75;127;128). Since LepRb is expressed normally in the LepRb^EGFP^ animals, they exhibit no detectable alterations in energy homeostasis. As

previously, fasting increased pS6-IR in the MB-ARC of LepRb^{EGFP} mice, and leptin treatment tended to increase the number of pS6-IR neurons (Figure 2.7). Furthermore, the fasting-stimulated pS6-IR neurons extensively colocalized with LepRb/EGFP neurons; approximately 40% of MB-ARC LepRb neurons demonstrated fasting-induced pS6-IR.

The similarity of the distribution of MB-ARC neurons that demonstrated fasting- (or leptin deficiency-) induced pS6-IR with the distribution of orexigenic NPY/AgRP-expressing MB-ARC neurons suggested the possibility that these neurons might represent a major locus of fasting-induced pS6-IR. We thus utilized animals heterozygous for *Agrp*^{*LacZ*} (in which the homologous targeting of the coding sequences for LacZ mediates the expression of β -galactosidase (β -gal) by the native AgRP promoter, and specifically in AgRP-expressing neurons) (126;129) in order to examine the potential induction of pS6-IR in AgRP neurons during fasting (Figure 2.8 and Figure 2.9). These *Agrp*^{*LacZ*} heterozygous animals have no detectable alteration in energy balance relative to control animals. While little pS6-IR colocalized with the detectable AgRP neurons in fed animals, pS6-IR was extensively colocalized with β-gal/AgRP neurons in fasted animals (20-30% of the total pS6 neurons in the MB-ARC of fasted animals were AgRP positive). Thus, fasting promotes mTORC1 activity in LepRb- and AgRP-expressing neurons in the MB-ARC. Similar results were noted throughout the rostal-caudal axis of the ARC (Figure 2.9).

Role of LepRb signaling in the regulation of pS6 by endogenous leptin in the MB-ARC

The finding of increased pS6-IR observed in the MB-ARC of *Lep*^{*ob/ob*} animals suggested that increased mTORC1 activity results from the absence of a LepRb-

mediated signaling pathway. In order to determine the potential mechanism by which endogenous leptin might act via LepRb in wild-type animals to regulate MB-ARC pS6-IR, we examined pS6-IR in the ARC of mouse models containing specific alterations in LepRb signaling (Figure 2.10). As in leptin-deficient $Lep^{ob/ob}$ animals, $Lepr^{db/db}$ animals (in which LepRb is disrupted by a splicing defect) displayed a large number of pS6-IR MB-ARC neurons compared to controls, suggesting that the appropriate regulation of mTORC1 activity in MB-ARC neurons by endogenous leptin requires intact LepRb. We also examined pS6-IR in mice containing mutant LepRb molecules with substitution mutations of tyrosine residues that control specific signaling pathways. In contrast to the $Lep^{ob/ob}$ and $Lepr^{db/db}$ animals, however, *ad libitum* fed mice containing a mutation of LepRb Tyr_{985} ($Lepr^{l985/l985}$ (l/l) mice) (130) or Tyr_{1138} ($Lepr^{s1138/s1138}$ (s/s) mice) (101) demonstrated little pS6-IR in the MB-ARC (Figure 2.10), suggesting that the signals controlled by these residues (SHP2/SOCS3 and STAT3, respectively) are not required for the normal modulation of mTORC1 activity by endogenous leptin in the MB-ARC. While their morbid obesity impairs the examination of the fasting response in *s/s* animals, we examined the effect of fasting in *l/l* mice, which revealed the fasting-mediated induction of pS6-IR in the MB-ARC in these animals, ruling out an effect of Tyr_{985} in the regulation of this effect.

Neuronal activation and S6 phosphorylation in the MB-ARC and VMH

As we sought to understand the mechanisms by which fasting and/or the absence of leptin action might activate mTORC1 in the MB-ARC and decrease mTORC1 in the VMH, we noted that the induction of MB-ARC pS6-IR correlated with the activation of

orexigenic neurons, such as AgRP neurons. These neurons are activated by fasting in wild-type mice and also in the *ad libitum*-fed state in *Lep^ob/ob* and in *Lepr^db/db* animals, but not in *l/l* or *s/s* mice (29;109;131;132)(Figure 2.11).

   To examine this correlation more closely, we examined the potential colocalization of pS6-IR and c-Fos-IR (a marker of neuronal activation) (133) in the MB-ARC and VMH during fasting and leptin treatment (Figure 2.12). In addition to demonstrating the expected induction of MB-ARC pS6 and c-Fos-IR by fasting, 54% of the pS6-IR neurons in fasted mice colocalized with c-Fos-IR, suggesting that the majority of the neurons of the MB-ARC that contain highly active mTORC1 represent actively firing neurons. Furthermore, fasting decreased VMH pS6-IR and tended to decrease VMH c-Fos-IR. Neither fasting nor leptin altered the percentage of MB-ARC or VMH pS6-IR neurons positive for c-Fos-IR, consistent with the idea that the regulation of c-Fos/neuronal activation is tightly coupled to pS6/mTORC1.

   It is, of course, possible that mTORC1 activation might promote neuronal activation, rather than the converse. In order to address this issue, we treated fasted animals with ICV rapamycin before sacrificing them for the examination of pS6-IR and c-Fos-IR in the MB-ARC (Figure 2.13). While, as expected, rapamycin treatment abrogated the mTORC1-dependent pS6-IR in the MB-ARC, c-Fos-IR remained high. Thus, while most pS6-IR neurons in the MB-ARC neurons of fasted mice are active by the criterion of c-Fos-IR, mTORC1 activity is not required for this neuronal activation, consistent with the notion that the depolarization of active neurons in the MB-ARC may induce mTORC1 activation.

In order to further examine the notion that depolarization and neural firing may promote mTORC1 activity in the MB-ARC, we examined the induction of pS6-IR and c-Fos-IR by treatment with ICV ghrelin, which rapidly and directly depolarizes orexigenic neurons in the MB-ARC (independently of any acute effects on circulating metabolites) (134) (Figure 2.14). As for fasting, ICV ghrelin treatment induced pS6-IR and c-Fos-IR and their colocalization in the MB-ARC, consistent with the activation of mTORC1 by depolarization of these neurons.

Insulin and pS6-IR in the hypothalamus

In order to understand the potential for additional mechanisms of hypothalamic mTORC1 activation, we examined pS6- and c-Fos-IR in the hypothalamus following insulin treatment, as insulin hyperpolarizes all studied ARC neurons, but activates mTORC1 via the PI 3-kinase pathway in cultured cells (135).

Indeed, while ICV insulin treatment dramatically increased pS6-IR in the ARC and VMH, c-Fos-IR did not change in the ARC and decreased in the VMH, with the result that the percent of pS6-IR neurons containing c-Fos decreased in each region with insulin treatment (Figure 2.15). Similarly, IP insulin injection increased pS6-IR in the MB-ARC (although not in the VMH or elsewhere in the brain), but without increasing c-Fos-IR or the percent of pS6-IR neurons containing c-Fos (Figure 2.16). These data are consistent with the notion that insulin activates mTORC1 widely, and independently of neuronal activity. Thus, the regulation of mTORC1 in the basomedial hypothalamus varies by cell type, nucleus, and nutritional status, as opposed to responding in a uniform manner to nutritional and hormonal perturbations.

DISCUSSION

Leptin activates a variety of signals in hypothalamic LepRb neurons, such as STAT3, STAT5, ERK, PI3K, AMPK, and mTORC1 (84;85;85;94-96;98;136-138). Many of these pathways (including STAT3, PI3K, and mTORC1) are known to play important roles in anorexia and other leptin-dependent responses (22;94;96;107;111;139). While the receptor motifs by which LepRb activates many signals (e.g. STAT3) have been revealed (117;137), the mechanisms by which LepRb modulates other signals, such as PI3K, AMPK, and mTORC1 remain obscure. The difficulty in dissecting these signals stems in large part from the inability to observe the regulation of these signals by LepRb in cultured cells, such as HEK293 cells, without the overexpression of multiple signaling proteins that likely result in the non-specific activation of some signals. Additionally, the opposite regulation of some signals by leptin in distinct types of LepRb neurons complicates the analysis of their regulation. For instance, while leptin promotes the activation of PI3K in POMC neurons, it inhibits PI3K in AgRP neurons (98;99;117). Thus, the analysis of the mechanisms by which leptin regulates these signals requires further studies *in vivo* and presumably in a cell-type specific manner, as we have done here for the regulation of mTORC1.

Our data reveal the IHC detection of pS6-IR in the MB-ARC to be largely dependent upon the major mTORC1 mediator, S6K1, and to be entirely dependent upon mTORC1 itself. Using this IHC methodology to explore the regulation of mTORC1 by leptin and nutritional manipulation (i.e. fasting) not only confirmed the expected enhancement of

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mTORC1 signaling upon leptin treatment, but also revealed increased mTORC1 activity

in LepRb and AgRP-expressing neurons in the MB-ARC by fasting and leptin-deficiency.

In contrast, fasting and leptin deficiency decrease mTORC1 activity in the VMH.

The finding that endogenous leptin prevents mTORC1 activation in the MB-ARC

suggested potential roles for specific LepRb signals in this process. We thus explored

the signaling mechanisms by which LepRb might modulate the accumulation of pS6-IR in

the MB-ARC. This analysis revealed that neither Tyr_{985} (the binding site for SHP-2 and

SOCS3) nor Tyr_{1138} (binding site for STAT3) participate in the regulation of MB-ARC pS6-

IR under conditions of endogenous leptin (in contrast to the activation observed in the

absence of leptin action in $Lep^{ob/ob}$ and $Lepr^{db/db}$ mice). While the obesity and high

circulating leptin levels of the $Lepr^{s1138/s1138}$ mice make it difficult to assess a role for

Tyr_{1138} in the leptin-stimulated activation of mTORC1 in the ARC, the $Tyr_{1138}\rightarrow$STAT3

pathway represents an unlikely mediator of the acute effects of leptin on pS6-IR, since

the regulation of this pathway is unlikely to be transcriptionally mediated.

Our data further suggest that the mechanism by which fasting and leptin-deficiency

mediate this activation is indirect, via the regulation of neural activity. This conclusion is

supported not only by the correlation between the induction of pS6-IR in the MB-ARC by

nutritional conditions and genetic environments ($Lepr^{db/db}$ mice) in which these

orexigenic neurons are activated (140;141), but also by the induction of pS6-IR primarily

in neurons that also demonstrate IHC evidence of activation (c-Fos-IR) both in the MB-

ARC and in the oppositely-regulated VMH.   Furthermore, ghrelin, which acutely

depolarizes the orexigenic neurons of the MB-ARC (134) without altering the overall

nutritional milieu, also promotes pS6-IR coincident with c-Fos-IR in the MB-ARC. Note that while one hour of stimulation, which generally represents the peak of mTORC1 activation/pS6 following an acute stimulus (and used in this study), precedes the peak of c-Fos-IR following neuronal depolarization; thus, the ultimate extent of c-Fos-IR colocalization in this experiment may be underrepresented.

Since leptin depolarizes and promotes the firing of some neurons within the MB-ARC (140;142), it is possible that the previously reported activation of mTORC1 during acute leptin treatment also takes place secondary to changes in neuronal activity. Another reasonable possibility is that leptin might promote mTORC1 signaling via the induction of PI3K, which is an important upstream activator of mTORC1 (118-120). Indeed, the observation that insulin (whose major signaling output is via PI3K (143)) promotes pS6-IR in the mediobasal hypothalamus without increasing c-Fos-IR suggests that robust PI3K activation in the hypothalamus stimulates mTORC1, as in cultured cells.

Overall, our present results demonstrate that leptin and nutritional cues do not modulate hypothalamic mTORC1 activity in a uniform manner, but rather control mTORC1 via multiple pathways and, in many cases, in opposite directions in different cell types and brain regions, via the regulation of neuronal activity.

FUTURE DIRECTIONS

In this study, we have completed the characterization of mTORC1 activation by different nutritional cues and its specific localization within the basomedial hypothalamus. However, it would be interesting to assess the activation of the mTORC1

pathway by neuronal activation more closely by performing KCl-induced depolarization of ARC explants. This could be done in the presence and absence of leptin in order to detect how specific activation of ARC neurons regulates pS6, which could be measured in the media. Moreover, examining the role of mTORC1 on neuronal regulation, including neuropeptide synthesis/release and neuronal plasticity would provide information on the specific function of mTORC1 in the CNS.

Also, given that only 30% of the pS6 neurons were AgRP positive, it would be very interesting to know the identity of the other neurons presenting S6 activation in the ARC, and determine if they are also activated by fasting. Most importantly, it would be crucial to determine if LepRb-Tyr$_{1077}$ is the pathway by which leptin regulates neuronal activation and thus, indirectly turns on the mTORC1 pathway in the ARC. And if so, what are the differences of the regulation of the mTORC1 pathway in the VMH, where mTORC1/pS6 regulation opposes what is observed in the ARC. More specifically, we could investigate the role of other signaling molecules, such as PI3K and how they might influence neuronal activation by leptin or how PI3K activity might differ by cell-type (within orexigenic neurons) and brain areas (ARC versus VMH).

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NOTES

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FIGURES: CHAPTER II
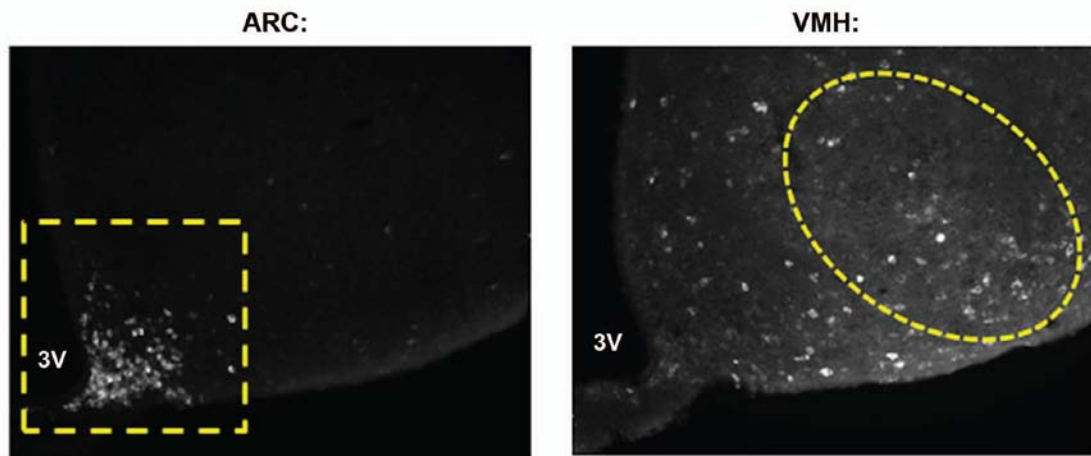
Figure 2.1: Regions of interest in the basomedial hypothalamus
Regions of interest utilized for counting cells in the ARC and VMH. Shown are pS6-IR images of the medial basal hypothalamus demonstrating (enclosed in yellow dashed lines) regions utilized for counting ARC (left panel; image from fasted wild-type animal) and VMH (right panel; image from *ad libitum*-fed wild-type animal) neurons in histochemical sections. Note that the indicated VMH section represents the ventrolateral VMH, which was the region in which the greatest changes were observed. 3V-third ventricle.
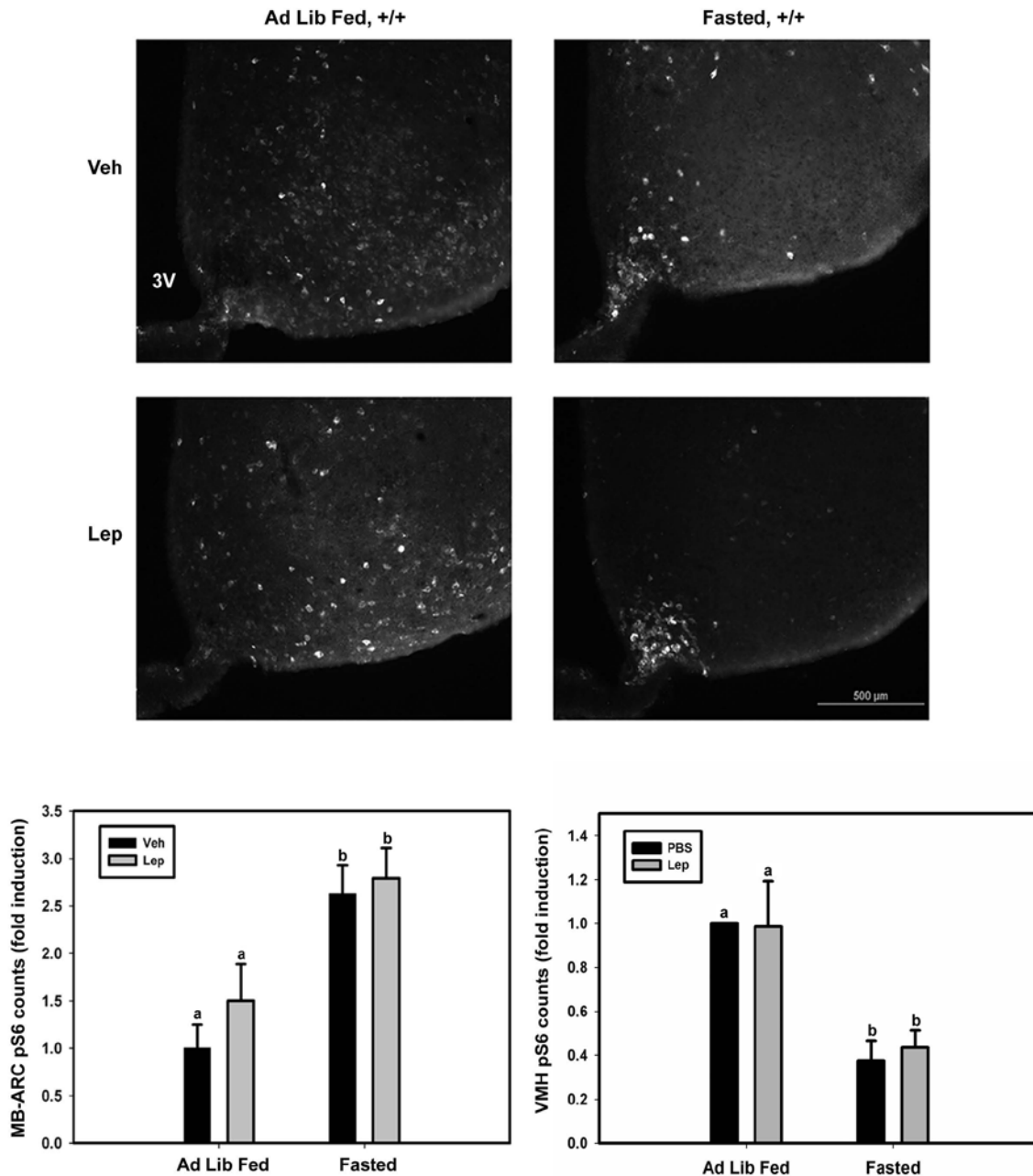
Figure 2.2: Phosphorylation of S6 in response to fasting and leptin in the MB-ARC
Wild-type (+/+) animals under *ad libitum* fed or 24-hour fasted conditions treated with leptin (Lep; 5mg/kg, ip) or vehicle (Veh) for 1 hour before sacrifice that were processed for immunohistochemical analysis of pS6-IR. Top panels show representative images of immunofluorescent detection of pS6-IR, while the graphs below show the fold induction of the number of pS6 cells in the MB-ARC, which were quantified and plotted as mean +/- SEM (n≥3 per condition; bars with different letters were significantly different by ANOVA, $p<0.05$). 3V-third ventricle.
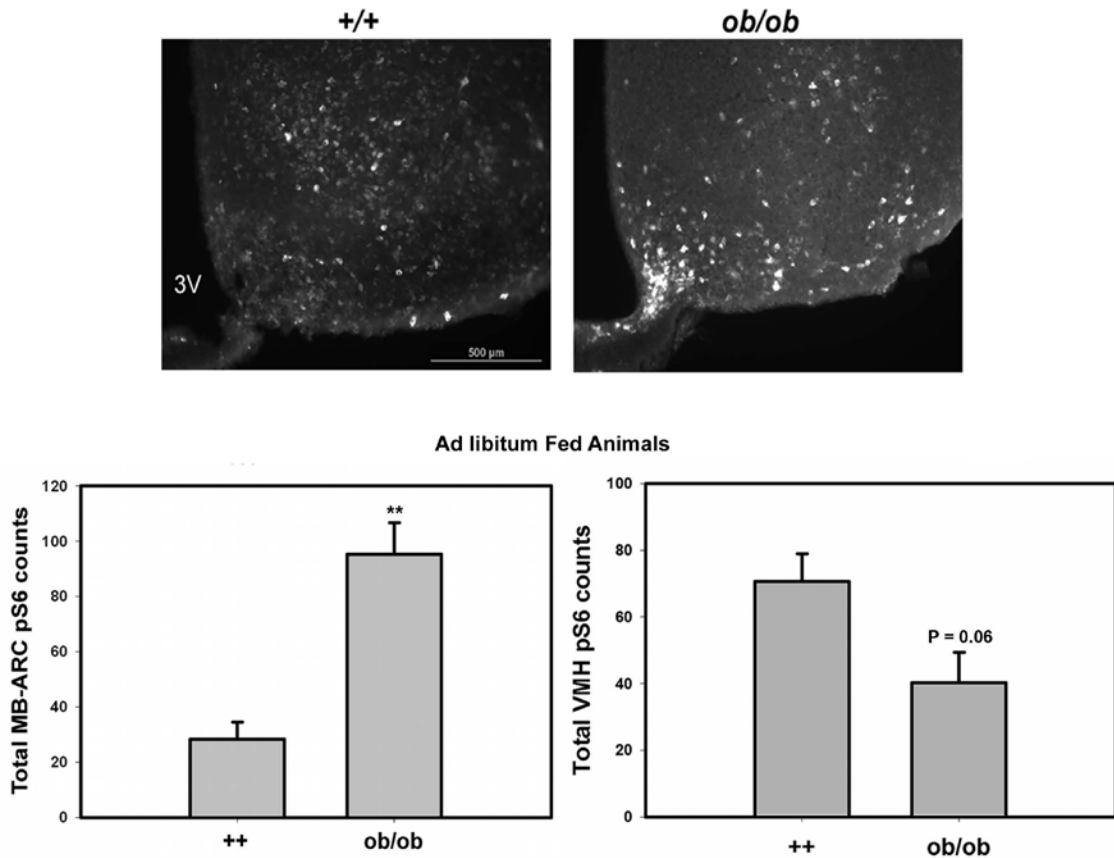
Figure 2.3: Phosphorylation of S6 in leptin-deficient *ob/ob* mice

Ad libitum fed *Lep^{ob/ob}* mice and *+/+* mice treated with leptin (Lep; 5mg/kg, ip) or vehicle (Veh) for 1 hour before sacrifice that were processed for immunohistochemical analysis of pS6-IR. Top panels show representative images of immunofluorescent detection of pS6-IR, while the graphs below show the total counts of the number of pS6 cells in the MB-ARC, which were quantified and plotted as mean +/- SEM (n≥3 per condition; bars with different letters were significantly different by ANOVA, p<0.05). 3V-third ventricle.

+/+, Veh   **+/+, Lep**

3V

500 µm
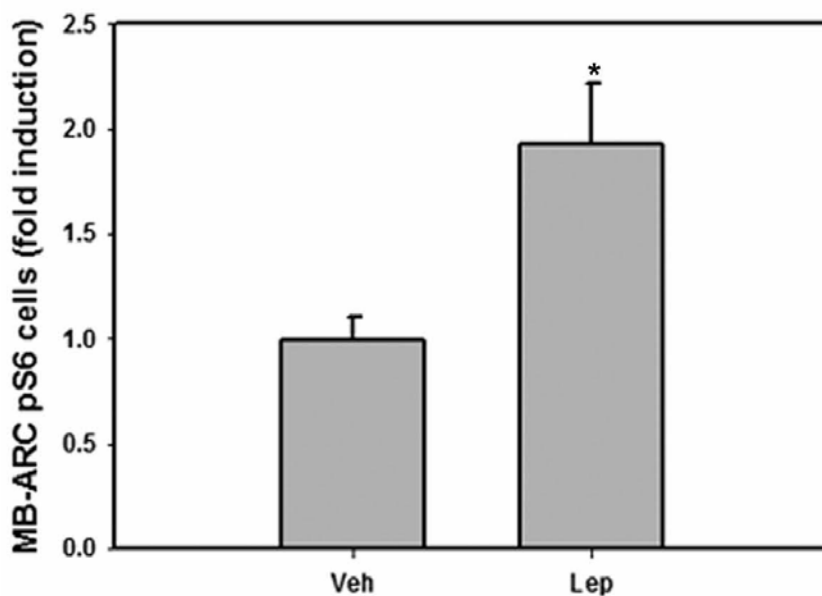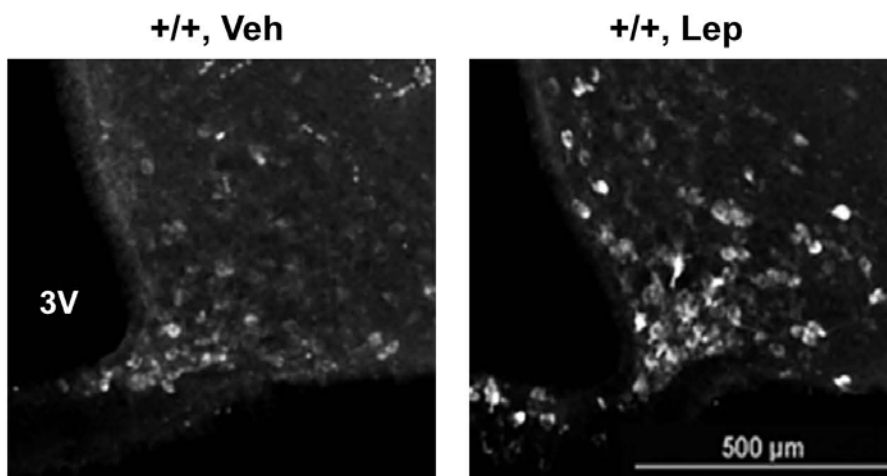
Figure 2.4: Leptin-induced phosphorylation of S6 in wild-type mice
Overnight (approximately 16-hour) fasted *+/+* mice treated with leptin (Lep; 5mg/kg, ip) or vehicle (Veh) for 1 hour before sacrifice that were processed for immunohistochemical analysis of pS6-IR. Top panels show representative images of immunofluorescent detection of pS6-IR, while the graphs below show the fold induction of the number of pS6 cells in the MB-ARC, which were quantified and plotted as mean +/- SEM (n≥3 per condition; bars with different letters were significantly different by ANOVA, p<0.05). 3V-third ventricle.
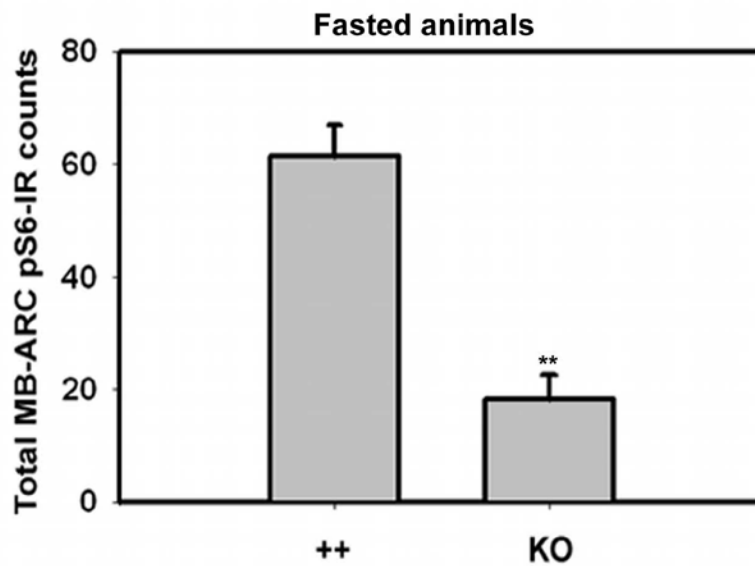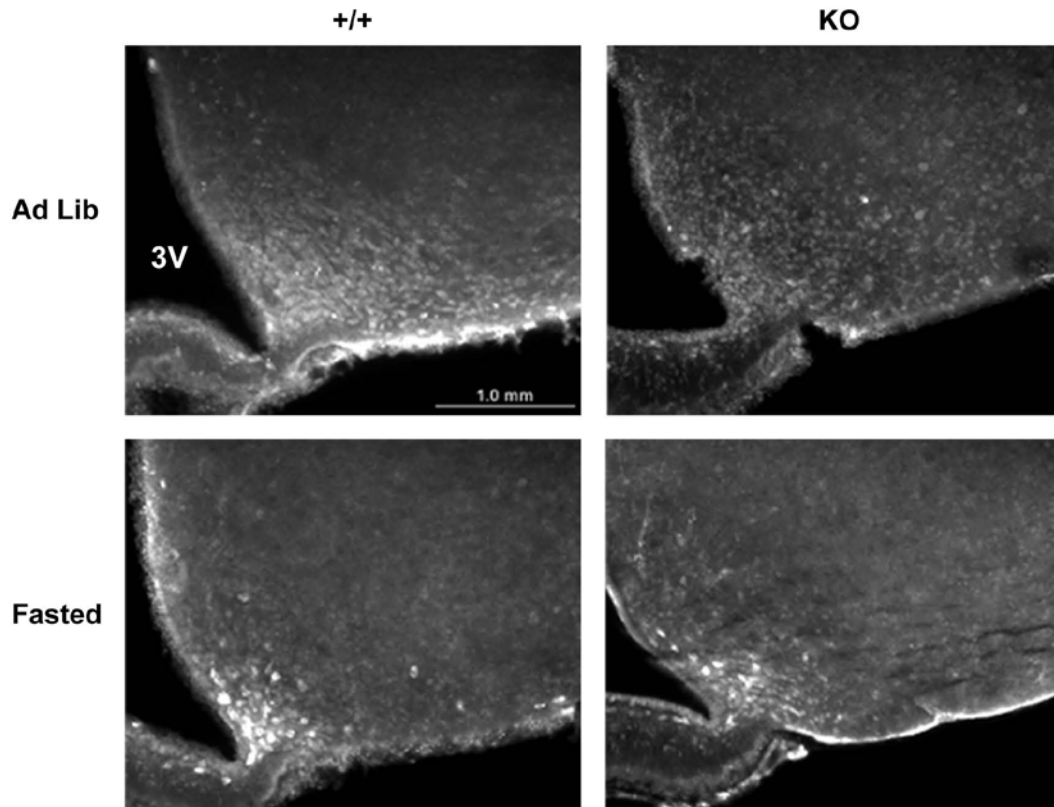
Figure 2.5: S6K1-dependence of pS6-IR in the MB-ARC

Wild-type (+/+) or S6K1$^{-/-}$ (KO) animals under *ad libitum* fed or 24-hour fasted conditions were perfused and processed for the immunofluorescent detection of pS6-IR. Top panel shows representative images. Total numbers of pS6-IR neurons in the MB-ARC (mean +/-SEM) for each genotype are presented in the graph. (n≥3 per condition; ** represents a p value <0.001 by Student's t-test). 3V-third ventricle.
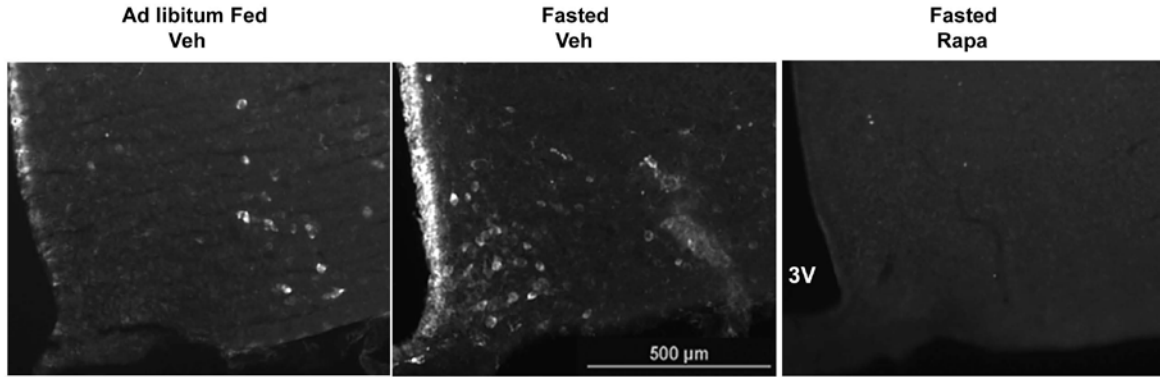
Figure 2.6: mTORC1-dependence of pS6-IR in the MB-ARC

Wild-type animals under the indicated *ad libitum* fed or fasted conditions were treated with icv vehicle or rapamycin (2µg/µl) overnight and 2 hours before sacrifice and processing for the immunofluorescent detection of pS6-IR. Representative images under each condition are shown. In addition to the MB-ARC pS6-IR that is the focus of this analysis, note some inflammatory induction of pS6-IR in the apendymal cells that line the ventricle, which is also blocked by treatment with rapamycin. 3V-third ventricle.
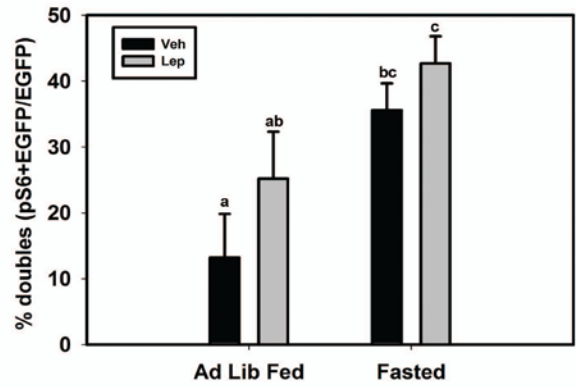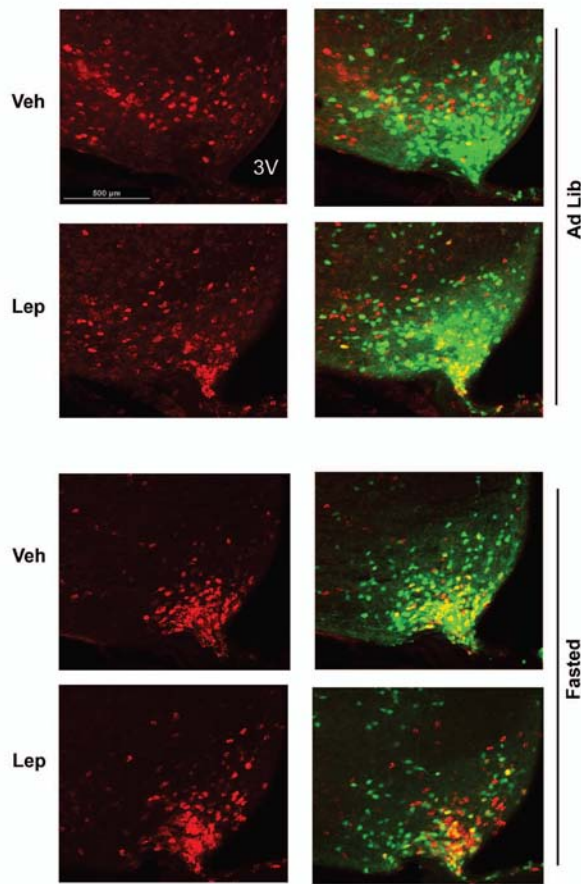
Figure 2.7: Fasting induces pS6-IR in LepRb-expressing neurons in the MB-ARC

LepRb^GFP mice were allowed to feed *ad libitum* or were fasted for 24 hours before treatment with leptin (Lep; 5 mg/kg, ip) or vehicle (Veh) for 1 hour prior to sacrifice and processing for the immunofluorescent detection of LepRb/GFP (green)-expression and pS6-IR (red). Top panels show representative images of pS6-IR alone (left) and merged pS6-IR and GFP-IR images (right). LepRb/GFP neurons that contain pS6-IR are plotted as mean +/-SEM (n≥3 per condition; bars with different letters were significantly different by ANOVA, $p < 0.05$). 3V-third ventricle.
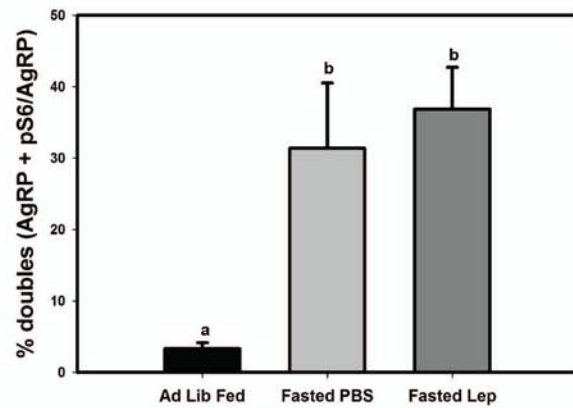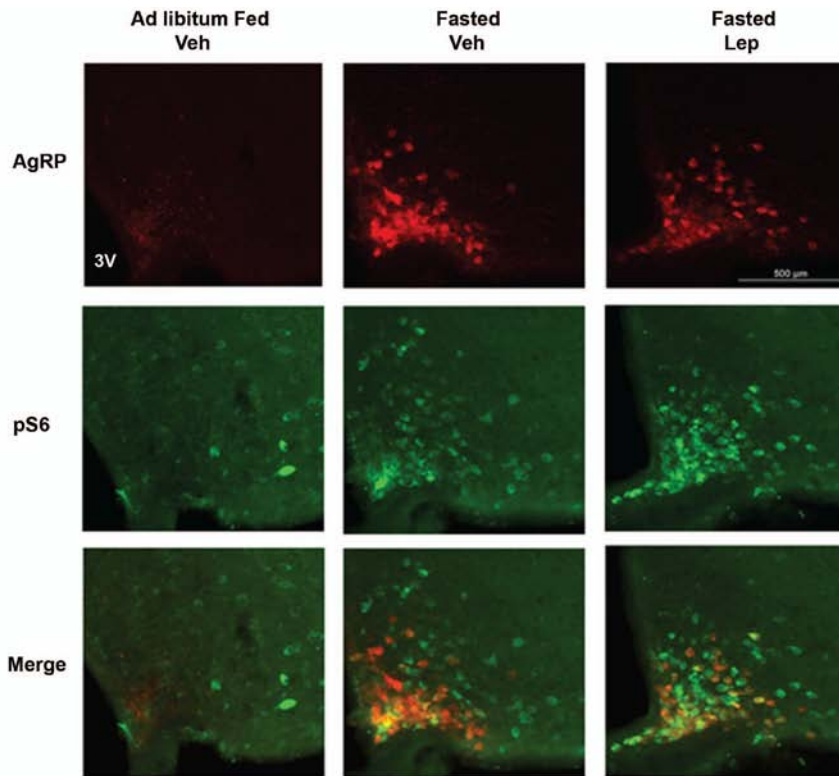
Figure 2.8: Fasting induces pS6-IR in AgRP-expressing neurons in the MB-ARC

Animals heterozygous for *Agrp^LacZ* were allowed to feed *ad libitum* or were fasted for 24 hours before treatment with leptin (Lep; 5 mg/kg, ip) or vehicle (Veh) for 1 hour prior to sacrifice and processing for the immunofluorescent detection of AgRP/LacZ (red)-expression and pS6-IR (green). Top panels show representative images of AgRP (β-gal) alone (top), pS6-IR alone (middle) and merged β-gal and pS6-IR images (bottom). AgRP (β-gal) neurons that contain pS6-IR are plotted as mean +/-SEM (n≥3 per condition; bars with different letters were significantly different by ANOVA, $p < 0.05$). 3V-third ventricle.
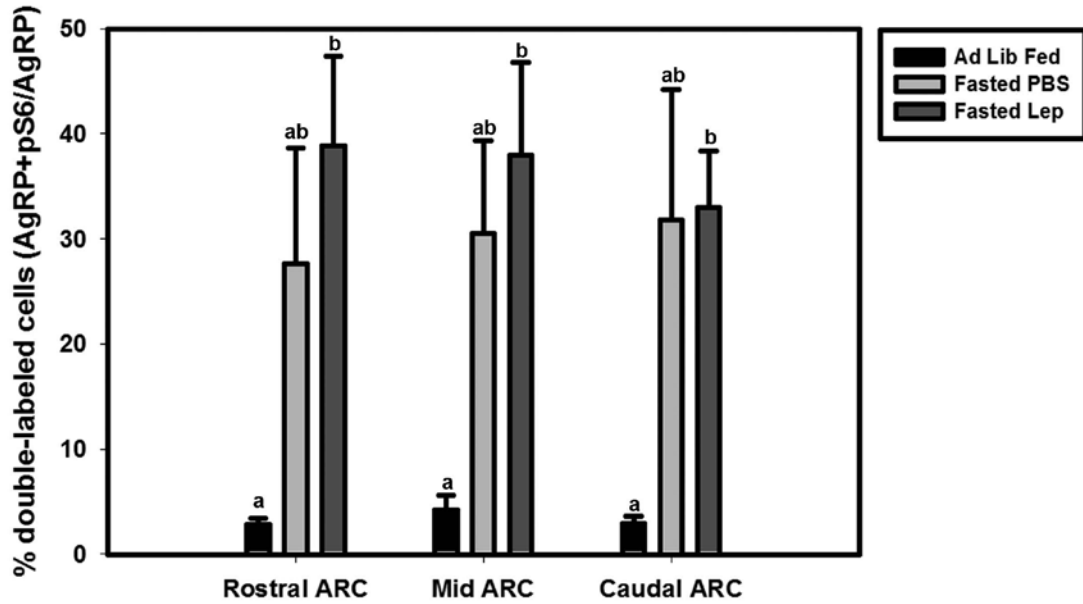
Figure 2.9: Distribution of AgRP/pS6 cells along the rostro-caudal axis of the MB-ARC

Animals heterozygous for *Agrp^LacZ* were allowed to feed *ad libitum* or were fasted for 24 hours before treatment with leptin (Lep; 5 mg/kg, ip) or vehicle (Veh) for 1 hour prior to sacrifice and processing for the immunofluorescent detection of AgRP/LacZ expression and pS6-IR. AgRP (β-gal) neurons that contain pS6-IR in each region of the MB-ARC are plotted as mean +/-SEM ($n \geq 3$ per condition; bars with different letters were significantly different by ANOVA, $P < 0.05$). These data represent data from Figure 3B, separately analyzed for each region of the rostrocaudal axis of the MB-ARC.
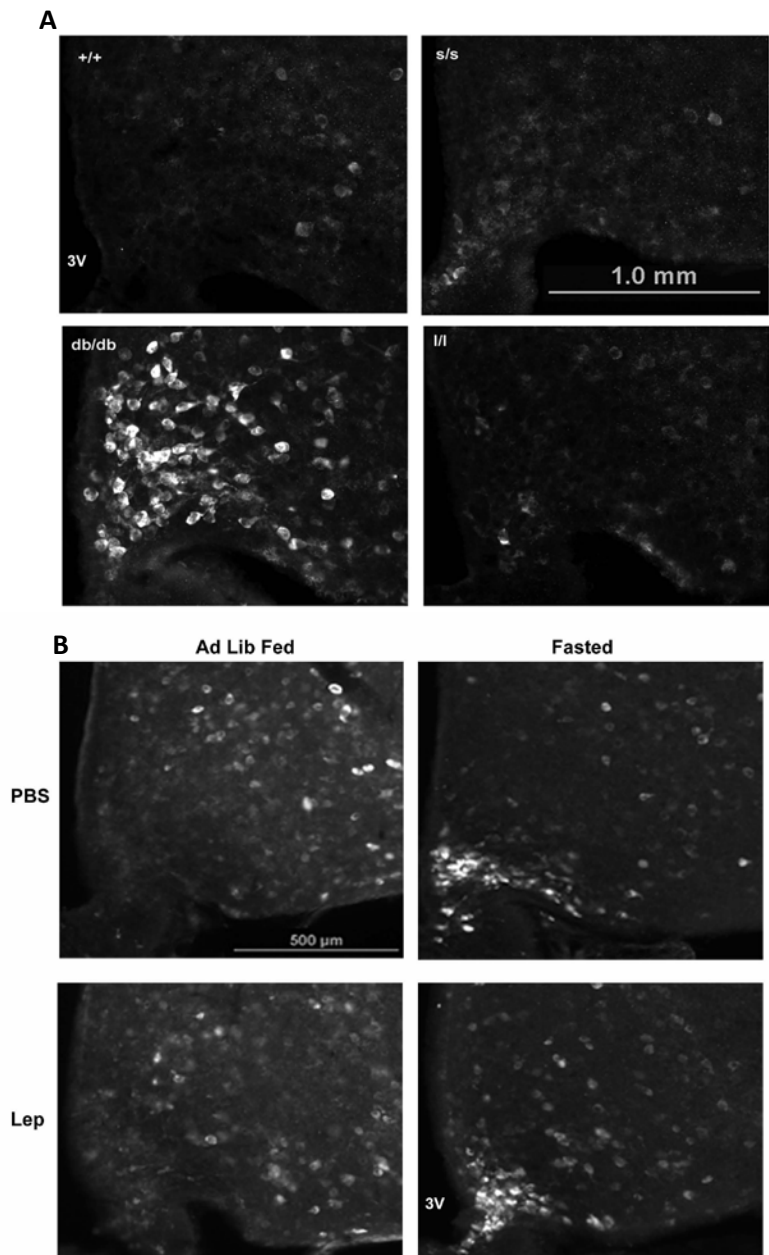
Figure 2.10: pS6-IR in the MB-ARC of mouse models with defects in LepRb signaling

A, *Ad libitum*-fed wild-type (*+/+*), $Lepr^{db/db}$ (*db/db*), $Lepr^{s1138/s1138}$ (*s/s*) and $Lepr^{l985/l985}$ (*l/l*) were perfused and processed for the immunofluorescent detection of pS6-IR. Representative images of pS6-IR from each genotype are shown. B, *Ad-libitum* fed and 24-hour fasted *l/l* mice were treated with leptin or PBS, as indicated, perfused, and processed for the immunofluorescent detection of pS6-IR. 3v-third ventricle.
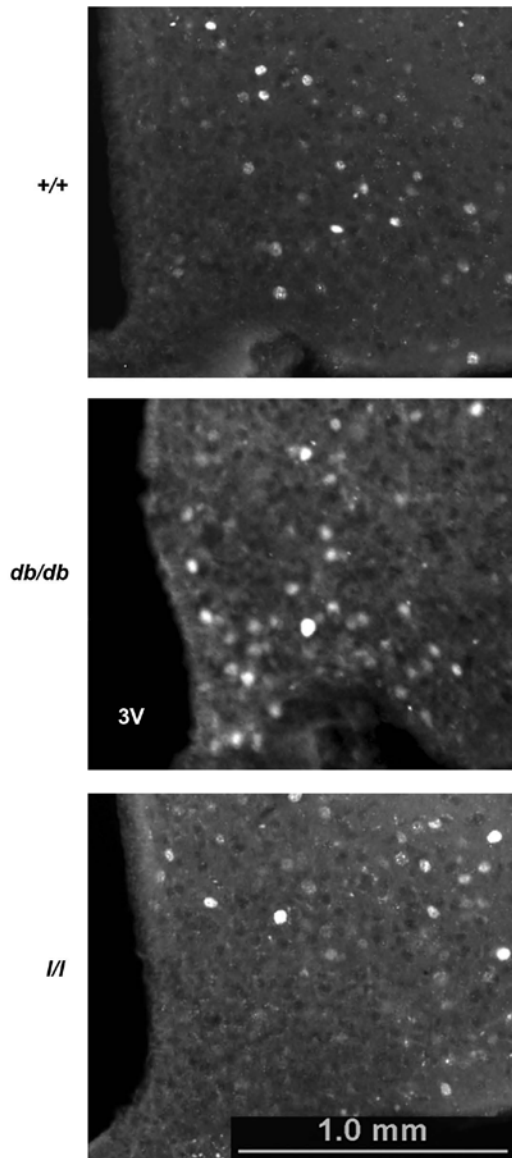
Figure 2.11: C-Fos-IR in the MB-ARC of *db/db* and *l/l* mice

Ad libitum-fed wild-type (*+/+*), *db/db*, or *l/l* mice were perfused and processed for the immunohistochemical detection of c-Fos-IR. Representative images are shown. Note that the extensive activation of c-Fos-IR in the MB-ARC of *db/db* animals is absent from *l/l* animals. 3V-third ventricle.

Figure 2.12: pS6-IR localization in activated neurons in the MB-ARC and VMH
Wild-type animals were allowed to feed *ad libitum* or were fasted for 24 hours before treatment with leptin (Lep; 5 mg/kg, ip) or vehicle (Veh) for 1 hour prior to sacrifice and processing for the immunofluorescent detection of c-Fos-IR (red) and pS6-IR (green). Top panels show representative images of merged images at each condition. Smaller panels beneath show digital zoom of each channel and merged images from the boxed area in the larger panels above. Graphs: Total numbers of pS6-IR, c-Fos-IR, and % double-labeled neurons are plotted as mean +/-SEM (n≥3 per condition; bars wi th different letters were significantly different by ANOVA, p<0.05). 3v-third ventricle.

**Figure 2.13: Rapamycin abrogates pS6-IR but not cFos-IR in the MB-ARC**

Wild-type animals under the indicated *ad libitum* fed or overnight (approximately 16 hours) conditions were treated with icv vehicle or rapamycin (2μg/μl) via indwelling catheters overnight and for additional 2 hours before sacrifice and processing for the immunofluorescent detection of pS6-IR (green) and c-Fos-IR (red). Representative images under each condition are shown. In addition to the MB-ARC pS6/c-Fos-IR that is the focus of this analysis, note some inflammatory induction of pS6-IR in the apendymal cells that line the ventricle, which is also blocked by treatment with rapamycin.
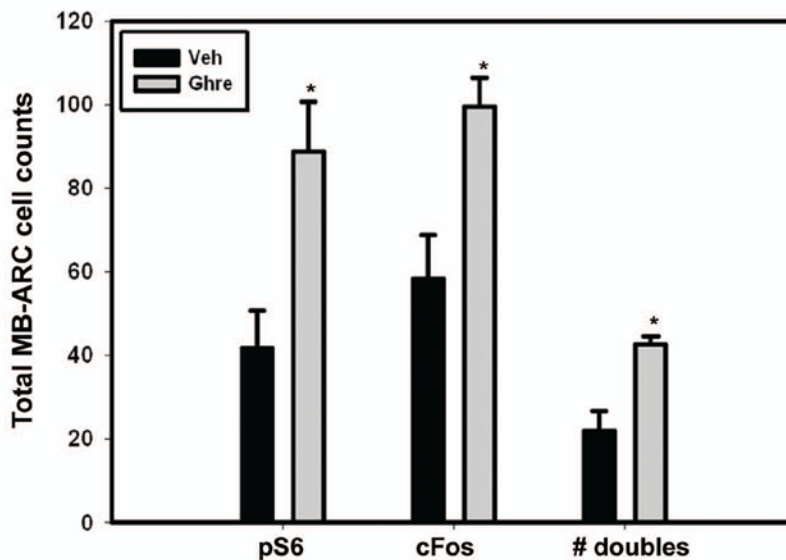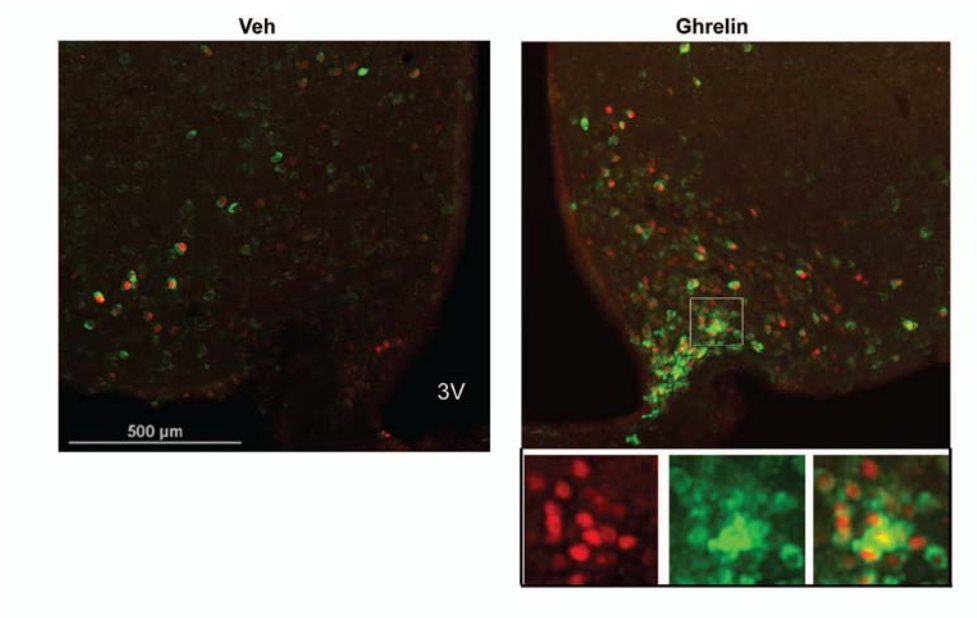
Figure 2.14: Ghrelin-induced neuronal firing promotes mTORC1 activation in the MB-ARC

Ad libitum fed wild-type animals with indwelling icv catheters were treated with vehicle (Veh) or ghrelin (2µg/µl) for 1 hour before sacrifice and processing for the immunofluorescent analysis of pS6-IR (green) and c-Fos-IR (red). Top panels: Representative merged images. Panels beneath the image from the ghrelin-treated sample represent digital zoom of individual channels and merged images from the boxed area in the larger image. Graph: Total numbers of pS6-IR, c-Fos-IR, and double-labeled neurons are plotted as mean +/-SEM (n≥3 per condition; * represent p values ≤ 0.01 by Student's t-test). 3V-third ventricle.
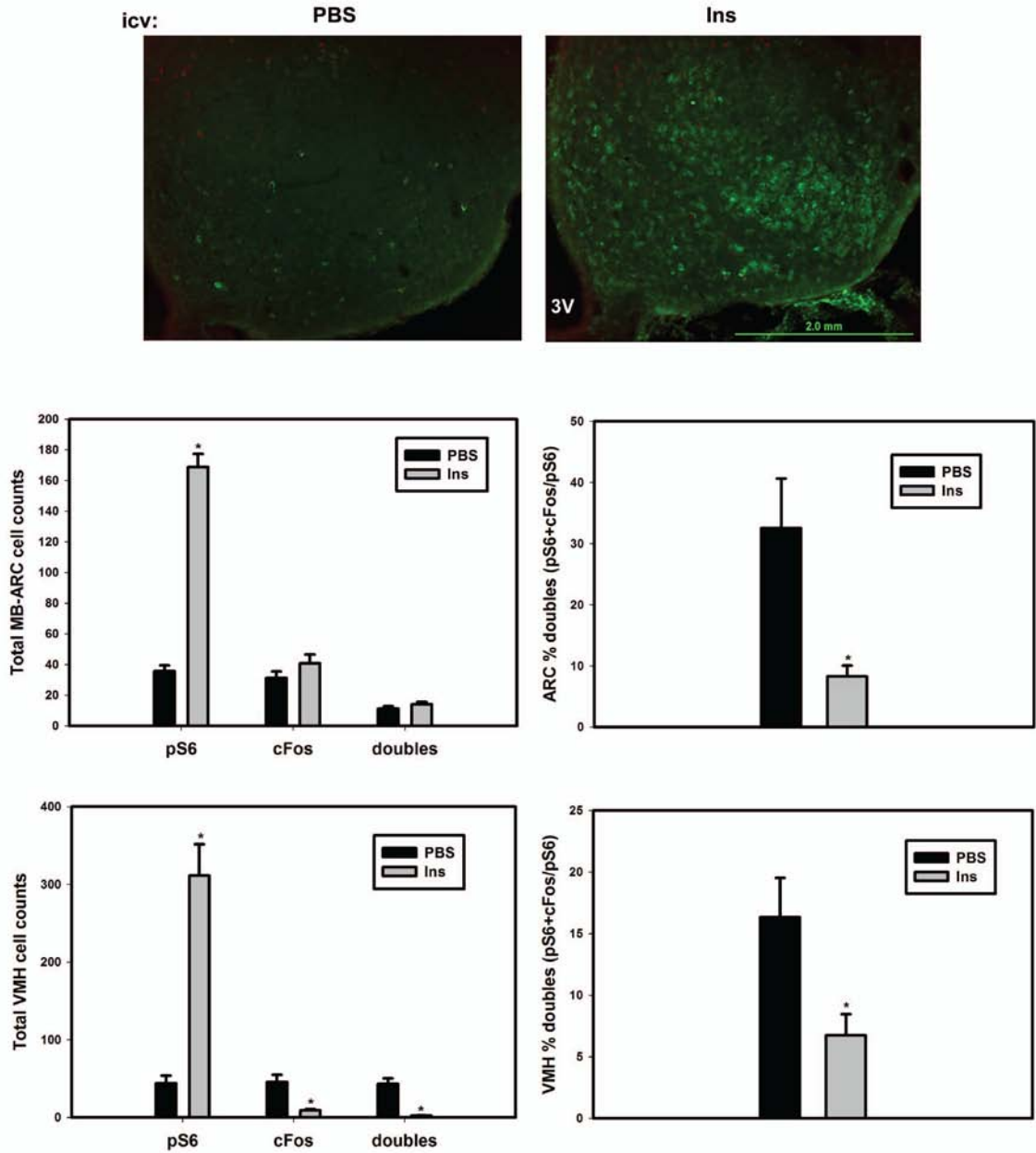
Figure 2.15: ICV insulin induces mTORC1 activation independently of c-Fos
Ad libitum-fed wild type animals with indwelling ICV catheters were treated with vehicle (Veh) or insulin (Ins) (300 mU/mL) for 1 h before the animals were killed and processing for immunofluorescent analysis of pS6-IR (green) and c-Fos-IR (red). Top panels show representative merged images. Graphs show total number of pS6-IR, c-Fos-IR, and double-labeled neurons, along with percent double-labeled neurons, are plotted as mean ± SEM (n ≥ 3 per condition; * P ≤ 0.01 by Student's t-test. 3V-third ventricle.
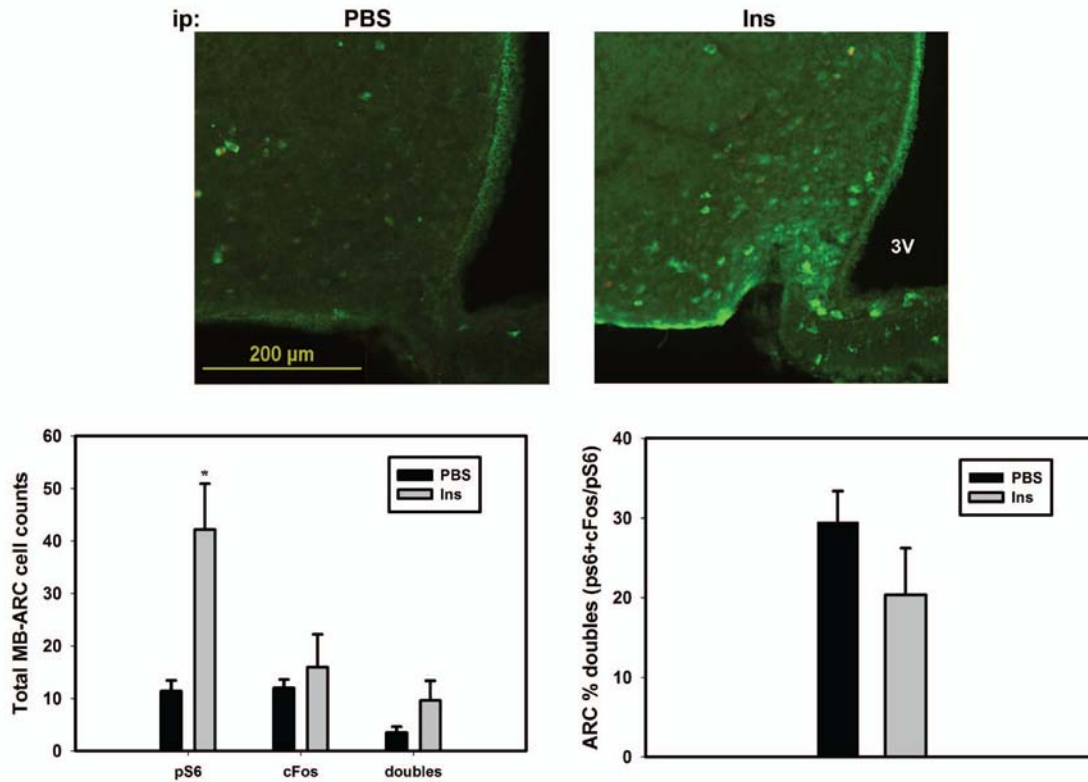
Figure 2.16: IP insulin induces mTORC1 activation independently of c-Fos in the MB-ARC

Ad libitum-fed wild type animals were treated with vehicle (Veh) or insulin (Ins) (400 mU/mL) for 1 h before the animals were killed and processing for immunofluorescent analysis of pS6-IR (green) and c-Fos-IR (red). Top panels show representative merged images. Graphs show total number of pS6-IR, c-Fos-IR, and double-labeled neurons, along with percent double-labeled neurons, are plotted as mean ± SEM (n ≥ 3 per condition; * P ≤ 0.01 by Student's t-test. 3V-third ventricle.

CHAPTER III

**LEPTIN RECEPTOR-TYR$_{1077}$→ STAT5 SIGNALING: THE MISSING LINK BETWEEN
LEPTIN AND REPRODUCTION**

SUMMARY

Adipocytes secrete leptin in proportion to triglyceride content to communicate the repletion of peripheral energy stores to the brain, thereby suppressing feeding and permitting energy expenditure. Leptin acts through its receptor (LepRb) - a cytokine receptor that is expressed in brain regions involved in energy homeostasis. Leptin binding to LepRb initiates a cascade of signaling events starting with the activation of Janus kinase (Jak2), which stimulates the tyrosine phosphorylation of three sites on LepRb. Each site mediates the recruitment of distinct downstream signaling proteins. While the signals mediated by LepRb Tyr$_{1138}$ and Tyr$_{985}$ control important aspects of energy homeostasis and LepRb signal attenuation, respectively, the signaling mechanisms by which LepRb modulates reproductive function, and aspects of glucose homeostasis have remained unclear. In order to examine the hypothesis that LepRb-Tyr$_{1077}$ (which mediates the recruitment and transcriptional activation of the signal transducer and activator of transcription factor-5 (STAT5)) might play a role in these and other leptin actions, we generated a "knock-in" mouse model containing a replacement of LepRb-Tyr$_{1077}$ (Lepr$^{f1077/f1077}$ mice). In contrast to the dramatic hyperphagia and obesity of mice mutant for LepRb-Tyr$_{1138}$, Lepr$^{f1077/f1077}$ mice demonstrate only modest increases in food intake and adiposity compared to controls. Lepr$^{f1077/f1077}$ females

display marked impairment of the reproductive axis, however, with absent or delayed estrous cycling. $Lepr^{f1077/f1077}$ mice also exhibit alterations in glucose homeostasis that are not attributable to increased adiposity, suggesting important roles for LepRb-Tyr$_{1077}$ in glycemic control, as well. Our results also suggest that signaling by LepRb-Tyr$_{1077}$ links body adiposity to the neuroendocrine reproductive axis as well as to the regulation of glucose homeostasis; impaired signaling via this pathway could potentially contribute to the processes linking altered metabolism and reproduction in disease states.

INTRODUCTION

The hormone leptin is secreted into the circulation by the adipose tissue and is transported into the brain where it acts on neuronal populations to inhibit feeding and induce energy expenditure. Leptin also modulates other physiological processes, including having a permissive action on reproduction (34;55-57), and regulating glucose homeostasis by regulating insulin production in the pancreatic β-cells (51) or by altering intra-hepatic glucose fluxes (144). Activation of the leptin receptor (LepRb) induces a phosphorylation cascade starting with the autophosphorylation of Jak2, which then phosphorylates residues on LepRb (Tyr$_{985}$, Tyr$_{1077}$ and Tyr$_{1138}$). LepRb-Tyr$_{1138}$ recruits the signal transducer and activator of transcription-3 (STAT3), and contributes a small amount to the recruitment of STAT5. The LepRb-Tyr$_{1138}$ →STAT3 pathway is crucial for the control of energy balance and some aspects of reproduction and glucose homeostasis because mice with a replacement of LepRb-Tyr$_{1138}$ (*s/s)* have preserved reproduction and improved diabetes compared to *db/db* mice. LepRb-Tyr$_{985}$ binds to

SHP-2 and SOCS3 and controls LepRb feedback inhibition. Thus, leptin action relies on the regulation of different pathways via LepRb, but some aspects of how leptin controls physiology remain unclear, including the control of reproduction and glucose homeostasis.

In addition to STAT3, STAT5a and STAT5b are highly expressed in specific populations of hypothalamic neurons in brain regions known to mediate the regulation of energy homeostasis (145;146). Leptin promotes the phosphorylation and nuclear localization of STAT5 in the ARC of rodents (83;147), demonstrating that leptin activates STAT5 *in vivo* as well as in cultured cells and suggesting that LepRb→STAT5 signaling may contribute importantly to physiologic leptin action.

Recent data from the Myers laboratory revealed a dominant role for LepRb-Tyr$_{1077}$ (which is also phosphorylated during receptor activation) and a secondary role for LepRb-Tyr$_{1138}$ in the acute phosphorylation of STAT5a and STAT5b (83). Although the two isoforms of STAT5 (STAT5a and STAT5b) represent the products of distinct genes, the two genes lie in close proximity, enabling the generation of a conditional *Stat5^{fl}* allele in which the coding regions for both STAT5 isoforms can be excised in a single event (148;149). Deletion of *Stat5^{fl}* in the hypothalamus or throughout the CNS of mice (via *RIP-cre* or *Nestin-cre*, respectively) results in late-onset obesity with hyperphagia, impaired thermal regulation in response to cold, hyperleptinemia and insulin resistance, suggesting an important role for brain STAT5 in the regulation of energy balance (150). It remains unclear whether the obesity of these mice reflects the role for STAT5 in leptin action *in vivo*, however, as many factors other than leptin also regulate STAT5. In fact, a

variety of other cytokines and growth factors, including granulocyte macrophage colony stimulating factor (GM-CSF), growth hormone, prolactin, erythropoietin, and others promote the phosphorylation and transcriptional activation of STAT5 in cells expressing the cognate receptors for these ligands (150). Indeed, the anorexic response to CNS GM-CSF treatment is attenuated in *Nestin-cre;Stat5^{fl}* animals, demonstrating that at least a portion of the phenotype of these animals likely follows from leptin-independent mechanisms (150).

Furthermore, the neuroanatomical site at which STAT5 acts to mediate energy balance is not clear: *RIP-cre* and *Nestin-cre* mediate deletion in some (but not all) LepRb-expressing neurons and mediate excision in many non-LepRb-expressing neurons. Absence of STAT5 in the CNS does not alter mRNA expression of leptin's known targets within the ARC (i.e. POMC, AgRP, and NPY) suggesting that other genes must represent the key transcriptional targets of STAT5 in the regulation of energy balance.

In order to directly assess the physiological role of the LepRb-Tyr$_{1077}$→ STAT5 signaling pathway, we generated a "knock-in" mouse model containing a replacement on LepRb-Tyr$_{1077}$ (Tyr$_{1077}$ to Phe$_{1077}$). We hypothesized that LepRb-Tyr$_{1077}$→STAT5 is an important leptin-dependent signaling pathway that contributes to reproduction and glucose homeostasis, but only modestly to energy balance.

MATERIALS AND METHODS

Reagents

Leptin was the generous gift of Amylin Pharmaceuticals (San Diego, CA), insulin was from Novo Nordisk (Novolin), and 30% dextrose was from Hospira Inc. High fat diet (45% Kcal fat) chow was from Research Diets, NJ. Rabbit anti-phospho-STAT3 (Y709) was purchased from Cell Signaling (Boston, MA), and donkey serum was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Donkey anti-rabbit Alexa-488 conjugated antibodies were purchased from Molecular Probes, Inc. (Eugene, OR). All other immunohistochemical supplies were purchased from Sigma-Aldrich (St. Louis, MO).

Animals

Unless otherwise stated, animals were bred in our colony in the Unit for Laboratory Animal Medicine (ULAM) at the University of Michigan and handled in accordance with The University Committee on Use and Care of Animals (UCUCA). *Tg(EIIa-cre)C5379Lmgd/J* (C57Bl6 nII) mice were purchased from Jackson Laboratories. This transgenic line carries a *cre* transgene under the control of the EIIa (adenovirus) promoter that targets expression of Cre recombinase to the mouse embryo. Cre-mediated recombination in these animals is widely spread, including in the germ cells that transmit the genetic alteration to the progeny (151).

Generation, backcrossing and deletion of "neo" cassette in LepRb$^{f1077/f1077}$ mice

Targeted 129Sv embryonic stem (ES) cell clones were used to produce chimeric animals, which were crossed with C57Bl/6J mice to generate heterozygous *lepr$^{f1077/+}$* (*f/+*) "knock-in" F1 mice expressing LepRb^{F1077} from the genomic context of endogenous LepRb. Heterozygous *f/+* mice were crossed with *B6.FVB-Tg(EIIa-cre)C5379Lmgd/J* mice

to remove the "floxed" neomycin (*neo*) cassette found in the original targeting vector (Figure 3.1) and used for screening purposes during the targeting of *lepr*^{F1077} in ES cells. Successful deletion of the *neo* cassette was tested by Southern blot analysis (of tail DNA samples) with DIG-labeled *neo* probe to confirm deletion of *neo*. These animals were bred to C57Bl/6J mice to eliminate the *cre* transgene. Interbreeding of the resulting n2 C57Bl/6J heterozygous animals (*Lepr*^{Phe1077}/+) yielded homozygous (*f/f*) and wild-type (*+/+*) mice for study.

Phenotyping studies

Mice were single housed with *ad libitum* access to food and water, and male and female mice were weighed weekly beginning at 4-weeks to 12-weeks of age. Food consumption was measured weekly and was determined by weighing the amount of food left in the cage and subtracted from the amount from the previous week. Blood was collected bi-weekly in heparanized capillary tubes for glucose measurements using a glucometer and for serum determination of insulin and leptin (ELISA, Crystal Chem, Inc.). Body composition was assessed in conscious mice by an NMR-based Minispec LF90II (Bruker Optics) analyzer (Figure 3.2).

For analysis of reproductive function, female mice were checked daily from the time of weaning (at 28 days) for vaginal opening and thereafter for vaginal oestrogenization by cellular histology. Mice were checked daily until a second estrous cycle was achieved, or for up to 61 days.

Intraperitoneal Glucose (ipGTT) and Insulin Tolerance Tests (ipITT)

For ipGTT, 13-week old mice were fasted overnight (16 hours), tails were nicked and blood glucose was measured using a glucometer. Time was recorded at time 0 and then mice received (IP) one unit (0.001cc) (per gram of body weight) of a 30% dextrose solution per gram of body weight. Blood glucose was sampled at 15, 30, 60 and 120 minutes using the glucometer.

For ipITT, 14-week old mice were fasted for 4-5 hours, tails were nicked and blood glucose measured at time 0. Insulin was diluted to 0.0025U/mL and each mouse received one unit per gram of body weight from the final dilution into their peritoneal cavity. Blood glucose was sampled at 15, 30, 45, and 60 minutes using the glucometer. Insulin clearance was calculated by taking the average of the percent of initial glucose value (at time 0).

Microdisections and tissue collection

15-week-old male and female mice received an overdose of pentobarbital (150 mg/kg, IP) and snout-anus length was measured with a micrometer. Terminal blood (from trunk) was collected for future analysis of hormones in the serum. Gross dissections were performed and tissues including hypothalamus, brown adipose tissue (BAT), liver, white adipose tissue (WAT), gonads, pancreas and muscle were collected. These tissue samples were weighed and either snap-frozen in dry ice or fixed in 10% formalin for further analysis of protein lysates and histology, respectively. In male mice, specific brain regions were collected by microdissection.

Perfusion and Immunohistochemistry (IHC)

For immunohistochemical analysis, 10 to 12-week old mice remained with food in the cage, and were treated with leptin (1mg/kg of body weight) or PBS for 2 hours and sacrificed between 9 a.m. and 12 p.m. Perfusion and IHC procedures were performed essentially as described previously (90). In brief, mice were deeply anesthetized with an overdose of pentobarbital (150 mg/kg, IP) and transcardially perfused with sterile PBS followed by 4% paraformaldehyde. Brains were removed, post-fixed and cryoprotected before sectioning into 30μm coronal slices, which were collected into 4 representative series and stored at -20°C until further use. For immunofluorescence, sections were pretreated in ice-cold methanol, 0.6% glycine and 0.03% SDS, and then blocked with donkey serum and incubated in the primary antibody (rabbit-anti-pSTAT3 [1:500]). Detection of primary antibody was done by immunofluorescence with donkey anti-rabbit-Alexa 488 [1:200].

Statistical Analysis

Mean ± SEM and differences were analyzed by unpaired Student's t-test to determine significant differences between groups (*f/f* versus *+/+*). The difference between curves was analyzed by repeated measures ANOVA. Differences were accepted for p values ≤ 0.05, with an n value ≥ 10 for all.

RESULTS

Normal leptin-stimulated STAT3 in Lepr$^{f1077/f1077}$ mice

To facilitate the study of LepRb-Tyr$_{1077}$ signaling to leptin action *in vivo*, we generated "knock-in" mice in which the Lepr was replaced by Lepr^{F1077}, where Phe

replaces Tyr_{1077} to abrogate phosphorylation of the site and block the majority of STAT5 recruitment, as observed in cultured cells (83). Using this approach, levels and patterns of pSTAT3-immunoreactivity (IR) expression for $Lepr^{F1077}$ are predicted to mimic those of wild-type mice (100;101). To confirm that this was the case in *f/f* animals, we treated *f/f* and *+/+* mice with leptin or PBS, and examined STAT3 phosphorylation (pSTAT3) in the basomedial hypothalamus by immunofluorescence. As expected, both *f/f* and *+/+* mice demonstrated similar patterns of leptin-activated pSTAT3-IR, consistent with the expression of functional $LepRb^{F1077}$ in *f/f* mice (Figure 3.3).

Lepr^{f1077/f1077} mice demonstrate modestly increased energy balance

We initially examined the regulation of body weight in *+/+* and *f/f* mice (of each sex) fed a normal chow (NC) or high fat (HF) diet. While body weight in *f/f* males was similar to controls, analysis by repeated measures ANOVA revealed modestly increased body weight in female *f/f* animals (Figure 3.4). Similarly, modest increases in body weight were observed in male and female animals subjected to a HF diet from 4-12 weeks of age (Figure 3.5).

On NC diet, food intake was detectably increased only in male *f/f* mice at the beginning of the study, but these small differences disappeared over the course of the study (Figure 3.4). In HF diet-fed animals, however, food intake was detectably higher in *f/f* animals of both sexes compared to controls (Figure 3.5). Thus, *f/f* animals on a HF diet display modestly increased in body weight and food intake, which suggests positive energy balance in *f/f* mice, although this effect is quite modest relative to *db/db* mice lacking LepRb or *s/s* mice lacking LepRb-$Tyr_{1138}\rightarrow$STAT3 signaling.

Consistent with this notion, body adiposity (% fat) and fat pad weight were significantly increased in both male and female *f/f* mice on NC diet, while circulating leptin levels were only significantly higher in *f/f* females. HF diet increased adiposity and fat pad weight in *f/f* mice of both sexes and both genotypes (although not to significant levels), while leptin levels did not change (Figure 3.6, Tables 3.1 and 3.2). While HF diet-fed *f/f* animals of both sexes trended to have higher adiposity and markers of adiposity, these differences were not significant; this may reflect the modest effect of the Leprb-Tyr$_{1077}$ motif on adiposity relative to the effect of HF diet. Furthermore, lean mass was not different between *f/f* and *+/+* mice of either sex or genotype on either diet (Figure 3.6).

Improved glucose clearance in Lepr$^{f1077/f1077}$ mice

We found that glucose and insulin levels were similar in wild-type and mutant mice of either sex (Table 3.1). Leptin contributes to glucose homeostasis in multiple ways: by modulating adiposity (and thus adiposity-dependent insulin resistance) and independently of body fat via an undefined pathway from the CNS to control hepatic glucose production, and potentially by the modulation of pancreatic insulin secretion. As we previously defined a Tyr$_{985}$/Tyr$_{1138}$-independent mechanism for the adiposity-independent regulation of glucose homeostasis by leptin, we carefully examined parameters of glycemic control in *f/f* mice.

Interestingly, while glucose clearance during an ipGTT in male *f/f* mice on NC diet was similar to controls, *f/f* females on NC diet displayed decreased glucose excursions (increased glucose clearance) following glucose challenge compared to controls.

Indeed, while ipGTT in HF-fed male *f/f* mice did not differ from controls, glucose excursions were dramatically lower in female HF-fed *f/f* animals compared to controls, especially at early time points (Figure 3.7). This improved glucose tolerance in female *f/f* mice is not likely to be attributable to altered insulin sensitivity as insulin tolerance tests (ipITT) in *f/f* females were not different from controls for either diet (Figure 3.8).

The improved glucose tolerance in female *f/f* animals independent of adiposity (increased adiposity would predict worsened glucose tolerance) suggests possible alterations in insulin secretion or hepatic glucose production in *f/f* females. Also, we observed a significant increase in pancreatic weight in *f/f* females on both diets (Table 3.2), which suggests a possible increase in β-cell mass and thus an increase in insulin secretion in *f/f* females. These data suggest an important role for LepRb-Tyr$_{1077}$ in this effect.

Impaired reproductive function in Lepr$^{f1077/f1077}$ females

Since important aspects of leptin action on reproductive function are mediated independently of Tyr$_{985}$ and Tyr$_{1138}$, we also examined the reproductive axis in female *f/f* mice since the energetic cost and thus leptin-dependence of reproduction is higher in females. Estrous cycling was monitored daily by studying vaginal cellular histology in NC-fed females from 28 days to 61 days of age. There was no significant difference on the time of vaginal opening (average of 31 days) or the time to the first estrous cycle (44 days) between *f/f* and *+/+* females (Figure 3.9). Interestingly, however, while the majority of *+/+* mice had a second estrous cycle by 61 days of age (84%), only 42% of the *f/f* mice did. Furthermore, the time between the first and the second estrous cycle

(E1 and E2) was significantly delayed in the *f/f* mice that underwent a second estrous (5 ± 0.4 days in *+/+* versus 9 ± 2 days in *f/f*) (Figure 3.10). Also, a trend to heavier ovaries was detected on *f/f* females on NC diet, and ovarian weight was significantly increased in female *f/f* mice on a HF diet compared to *+/+* females (Table 3.2), which suggests that the observed reproductive defect in *f/f* females may also cause localized defects in the ovaries. Thus, LepRb-Tyr$_{1077}$ mediates an important component of leptin action on the reproductive axis.

DISCUSSION

Leptin-deficient *ob/ob* mice and LepRb-deficient *db/db* mice have provided great insight into the importance of leptin and LepRb signaling. In order to understand the mechanisms by which LepRb mediates the physiological actions of leptin, we have previously examined the phenotype of mice mutant for LepRb-Tyr$_{985}$ and LepRb-Tyr$_{1138}$. Mice lacking LepRb-Tyr$_{985}$ (*l/l*) are leaner and more sensitive to leptin than littermate controls, but otherwise normal. In contrast, mice lacking LepRb-Tyr$_{1138}$ (*s/s*) have increased body weight, adiposity and food intake- approaching but slightly less dramatic than in *db/db* mice. Thus, while Tyr$_{1138}$ is required for most leptin action on energy balance, Tyr$_{1138}$-independent pathways presumably mediate at least some effects. Furthermore, while *db/db* animals are completely infertile, *s/s* females ovulate and bear normal-sized litters (although they fail to lactate following delivery); *s/s* animals also display improved glucose homeostasis in relation to *db/db* animals. Thus, LepRb signals other than those emanating from Tyr$_{985}$ and Tyr$_{1138}$ must contribute to the control of

glucose homeostasis and reproduction, as well as to a minor component of energy balance.

LepRb-Tyr$_{1077}$ plays a minor role in the regulation of energy balance

In this study, we explored the function of LepRb-Tyr$_{1077}$ by using a novel mouse model with defective LepRb-Tyr$_{1077}$ signaling (Lepr$^{f1077/f1077}$); based upon phenotypes of mice with defective LepRb signaling, such as those lacking LepRb-Tyr$_{985}$ and LepRb-Tyr$_{1138}$, we postulated minor defects in energy balance and predicted changes in reproduction and glucose homeostasis in the *f/f* mice. Indeed, *f/f* animals exhibited increased adiposity on NC diet, as well as displayed increased body weight and food intake on a HF diet. The increased adiposity of *f/f* animals on NC diet was modest compared to that of age-matched *db/db* and *s/s* mice, however, and minimal compared to the increase in adiposity observed with HF-feeding.

Impaired reproduction and enhanced glucose tolerance in female Lepr$^{f1077/f1077}$ mice

Female *f/f* mice entered puberty normally, as revealed by normal vaginal opening and first vaginal estrous, but exhibited absent or delayed estrous subsequently. Thus, the lack of LepRb-Tyr$_{1077}$ signaling impairs the normal post-pubertal cycling and presumably ovulation in females. These results reveal the importance of LepRb-Tyr$_{1077}$ to the permissive action of leptin on the reproductive axis. Since leptin acts in the CNS to promote neuroendocrine reproductive function, this presumably reflects the importance of this signal within the CNS, but this will require further analysis.

Although no differences were observed in glucose and insulin levels in *ad libitum* fed animals, female *f/f* mice displayed a pronounced increase in glucose clearance (in

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ipGTT); this effect was more noticeable in mice fed a HF diet. Interestingly, insulin tolerance during an ipITT was normal in female *f/f* animals, suggesting that increased glucose tolerance in *f/f* female mice is not due to increased insulin sensitivity. This suggests that *f/f* females may display increased insulin secretion in the face of an acute glucose challenge, or may have other alterations in glucose production. Given the increased weight of pancreata from *f/f* mice, we postulate that insulin secretion may be increased. Indeed, a number of studies have suggested that leptin attenuates glucose-stimulated insulin secretion and it is possible that Tyr_{1077} contributes to this effect. We plan to perform glucose-stimulated-insulin-secretion (GSIS) as well as insulin clamp studies to address this issue. We will also examine pancreatic histology to detect any possible differences in islet size or morphology. Overall, these results suggest that LepRb-Tyr_{1077} is important for the regulation of glucose homeostasis by leptin.

Downstream signaling by LepRb-Tyr_{1077}

Leptin treatment activates STAT5 in cultured cells and *in vivo*, and LepRb-Tyr_{1077} mediates the majority of leptin-stimulated STAT5 activation in cultured cells. While technical limitations have prevented us from examining leptin-stimulated STAT5 activation in the hypothalamus of *f/f* mice, it is reasonable to believe that Tyr_{1077} functions similarly to recruit STAT5 *in vivo*. While the effect of mutating Tyr_{1077} on leptin action in *f/f* mice may be secondary to altered STAT5 signaling, a number of observations suggest caution regarding this conclusion. Deletion of STAT5 in the CNS of *Nestin-cre;Stat5^{fl}* mice results in modestly increased body weight and adiposity at young ages (150), similar to *f/f* mice, but without apparent defects in reproduction or

enhancements in glucose tolerance as observed in *f/f* mice. Unfortunately, however, reproduction and glucose homeostasis were not carefully studied in the *Nestin-cre;Stat5^{fl}* mice and it is not possible to know whether alterations in these pathways exist. The defect in energy balance (obesity) in older *Nestin-cre;Stat5^{fl}* mice is much more pronounced than in our *f/f* mice, but it is not clear whether this difference reflects only differences in age, or also blockade of STAT5 signaling by other factors (such as GM-CSF) and the more widespread deletion of STAT5 in these mice than in just LepRb neurons.

Also, some evidence suggests that leptin induces phosphorylation of some members of the insulin receptor substrate (IRS) protein family, leading to phosphorylation of PI3K (152). Among other IRS proteins, IRS4 is highly expressed in many areas of the hypothalamus (153;154). Mice lacking IRS4 display impaired reproduction, and slightly lower blood glucose concentration (both fed and fasted) while plasma insulin concentrations remain normal, and only modest defects in energy balance are found (155). While some of the IRS4-null phenotype may reflect altered pituitary function, one study suggests that IRS4 may interact with LepRb via Tyr$_{1077}$ (156). Although this study has many limitations, we must at least consider the possibility that LepRb-Tyr$_{1077}$ may act in part via this pathway.

Neurophysiological basis and neuroanatomical localization of Tyr$_{1077}$ action

The absence of STAT5 in the CNS (in *Nestin-cre;Stat5^{fl}* mice) did not alter mRNA expression of some of leptin's known targets in the ARC (i.e. POMC, AgRP, NPY) (150) suggesting that other genes (and possibly, other brain areas) must represent the key

transcriptional targets of STAT5 in the regulation of energy balance. In fact, STAT5 colocalizes with orexin (OX) neurons (which are cells devoid of LepRb) in the LHA in wild-type mice, and *Nestin-cre;Stat5^{fl}* mice exhibited mild dysregulation of OX in the LHA, suggesting that STAT5 may play a role in the regulation of these neurons. In order to understand the neuronal populations potentially affected in *f/f* mice, it will be important to assess mRNA expression of these (POMC, AgRP, NPY, OX) and other neuropeptides, and the regulation of neuronal firing in the hypothalamus of *f/f* mice.

FUTURE DIRECTIONS

Our present results with the *f/f* mice reveal that LepRb-Tyr$_{1077}$ contributes little to the regulation of body weight, but is important for the control of some aspects of glucose homeostasis and reproduction by leptin. Many questions remained to be answered, however.

So far, we have no reliable antibody to detect STAT5 phosphorylation in brain sections; therefore, we are currently testing if regulation of STAT5 is different in *f/f* mice by looking at STAT5 nuclear localization by immunohistochemistry (together with DAPI staining). This would be crucial as we seek to determine if STAT5 activation is decreased in *f/f* mice, as we expect. We also plan to study neuronal activation in these mutant mice by examining cFos- and pS6-IR, as LepRb-Tyr$_{1077}$ might be important for the activation of some neurons (and possibly the mTORC1 pathway) in the basomedial hypothalamus. Moreover, we will use IHC techniques to study other mechanisms via

pathways such as IRS/PI3K and determine if these signaling pathways are regulated differently in *f/f* mice.

Given that we observed improved glucose tolerance in *f/f* females, it will be important to determine whether this effect is due to increased insulin production and secretion from the β-cells, or if there are other signals from the hypothalamus that are controlling glucose transport to the liver independently of insulin. We will thus subject 13-week old *f/f* and *+/+* female mice fed a HF diet to glucose-stimulated-insulin-secretion (GSIS) studies, and insulin clamps. GSIS analysis will help us determine if glucose clearance is due to an increased acute insulin secretion and insulin clamps will provide information on glucose production and uptake in the periphery.

Female *f/f* mice had absent or delayed estrous cycling and to study this reproductive defect even further, we are currently monitoring estrous cycling in females on a HF diet to examine if an increase in fat affects cycling compared to a NC diet. We also observed increased ovarian weight in *f/f* females compared to *+/+* mice and we plan to study the histology of these ovaries in order to determine any differences in follicle morphology, the presence of cysts, increased adipose tissue and/or increased corpus luteum size. Also, we want to assess if stimulation of estrous could be achieved in young *f/f* mice with gonadotropin treatment, which would help dissect if the phenotype observed is due to a direct impact at the ovarian level (independent of the hypothalamic-pituitary-adrenal (HPA) axis). Furthermore, we will study the potential defects in the HPA axis of *f/f* mice by examining LH secretion in ovariectomized mice and determine leptin-stimulated LH secretion during the fasted and fed states.

While *db/db* mice exhibit essentially complete blockade of the reproductive axis, both *s/s* and *f/f* animals display partial reproductive function. What then are the contributions of Tyr_{1138} and Tyr_{1077}? One important variable in the *s/s* mice is their other endocrine alterations, including hypercorticosteronemia and hypothyroidism-both which are likely to contribute to decreased fertility. It is also possible that the disruption of a portion of STAT5 signaling by Tyr_{1138} in these *s/s* animals blunts reproductive function.

The extension of this study will be accomplished by determining the phenotype of mice containing a double mutation on LepRb (Lepr$^{f1077/s1138}$ mice) which would help elucidate and clarify many aspects of LepRb-dependent signaling that still remain poorly defined, specifically via LepRb→STAT5 signaling. Also, a mouse model lacking STAT5 in all LepRb-expressing neurons is currently being developed and studied in the Myers laboratory (LepRb-cre;Stat5^{fl} mice). This novel mouse model will help examine the specific role of STAT5 to the overall leptin action *in vivo*, including in the regulation of reproduction and glucose homeostasis.

FIGURES: CHAPTER III

Figure 3.1: Targeting strategy and deletion of "neo" cassette in LepRb$^{f1077/f1077}$ mice

Diagram of gene-targeting strategy to replace Lepr with Lepr^{F1077}, where Phe replaces Tyr$_{1077}$ to abrogate phosphorylation of the site. In summary, targeted 129Sv embryonic stem (ES) cell clones were used to produce chimeric animals, which were crossed with C57Bl/6J mice to generate heterozygous *lepr$^{f1077/+}$* (*f/+*) "knock-in" F1 mice expressing LepRb^{F1077} from the genomic context of endogenous LepRb. Heterozygous *f/+* mice were crossed with *B6.FVB-Tg(EIIa-cre)C5379Lmgd/J* mice to remove the "floxed" neomycin (*neo*) cassette found in the original targeting vector and used for screening purposes during the targeting of *lepr^{F1077}* in ES cells.
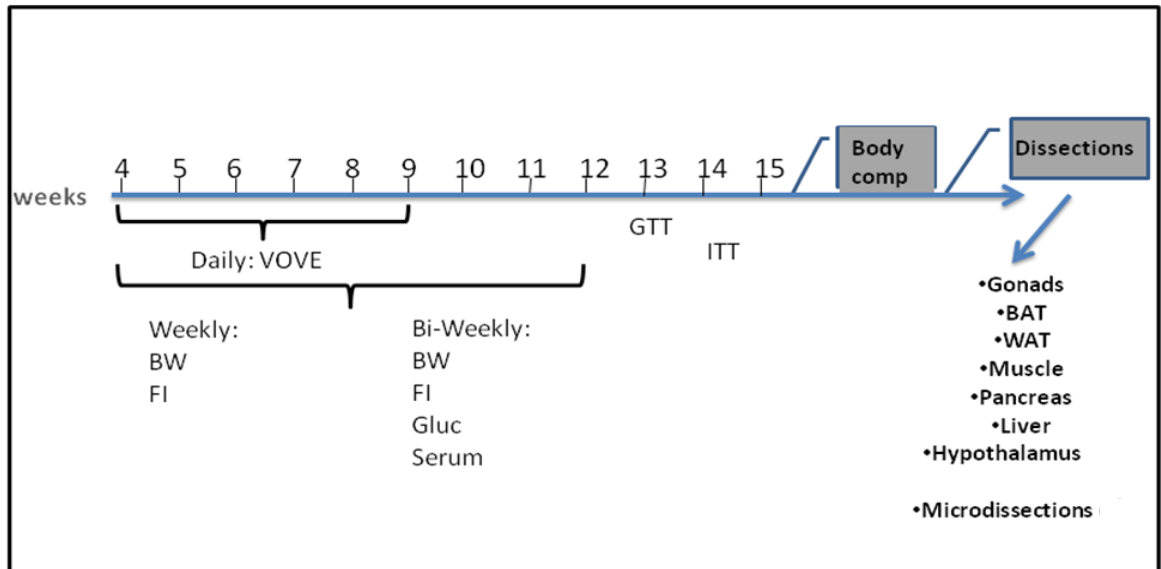
Figure 3.2: Phenotypical analysis of LepRb$^{f1077/f1077}$ mice
Mice were single housed with *ad libitum* access to food and water. For measurement of the phenotypic parameters, male and female mice were weighed weekly beginning at 4-weeks to 12-weeks of age. Food consumption was measured weekly and blood was collected bi-weekly for glucose measurements and for serum determination of insulin and leptin. For analysis of reproductive function, female mice were checked daily from the time of weaning (at 28 days) for vaginal opening and thereafter for vaginal oestrogenization by cellular histology. After 12-weeks, mice were subjected to glucose tolerance tests (GTT) and insulin tolerance tests (ITT). After recovery, body composition was assessed in conscious mice by an NMR-based Minispec analyzer. Finally, mice were dissected, tissues were collected and harvested for future analyses.
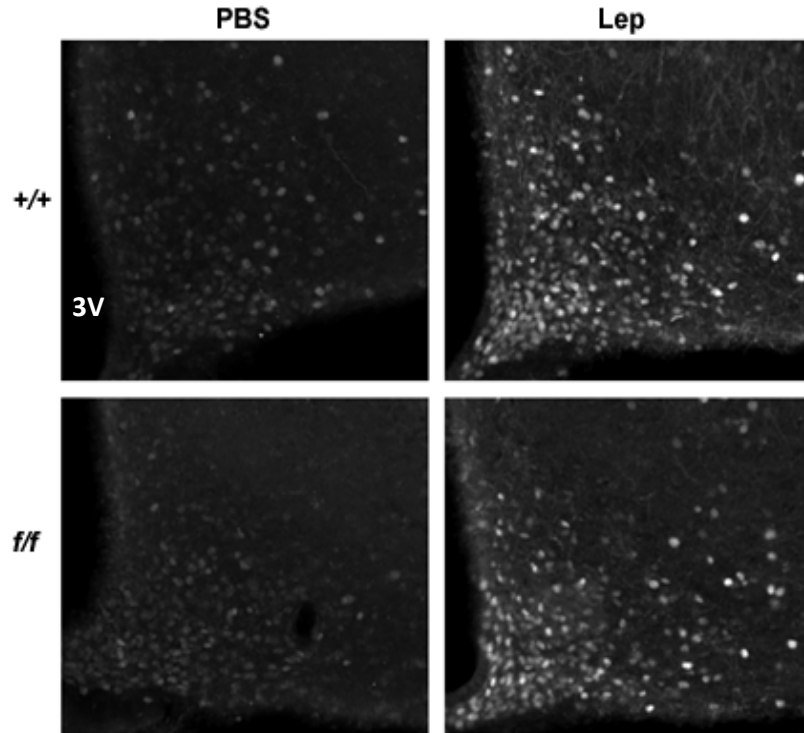
Figure 3.3: Normal leptin-stimulated pSTAT3-IR in LepRb$^{f1077/f1077}$ mice

To confirm that the levels and patterns of pSTAT3-IR expression for *Lepr^{F1077}* mimic those of wild-type mice, we treated *f/f* and *+/+* mice with leptin (5mg/kg) or PBS for 2 h, and examined STAT3 phosphorylation (pSTAT3) in the basomedial hypothalamus by immunofluorescence. As expected, both *f/f* and *+/+* mice demonstrated similar patterns of leptin-activated pSTAT3-IR, consistent with the expression of functional LepRb^{F1077} in *f/f* mice. 3V-third ventricle.
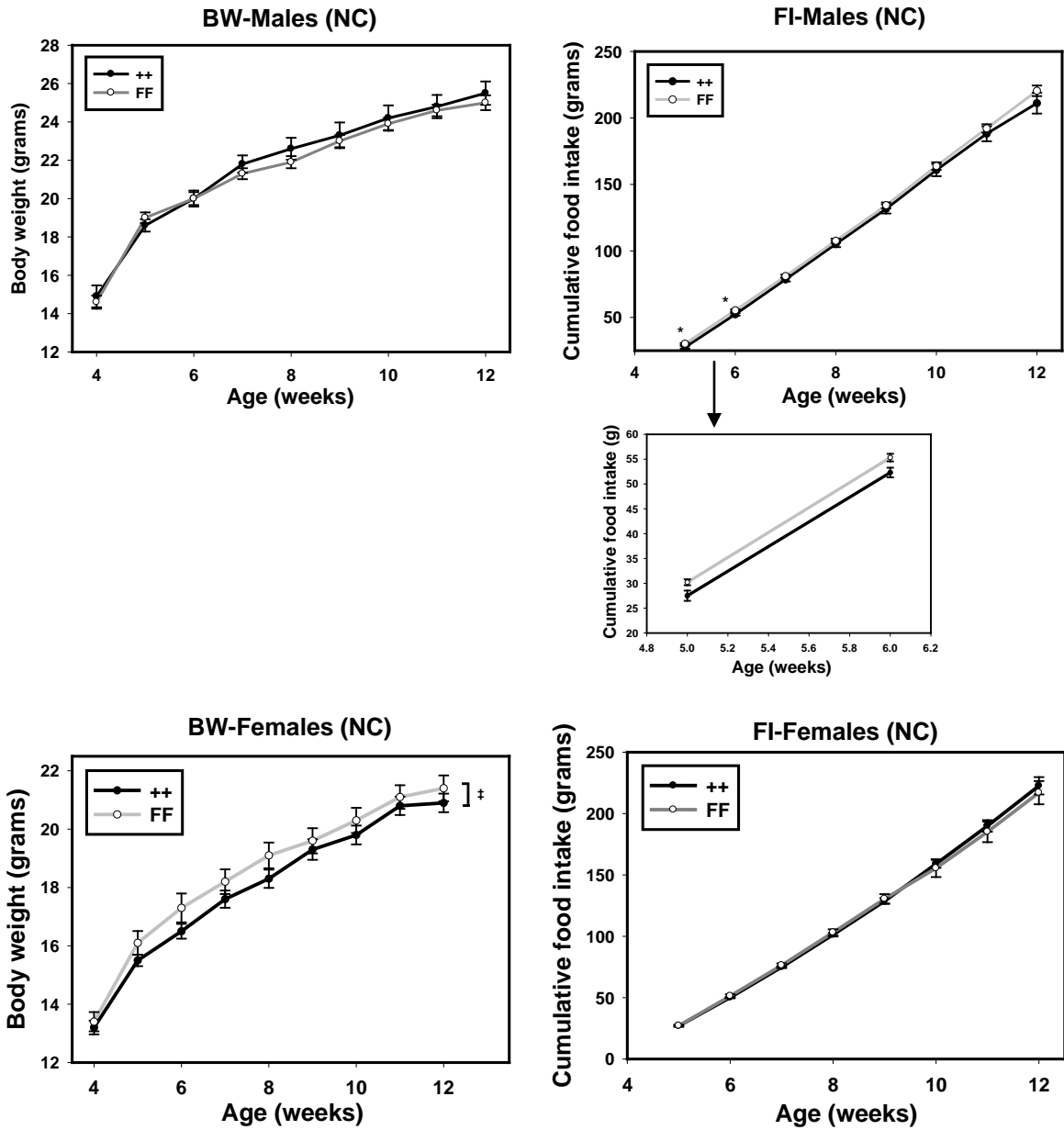
Figure 3.4: Modest increase in body weight and food intake in LepRb$^{f1077/f1077}$ mice (NC diet)
Mice were fed a normal chow (NC) throughout the study and body weight and food intake measured from 4-12 weeks of age. Body weight was similar to controls in males and food intake was only significantly different during the first two weeks of the study (smaller graph). Body weight was modestly increased in female *f/f* mice, while food intake remained unchanged. Significant differences were analyzed by Student's T-test at each time point (* p≤ 0.05) and differences between curves by repeated measures ANOVA (‡ p= 0.001), n≥ 10 per gender, per genotype.
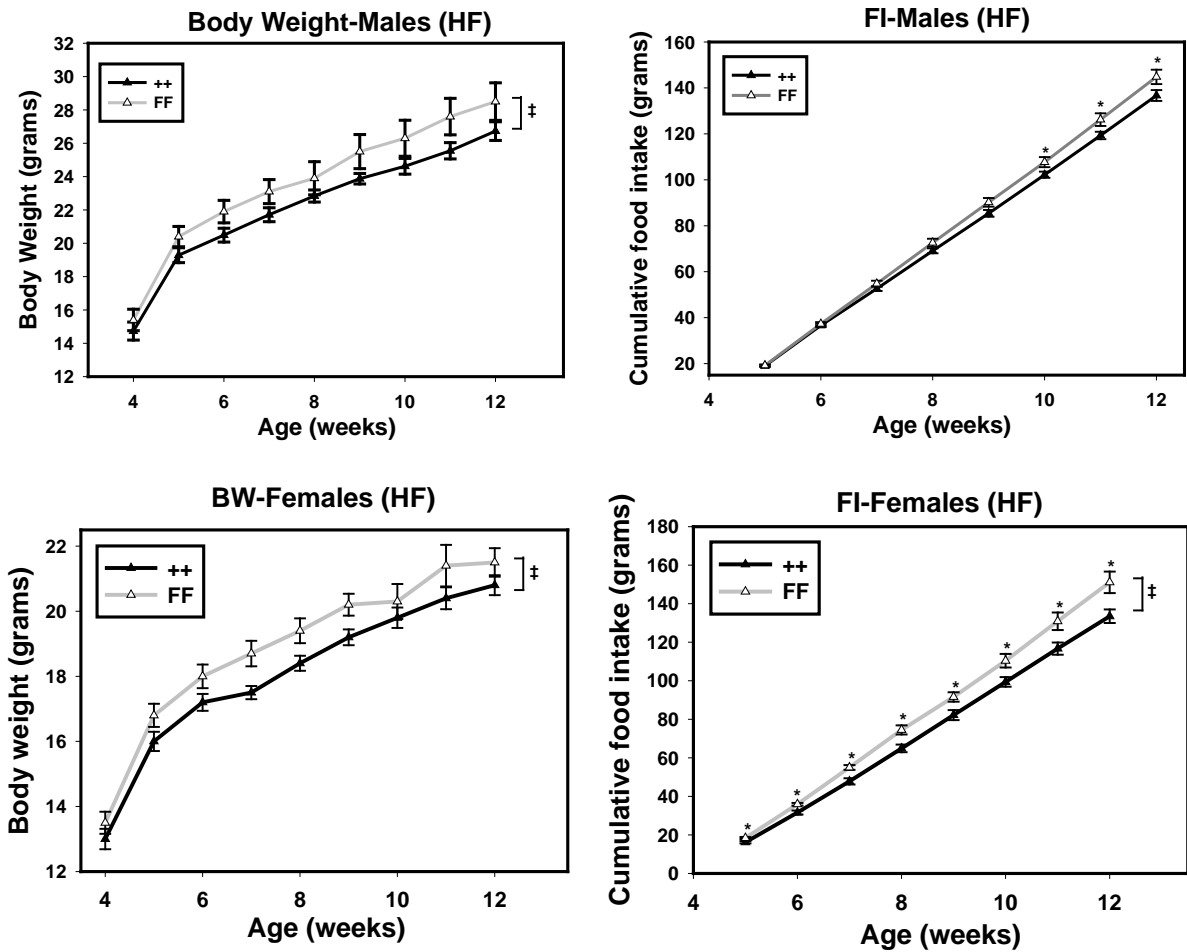
Figure 3.5: Modest increase in body weight and food intake in LepRb$^{f1077/f1077}$ mice (HF diet)
Mice were fed a high fat (HF) diet (45 % fat) throughout the study and body weight and food intake measured from 4-12 weeks of age. We detected a modest increase in body weight in male and female *f/f* mice and food intake was detectably higher in *f/f* animals of both sexes compared to *+/+* controls. Significant differences were analyzed by Student's T-test at each time point (* p≤ 0.05) or by repeated measures ANOVA (‡ p= 0.001), n≥ 10 per gender, per genotype.
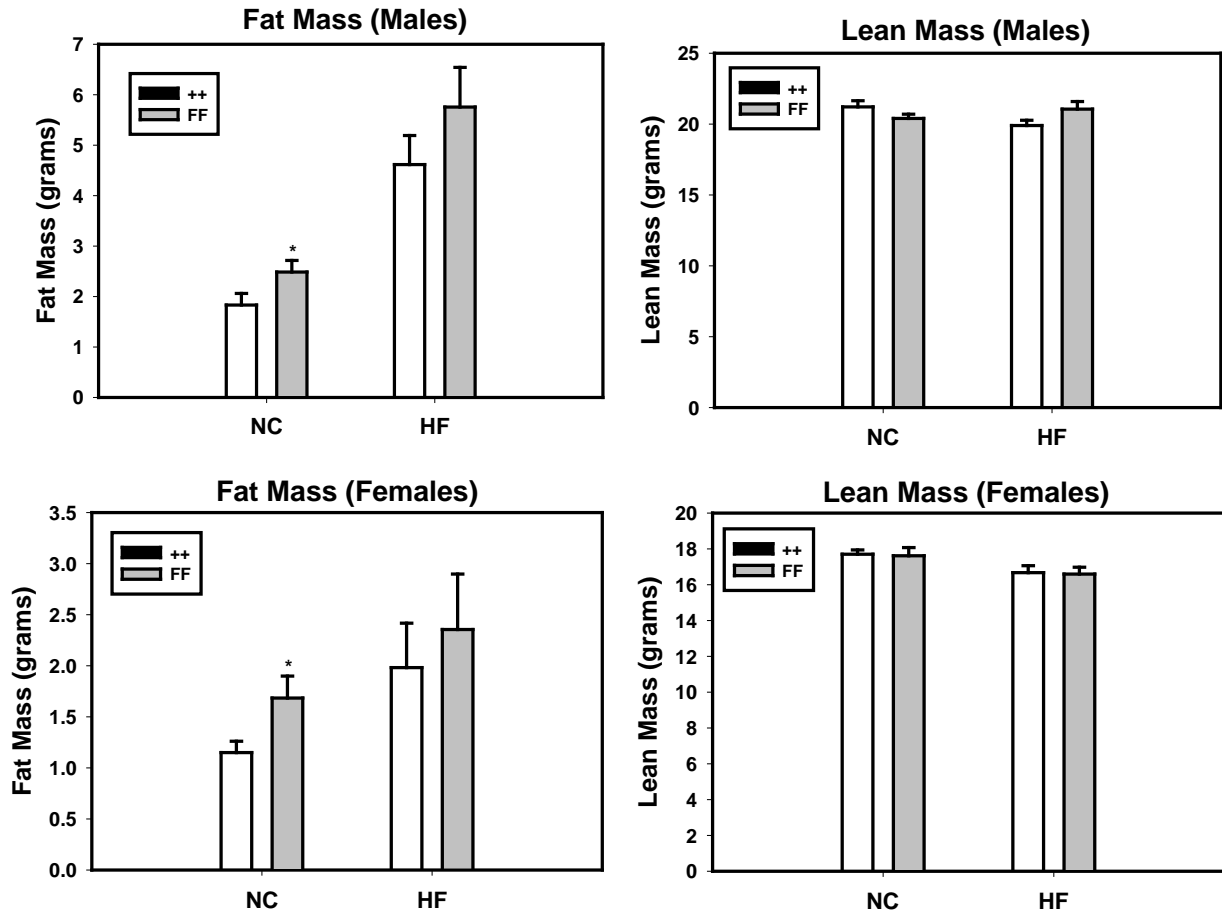
Figure 3.6: LepR*b*^{*f1077/f1077*} mice present mild increase in body adiposity and no changes in lean mass

Mutant (*f/f*) and wild-type (*+/+*) mice were fed a normal chow (NC) or a high fat (HF) diet from 4 to 12-weeks of age. Body composition of 15-week old mice was determined and revealed that fat mass was significantly increased in *f/f* male and female mice on a NC diet. However, although mice on HF diet trended to have higher adiposity, these differences were not significant. Lean mass remained normal in mice for all the groups. Significant differences were analyzed by Student's T-test at each time point (* p ≤ 0.05), n≥ 10 per gender, per genotype.
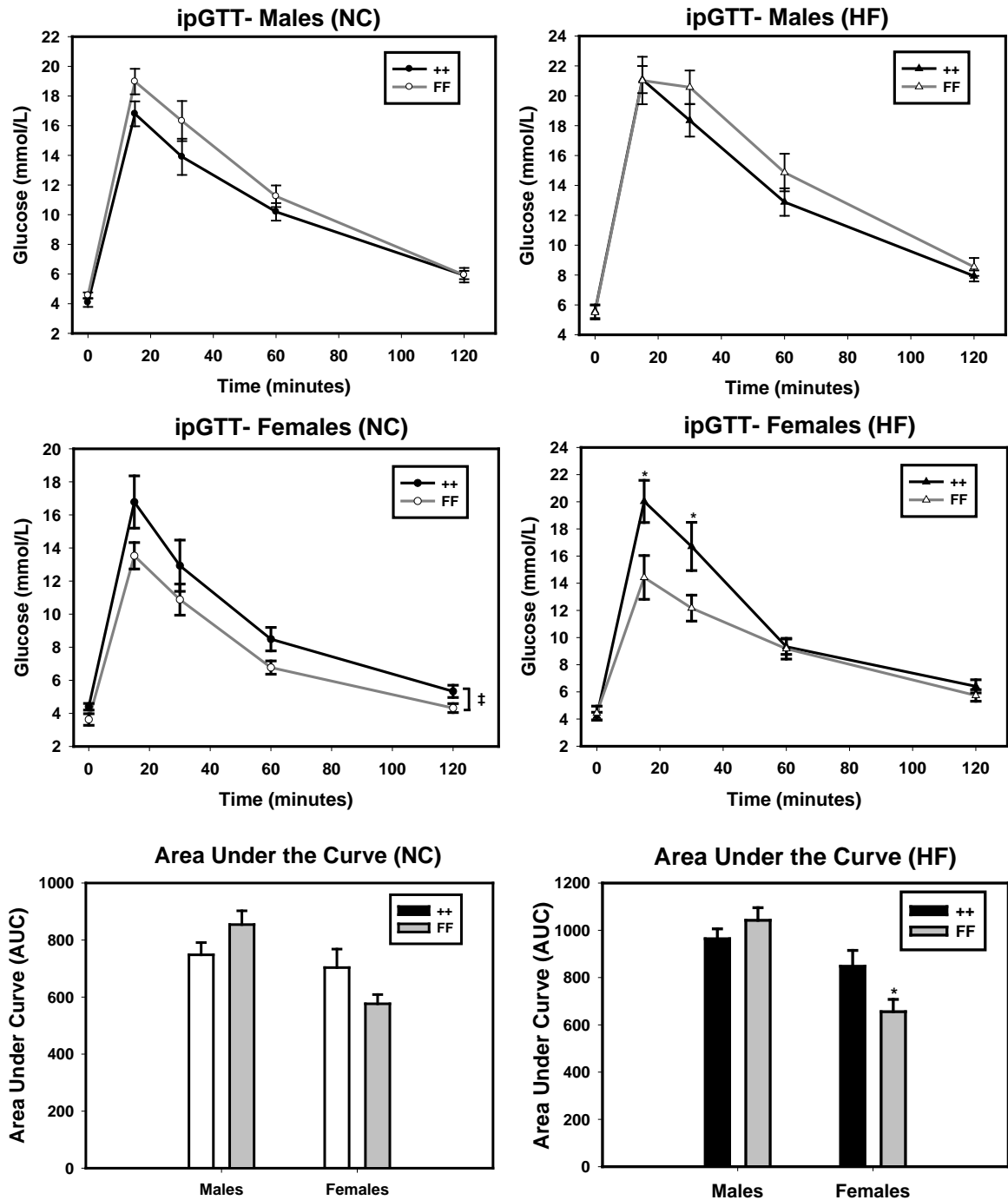
Figure 3.7: LepRb$^{f1077/f1077}$ **female mice have improved glucose clearance**

Mice were subjected to an intraperitoneal glucose tolerance test (ipGTT) at 13-weeks of age. While glucose clearance in male mice on NC diet was similar to controls (*+/+*), female *f/f* mice on ND diet had increased glucose clearance. These glucose excursions were dramatically decreased in female *f/f* mice fed a HF-diet at early time points (15 and 30 minutes following a glucose challenge) as demonstrated in the ipGTT curve and determination of the area under the curve (AUC). Significant differences were analyzed by Student's T-test at each time point (* p≤ 0.05)   or by repeated measures ANOVA (‡ p= 0.01), n≥ 10 per gender, per genotype.
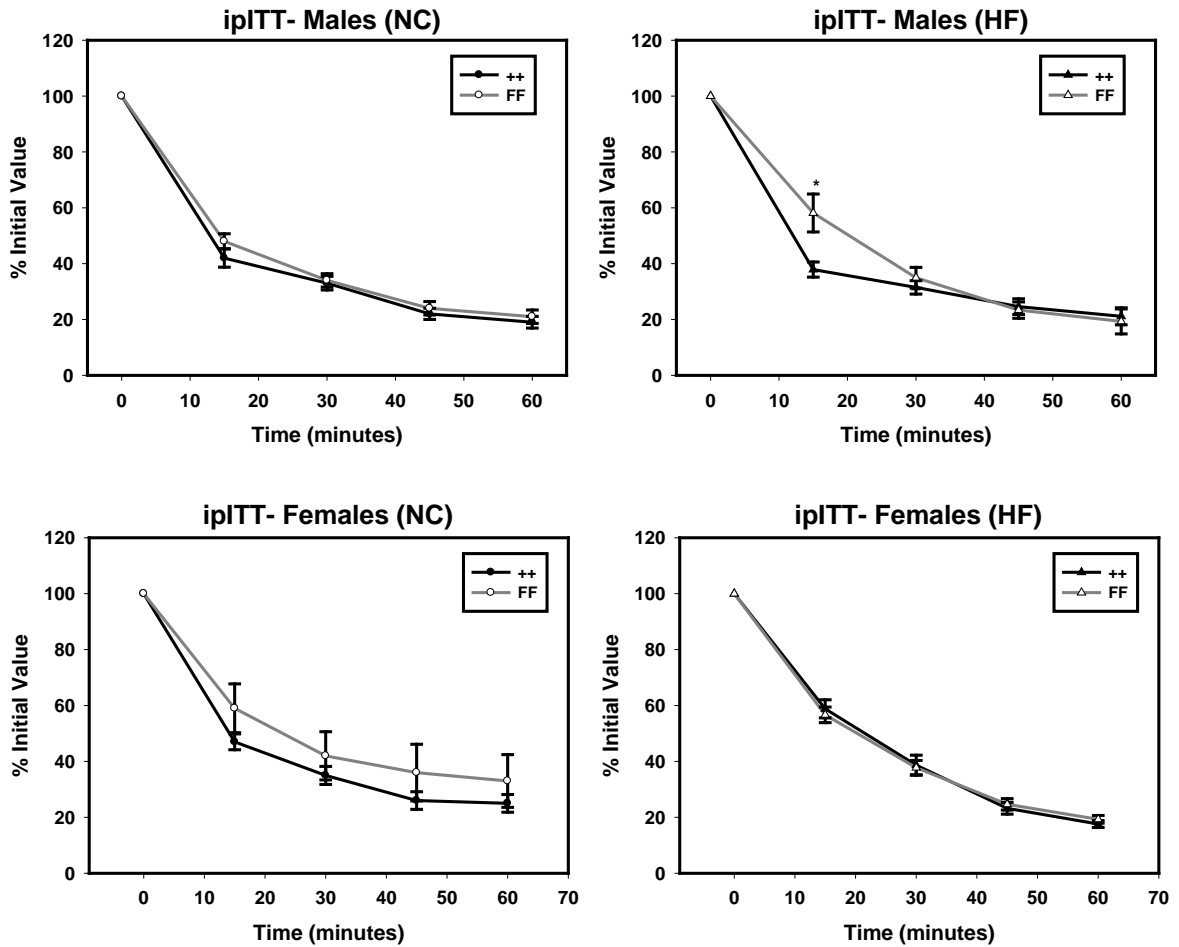
Figure 3.8: Normal insulin tolerance in LepRb$^{f1077/f1077}$ mice
Mice were subjected to an intraperitoneal insulin tolerance test (ipITT) at 14-weeks of age. Insulin tolerance was determined by calculating the percent of initial glucose value. There were no significant differences detected in *f/f* animals of sex or diet, compared *+/+* controls (with the exception of male mice fed a HF diet, which showed a slight insulin resistance at 15 minutes). Significant differences were analyzed by Student's T-test at each time point (* $p \leq 0.05$), n≥ 10 per gender, per genotype.

Vaginal Opening

First Estrous Cycle
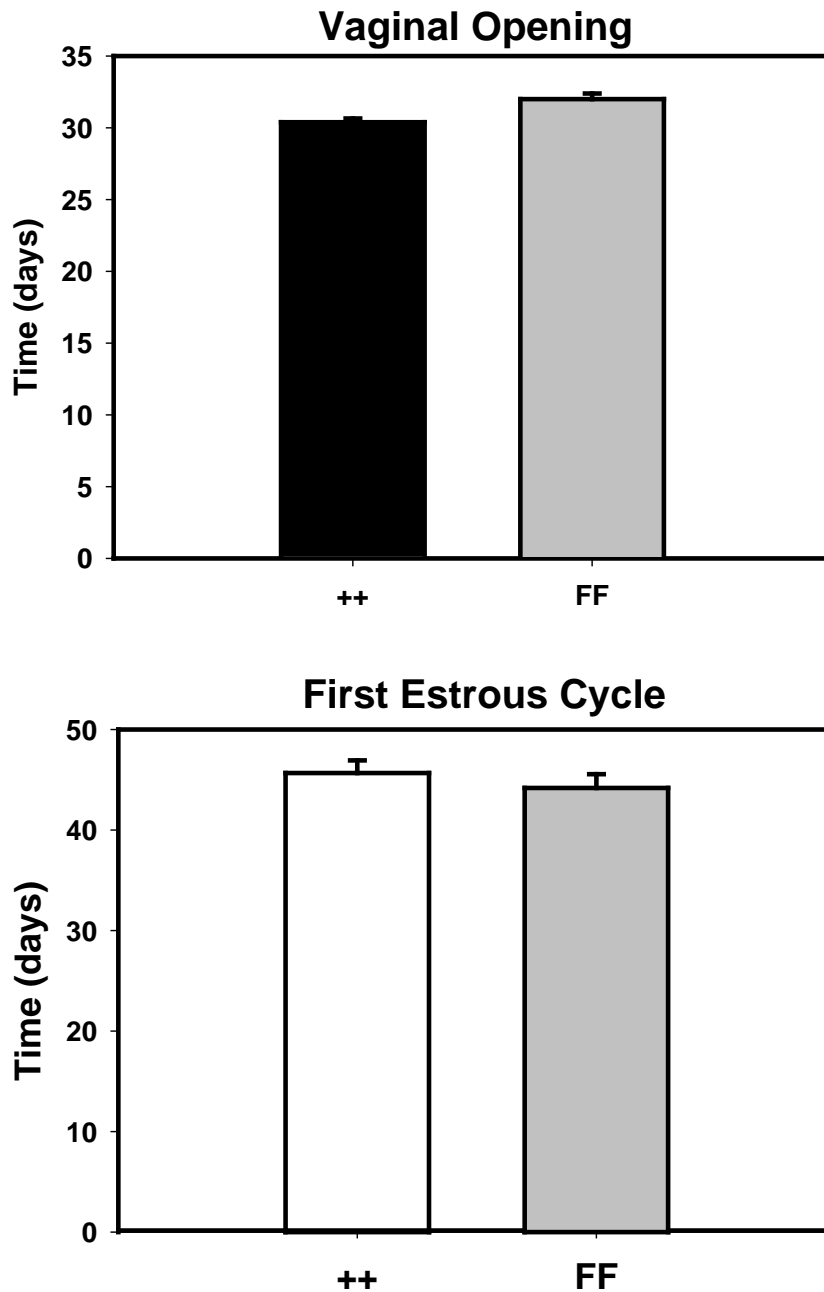
Figure 3.9: LepRb$^{f1077/f1077}$ female mice have normal onset of puberty
Vaginal opening (VO) and vaginal estrous (VE) was checked daily in *f/f* and *+/+* female mice from 28 days to 61 days of age. There was no difference on the time of vaginal opening or the time (average of 31 days) to the first estrous cycle (average of 44 days) in *f/f* mice and controls, n≥ 10 per gender, per genotype.

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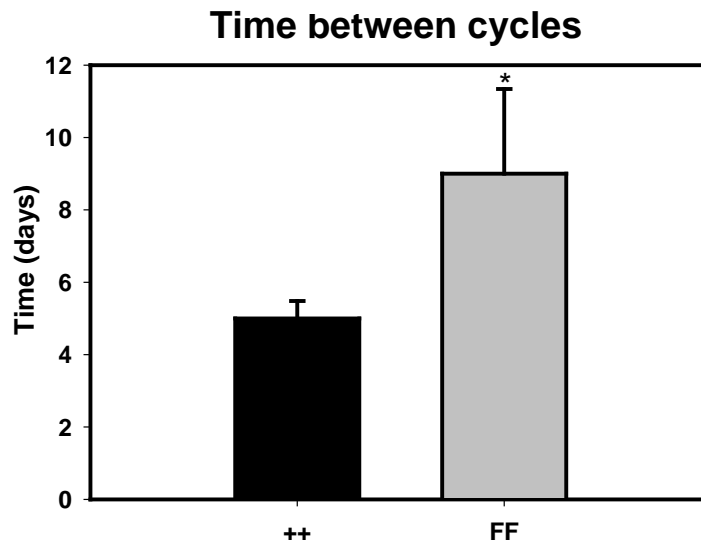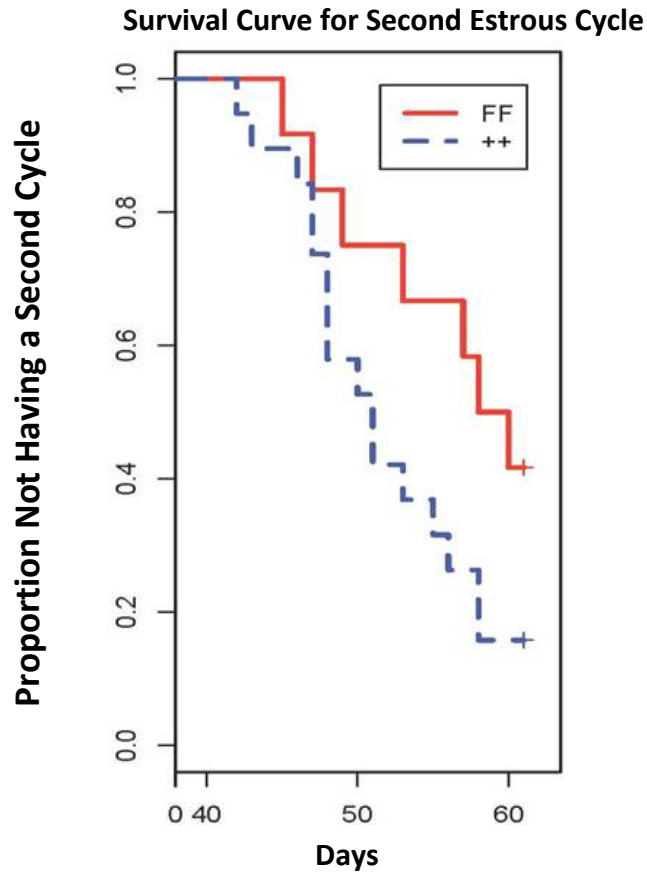
Figure 3.10: Impaired estrous cycling in LepRb$^{f1077/f1077}$ females

Vaginal opening and vaginal estrous was checked daily in *f/f* and *+/+* female mice from 28 days to 61 days of age (or up to a second estrous cycle was achieved). While only 16% of *+/+* mice did not have a second estrous cycle, 42% of *f/f* never underwent a second cycle. Also, the time between the first and the second estrous cycle was significantly delayed in the *f/f* mice that underwent a second estrous cycle. Significant differences were analyzed by Student's T-test at each time point (* $p \leq 0.05$), n≥ 10 per gender, per genotype.

TABLES: CHAPTER III

NC	Males		Females	
	+/+	*f/f*	*+/+*	*f/f*
Glucose	6.9 ± .22	6.8 ± .20	6.0 ± .20	6.0 ± .20
Leptin	2.37 ± .39	2.92 ± .36	1.14 ± .10	1.64 ± .29 *
Insulin	1.89 ± .26	2.32 ± .35	1.14 ± .12	.95 ± .10

HFD	Males		Females	
	+/+	*f/f*	*+/+*	*f/f*
Glucose	6.8 ± .19	7.2 ± .30	6.5 ± .20	6.7 ± .20
Leptin	7.07 ± .98	11.07 ± 2.4	5.97 ± 1.29	4.89 ± .77
Insulin	3.26 ± .42	2.99 ± .45	2.07 ± .36	1.86 ± .43

Table 3.1: Glucose, leptin and insulin levels in LepRb$^{f1077/f1077}$ mice on NC- and HF- diets

Fed glucose levels for male and female *f/f* and *+/+* mice on normal chow (NC) and high fat (HF) diet. Leptin and insulin concentrations were determined by ELISA from serum samples. All measurements from mice at 12-weeks of age, values ± SEM (n≥ 10, * P ≤ 0.05).

NC	Males			Females	
	+/+	*f/f*		*+/+*	*f/f*
S/A	92.2 ± .717	91.7 ± .498		88.2 ± .565	87.8 ± .717
Liver	.854 ± .025	.876 ± .021		.754 ± .020	.784 ± .025
BAT	.057 ± .003	.056 ± .002		0.043 ± .002	.046 ± .003
WAT	.400 ± .023	.503 ± .035 *		.142 ± .013	.293 ± .041 *
Gonads	.239 ± .010	.260 ± .010		.022 ± .001	.026 ± .002 †
Pancreas	.444 ± .022	.476 ± .021		.346 ± .009	.397 ± .027 *
Spleen	.087 ± .005	.079 ± .003		.1050 ± .006	.0938 ± .004
Muscle	.138 ± .004	.131 ± .002		.109 ± .002	.106 ± .003

HFD	Males			Females	
	+/+	*f/f*		*+/+*	*f/f*
S/A	91.1 ± .850	91.5 ± 1.360		87.8 ± .710	87.5 ± .746
Brain	.457 ± .007	.463 ± .005		.461 ± .005	.460 ± .005
Liver	.695 ± .030	.785 ±.049		.554 ± .020	.580 ± .025
BAT	.087 ± .006	.082 ± .010		0.052 ± .003	.057 ± .006
WAT	1.074 ± .105	1.365 ± .183		.300 ± .054	.365 ± .066
Gonads	.233 ± .007	.252 ± .014		.0182 ± .001	.0215 ± .001 *
Pancreas	.531 ± .036	.600 ± .049		.333 ± .024	.425 ± .034 *
Spleen	.092 ± .006	.086 ± .003		.127 ± .025	.099 ± .005
Muscle	.135 ± .003	.130 ± .003		.107 ± .002	.108 ± .003

Table 3.2: Physiological parameters studied in LepRb$^{f1077/f1077}$ mice
At the end of the study, mice were sacrificed and tissues collected, weighted and harvested. S/A length was also measured. All measurements from mice at 15-weeks of age, values ± SEM (n ≥ 10, *p ≤ 0.05; †p = 0.06).

CHAPTER IV

LEPTIN-DEPENDENT SIGNALING PATHWAYS REVISITED

<u>**SUMMARY AND CONCLUSIONS**</u>

The study of energy homeostasis and leptin neurophysiology

For years, scientists have struggled to understand the pathways regulating body weight and energy homeostasis. The discovery of leptin advanced our understanding of energy balance tremendously because it provided a mechanism to coordinate energy intake, energy storage and energy expenditure in mammals. Leptin is a peptide hormone secreted in rough proportions to body adiposity, which acts on brain LepRb-expressing neurons to inhibit appetite and increase energy utilization. While leptin acts in many locations throughout the brain, in the ARC, leptin induces the activation and release of proopiomelanocortin (POMC) while inhibiting neuronal activation of Agouti related peptide (AgRP) and Neuropeptide-Y (NPY) cells in the arcuate nucleus (ARC) of the hypothalamus. Also in the ARC, there are populations of neurons that express kisspeptin, which appears to express LepRb and likely project to GnRH-containing neurons to induce the release of gonadotropin-releasing hormone and control reproduction. However, not all leptin's effects are mediated by neurons located in the ARC, many other neuronal populations and brain regions are also important in mediating leptin action. In the ventromedial hypothalamus (VMH), steroidogenic factor-1 (SF-1)-expressing neurons are required for normal body weight homeostasis (80). The ventral premammillary nucleus (PMv) is another site that contains a large population of

LepRb neurons and is well known to regulate sexual and aggressive behaviors (157;158). Also, areas within the mesolimbic dopamine system, such as the ventral tegmental area (VTA) and the lateral hypothalamic area (LHA), contain LepRb-expressing neurons that seem to be important for the control of motivational processes (159). Thus, leptin is not only an adiposity signal but also a signal that plays roles in physiological functions such as growth, reproduction, glucose control, and immune response; and its action is not localized to one specific brain center but many regions are involved in the regulation of these leptin-dependent functions.

Leptin receptor-dependent signaling pathways

Leptin acts through a cytokine receptor, LepRb, which is expressed in areas of the hypothalamus and brain regions that respond to and effect energy balance. Upon LepRb activation, Jak2 autophosphorylates and activates, promoting the phosphorylation of downstream targets, including: LepRb-Tyr_{985}, Tyr_{1077} and Tyr_{1138}. Based on cell culture studies, we know that Tyr_{985} is responsible for the recruitment of the SH2-containing tyrosine phosphatase-2 (SHP2) to mediate the first step in the activation of the extracellular signal-regulated kinase (ERK) cascade (83-85). Also, phosphorylated Tyr_{985} binds the suppressor of cytokine signaling-3 (SOCS3), which acts as a negative regulator of LepRb signaling (86). Many different studies have shown that Tyr_{1138} recruits the signal transducer and activator of transcription-3 (STAT3), which mediates the regulation of gene expression, including SOCS3 (84;87). The phosphorylation of Tyr_{1077} promotes the recruitment, tyrosine phosphorylation, and

transcriptional activation of STAT5, although Tyr$_{1138}$ may also play a minor role in the regulation of STAT5 phosphorylation (83;91).

The knowledge of the pathways controlled by LepRb is based on both *in vitro* and *in vivo* evidence, and studies have shown that leptin controls many pathways other than those controlled directly by LepRb-Tyr residues. Among the indicators of cellular energy status implicated in the regulation of leptin-dependent circuits are the AMP-dependent protein kinase (AMPK, which is activated in response to increased AMP/ATP ratios, among other signals of decreased cellular energy) (95;116), and the mammalian target of rapamycin (mTOR).

In vitro studies are a great tool to study these aforementioned pathways but there is some difficulty in dissecting these signals by LepRb because of the inability to observe their regulation in cultured cells without overexpressing multiple signaling proteins that likely result in the non-specific activation of some signaling pathways. Additionally, the opposite regulation of some signals by leptin in distinct types of LepRb neurons complicates the analysis of their regulation *in vivo*. Thus, in order to circumvent these limitations, we have developed different mouse models of LepRb deficiency to study the specific contribution of individual signaling pathways to the overall physiological action of leptin.

The study of leptin-dependent functions in vivo

The information obtained from studying different mouse models is crucial to help explain regional regulation as well as the contribution of specific signaling pathways (from Jak2, Tyr residues in LepRb and others such as AMPK, mTOR, PI3K, etc).

Among the most important mouse models that made the studies mentioned in this dissertation possible, *ob/ob* and *db/db* mice are fundamental models mainly because the increased leptin sensitivity in *ob/ob* mice allows for the immediate detection of activated pathways by leptin, and lack of LepRb in *db/db* mice allows an easy comparison to other mouse models of LepRb-deficiency.

Mouse models developed in the Myers laboratory in the past years have provided valuable information and enhanced our understanding of LepRb signaling. "Knock-in" mice expressing mutant LepRb molecules from the genomic context of endogenous LepR have been generated for different LepRb motifs. Phenotypical analysis of mice expressing truncated LepRb containing the binding site for Jak2 but devoid of other LepRb intracellular sequences (LepRb$^\Delta$/LepRb$^\Delta$ mice) demonstrated a phenotype that closely resembles *db/db* mice in terms of energy homeostasis, neuroendocrine and immune function as well as the regulation of the ARC, but demonstrated modest improvements in glucose homeostasis. Thus, the fact that Jak2-autonomous LepRb signals failed to mediate leptin action suggests that leptin action is primarily mediated by pathways controlled by the phosphorylation of tyrosine residues within LepRb (105). Studies in mice with mutated LepRb-Tyr$_{1138}$ (*s/s*) have proven that this Tyr$_{1138}$ recruits STAT3 and plays a major role in regulating body weight and adiposity. The analysis of *s/s* mice also suggested that there are LepRb$_{1138}\rightarrow$STAT3 independent signals that control reproduction and glucose homeostasis, given that *s/s* mice have an improved reproductive response, and greatly improved glycemic control compared to *db/db* animals.

On the other hand, LepRb-Tyr$_{985}$ appears to play an important role in the attenuation of LepRb signaling (possibly via SOCS3) *in vivo* as well as in cultured cells: mice containing a replacement of Tyr$_{985}$ (*Lepr*$^{l985/l985}$ *or l/l*), have decreased body weight and increased leptin sensitivity but are otherwise normal compared to *+/+* controls (Table 4.1). However, the contributions of other LepRb-dependent and -independent signaling pathways should be considered as we seek to determine how leptin controls physiological functions via LepRb.

In this dissertation, we have extended our understanding of the mechanisms that contribute to the regulation of hypothalamic signals and physiological control by LepRb. We initially studied the regulation of the mammalian target of rapamycin complex-1 (mTORC1) pathway in the basomedial hypothalamus by examining the regulation of mTORC1 by nutritional and metabolic cues such as leptin, LepRb signals, insulin, ghrelin, and fasting, and by investigating the specific site (neuronal populations) of mTORC1 activation in the arcuate nucleus (ARC) in response to the above cues. We also investigated the role of LepRb-Tyr$_{1077}$ *in vivo* by examining leptin action and the physiologic phenotype of mice with replacement of Tyr$_{1077}$ (*Lepr*$^{f1077/f1077}$ or *f/f* mice).

Leptin-independent regulation of mTORC1 in vivo

Using an immunohistochemistry methodology to detect pS6-IR, we explored the regulation of mTORC1 by leptin and nutritional manipulation (i.e. fasting) in the ARC and confirmed the expected enhancement of mTORC1 signaling upon leptin treatment. However, these studies also revealed increased mTORC1 activity in LepRb and AgRP-expressing neurons in the MB-ARC by fasting and leptin-deficiency. In contrast, fasting

and leptin deficiency decrease mTORC1 activity in the VMH. Our data reveal the IHC detection of pS6-IR in the MB-ARC to be largely dependent upon the major mTORC1 mediator, S6K1, and to be entirely dependent upon mTORC1 itself.

We explored the signaling mechanisms by which LepRb might modulate the accumulation of pS6-IR in the MB-ARC by studying mTORC1 regulation in *l/l* and *s/s* mice, and found that neither Tyr_{985} nor Tyr_{1138} participate in the regulation of MB-ARC pS6-IR under conditions of endogenous leptin.

Our most interesting finding was the mechanism by which fasting and leptin-deficiency mediate mTORC1 activation. We found that mTORC1 activation in the ARC is indirect, via the regulation of neural activity. This conclusion was supported by the correlation between the induction of pS6-IR in the MB-ARC under nutritional conditions and genetic environments in which orexigenic (AgRP) neurons are activated (140;160), and by the induction of pS6-IR primarily in neurons that also demonstrate evidence of activation (c-Fos-IR) both in the MB-ARC and in the oppositely-regulated VMH. Also, ghrelin, which acutely depolarizes the orexigenic neurons of the MB-ARC (134), promotes pS6-IR and c-Fos-IR in the MB-ARC. Since leptin depolarizes and promotes the firing of some neurons within the MB-ARC (140;142), it is possible that the previously reported activation of mTORC1 during acute leptin treatment also takes place secondary to changes in neuronal activity. Another reasonable possibility is that leptin might promote mTORC1 signaling via the induction of PI3K, which is an important upstream activator of mTORC1 (118-120). Indeed, the observation that insulin (whose major signaling output is via PI3K (143)) promotes pS6-IR in the mediobasal hypothalamus

without increasing c-Fos-IR suggests that robust PI3K activation in the hypothalamus stimulates mTORC1, as in cultured cells.

Some studies suggest that mTORC1 is required to control leptin-induced anorexia during re-feeding conditions. However, deletion of the mTORC1 inhibitor TSC1 from POMC neurons (in *Pomc*-Tsc1cKO mice) (161) suggest that hypothalamic mTORC1 mediates an orexigenic signal, since these mice with mTORC1 activation demonstrate pronounced dysregulation of POMC cells and hyperphagic obesity. Overall, we believe that neuronal activation is the main regulator of mTORC1 activity in the hypothalamus in response to metabolic perturbations. Leptin and nutritional cues do not modulate hypothalamic mTORC1 activity in a uniform manner, but rather control mTORC1 via multiple pathways and, in many cases, in opposite directions in different cell types and brain regions, and the function of mTORC1 is likely to vary from cell type to cell type.

LepRb-Tyr$_{1077}$ contributes to the regulation of reproduction and glucose homeostasis by leptin

In order to understand the mechanisms by which LepRb mediates the physiological actions of leptin, we have previously examined the phenotype of mice mutant for LepRb-Tyr$_{985}$ and LepRb-Tyr$_{1138}$. In this study, we explored the function of LepRb-Tyr$_{1077}$ by using a novel mouse model with defective LepRb-Tyr$_{1077}$ signaling (Lepr$^{f1077/f1077}$). Based on phenotypes of mice lacking LepRb-Tyr$_{985}$ and LepRb-Tyr$_{1138}$, we postulated minor defects in energy balance and substantial changes in reproduction and glucose homeostasis in these mice. Indeed, *f/f* animals displayed modest increases in body weight and food intake, especially on a HF diet, as well as exhibiting increased

measures of adiposity on NC diet. The increased adiposity of *f/f* animals was mild compared to that of *db/db* and *s/s* mice, however, and small compared to the increase in adiposity observed with HF-feeding, so that differences in adiposity were not statistically significant on HF diet.

Interestingly, female *f/f* mice entered puberty normally, but exhibited absent or delayed estrous cycling. Thus, the lack of LepRb-Tyr$_{1077}$ signaling impairs the normal post-pubertal cycling and presumably ovulation in females. These results reveal the importance of LepRb-Tyr$_{1077}$ to the permissive action of leptin on the reproductive axis. Since leptin acts in the CNS to promote neuroendocrine reproductive function, this significant finding presumably reflects the importance of Tyr$_{1077}$-mediated signaling within the CNS.

Although no differences were observed in glucose and insulin levels in *ad libitum* fed animals, female *f/f* mice displayed a pronounced increase in glucose clearance together with normal insulin tolerance. Apparently, *f/f* females may display increased insulin secretion in the face of an acute glucose challenge, or may have other alterations in glucose production. Given the increased weight of pancreata from *f/f* mice, we postulate that insulin secretion may be increased. Overall, these results suggest that LepRb-Tyr$_{1077}$ is important for the regulation of glucose homeostasis by leptin.

Leptin treatment activates STAT5 in cultured cells and *in vivo*, and LepRb-Tyr$_{1077}$ mediates the majority of leptin-stimulated STAT5 activation in cultured cells. While technical limitations have prevented us from examining leptin-stimulated STAT5 activation in the hypothalamus of *f/f* mice, it is reasonable to believe that Tyr$_{1077}$

functions similarly to recruit STAT5 *in vivo*. In fact, deletion of STAT5 in the CNS of *Nestin-cre;Stat5*^{fl} mice results in modestly increased body weight and adiposity at young ages, similar to *f/f* mice, but without apparent defects in reproduction or enhancements in glucose tolerance as observed in *f/f* mice. Unfortunately, reproduction and glucose homeostasis were not carefully studied in the *Nestin-cre;Stat5*^{fl} mice and it is not possible to know whether alterations if these pathways exist. The obesity observed in older *Nestin-cre;Stat5*^{fl} mice is much more pronounced than in our *f/f* mice, but it is not clear whether this difference reflects only differences in age, or also blockade of STAT5 signaling by other factors and the more widespread deletion of STAT5 in these mice. Also, the possibility of leptin inducing phosphorylation of IRS proteins and PI3K together with some evidence that suggests that IRS4 may interact with LepRb via Tyr$_{1077}$ (and the phenotypical similarity between IRS4-null mice and *f/f* mice) dictates that we must at least consider the possibility that LepRb-Tyr$_{1077}$ may act in part via the IRS4-PI3K pathway. These and other LepRb-Tyr$_{1077}$–dependent pathways are worth examining, as well as the hypothalamic neuronal regulation in order to understand the specific neuronal populations potentially affected in *f/f* mice.

Importance of the study and clinical applications

In this thesis, we have identified the mechanisms of hypothalamic mTORC1 regulation and demonstrated its place in signaling downstream of neuronal activation. This mTORC1 signal is crucial for energy balance (94;161). Additionally, we identified a major pathway by which leptin mediates glucose homeostasis and reproductive function (Figure 4.1).

It is important to understand the specific pathways that regulate different physiological functions via LepRb, such as energy balance, because dissecting these specific signaling pathways could provide possible targets for the development of pharmacological therapies for the treatment of obesity and diabetes. However, leptin also regulates some aspects of reproduction and glucose homeostasis, and examining the mediators involved in these signaling pathways could provide a basis to better understand, and possibly, treat conditions linking metabolism to reproductive defects such as polycystic ovarian syndrome (PCOS), ovulatory infertility, menstrual irregularity and amenorrhea.

FIGURES: CHAPTER IV
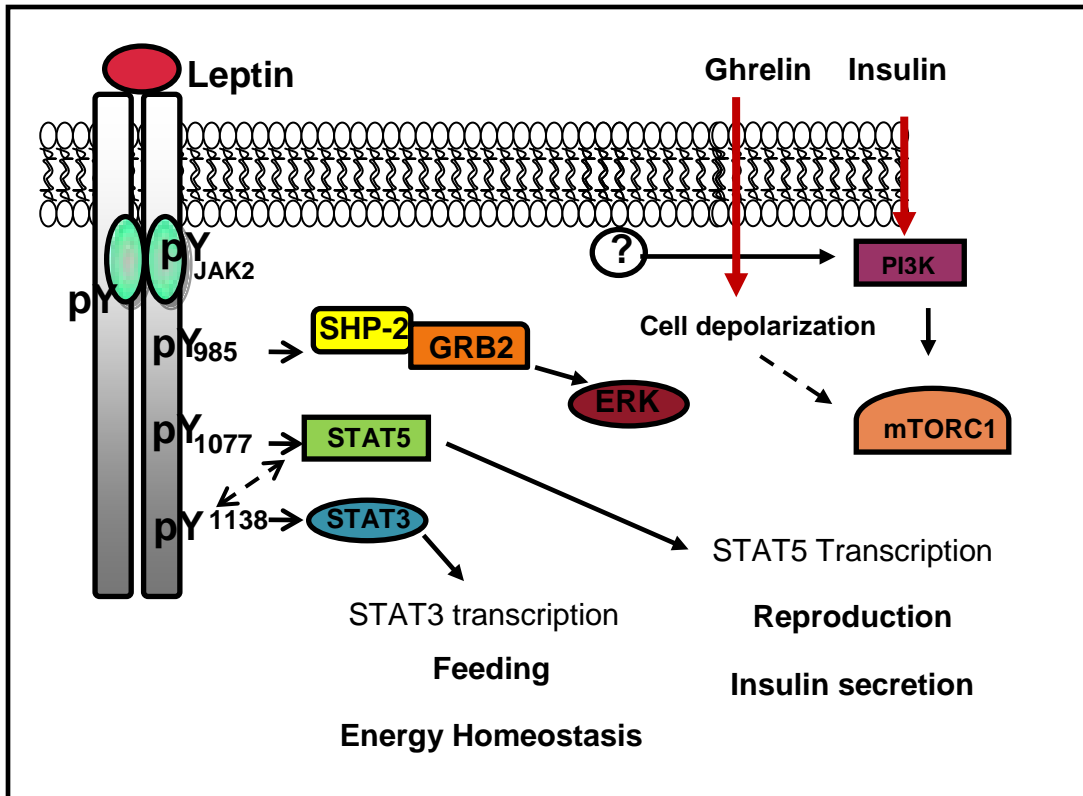
Figure 4.1: Proposed model for the mechanisms of LepRb signaling *in vivo*
LepRb activates Jak2 to initiate tyrosine-phosphorylation-dependent signal transduction pathways. In cultured cells, Tyr_{985} activates the SHP2/ERK cascade. Activation of Tyr_{1077} and Tyr_{1138} induces phosphorylation of STAT5 and STAT3, respectively (although Tyr_{1138} may also mediate a minor component of STAT5 phosphorylation), stimulating the translocation of STAT3 and STAT5 to the nucleus. Although the pertinent transcriptional targets of STAT5 remain unknown, several STAT3 transcriptional targets have been identified, including SOCS3 (which inhibits LepRb signaling). Tyr_{1077} seems to play an important role in the regulation of reproduction and glucose homeostasis and/or insulin secretion (presumably via STAT5). We do not yet fully understand the mechanisms by which LepRb activates other signals, such as IRS and PI3K in the hypothalamus, but we now know that cell depolarization activates mTORC1, at least in some cell types (Adapted from Villanueva, E, and Myers, MG, Leptin receptor signaling and the regulation of mammalian physiology, *International Journal of Obesity*, 2008).

TABLES: CHAPTER IV

	+/+	*db/db*	*s/s*	*l/l*	*f/f*
	Wild-type	No LepRb	No Tyr-1138	No Tyr-985	No Tyr-1077
Body Weight	↔	↑↑↑	↑↑	↓♀	↑
Food Intake	↔	↑↑	↑↑	↔	↑♀
Fat Mass	↔	↑↑↑	↑↑	↔	↑
Lean Mass	↔	↑	↑↑	↔	↔
Linear Length	↔	↓	↑	↔	↔
Glucose	↔	↑↑↑	↑↑	↔	↔
Leptin	↔	↑↑↑	↑↑	↓	↑
Insulin	↔	↑↑	↑↑	↓♀	↔
Glucocorticoids	↔	↑↑	↑↑	ND	ND
Fertility	↔	X	↓↓	↔	↓

Table 4.1: Comparison between different models with LepRb defects
Phenotypical parameters studied in different mouse models with LepRb defects. Note
the pronounced increased in adiposity of *db/db* and *s/s* mice, which is very mild
compared to *f/f* mice (↔, normal; ↑, increased; ↓, decreased, X, none; ND, not
determined).

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