MAPPING THE NEURAL MECHANISMS BY WHICH LEPTIN REGULATES DISTINCT PHYSIOLOGICAL FUNCTIONS

by

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To my charming husband, Stan

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LIST OF ABBREVIATIONS

3V, third ventricle
AgRP, agouti related peptide
AMPK, AMP-dependent protein kinase
ARC, arcuate nucleus of the hypothalamus
AVPV, anteroventral periventricular nucleus
BBB, blood brain barrier
BDNF, brain-derived neurotrophic factor
BL, barley lectin
CART, cocaine- and amphetamine-regulated transcript
CCK, cholecystokinin
CeA, central amygdala
CNS, central nervous system
CRH, corticotropin releasing hormone
DA, dopamine
DMH, dorsomedial hypothalamic nucleus
DR, dorsal raphe
Dyn, Dynorphin A
EGFP, enhanced green fluorescent protein
EGFPF, farnesylated green fluorescent protein
ERK, extracellular signal-regulated kinase
FG, fluorogold
FSH, follicle stimulating hormone
GABA, gamma aminobutryic acid
GLP-1, glucagon-like protein 1
GnRH, gonadotropin releasing hormone
GPR54, G-protein coupled receptor 54
ICV, intracerebroventricular
IF, immunofluorescence
IHC, immunohistochemistry
IP, intraperitoneal
IR, immunoreactivity
Jak2, Janus kinase-2
kDA, kilodalton
Kiss1, kisspeptin
LepRb, leptin receptor-b
LH, luteinizing hormone
LHA, lateral hypothalamic area
MC3R/MC4R, melanocortin 3/4 receptor

MCH, melanin concentrating hormone
ME, median eminence
NAC, nucleus accumbens
NKB, neurokinin B
NPTX2,(NARP) neuronal pentraxin-2
NPY, neuropeptide-Y
NTS, nucleus of the solitary tract
OVX, ovarectomize
OX, orexin
OX2R, orexin receptor 2
PBS, phosphate buffered saline
pCREB, phosphorylated cAMP response element-binding protein
PI3K, phosphatidyl-inositol-3-kinase
PMv, ventral premammillary nucleus
POA, preoptic area
POMC, pro-opiomelanocortin peptide
PVN, paraventricular nucleus of the hypothalamus
RT-PCR, reverse transcriptase polymerase chain reaction
SHP2, Src homology domain-containing tyrosine phosphatase-2
SNS, sympathetic nervous system
SOCS3, suppressor of cytokine signaling-3
STAT3/5, signal transducer and activator of transcription-3 or -5
Tac2, tachykinin 2
TH, tyrosine hydroxylase
TRH, thyrotropin releasing hormone
Tyr, tyrosine
VMH, ventromedial hypothalamic nucleus
VTA, ventral tegmental area
WGA, wheat germ agglutinin

ABSTRACT

MAPPING THE NEURAL MECHANISMS BY WHICH LEPTIN REGULATES DISTINCT PHYSIOLOGICAL FUNCTIONS

Body fat stores are essential to provide energy for physiological functions. When body fat levels are extremely low (as in starvation, fasting, lipodystrophy and anorexia), a variety of energy-sparing changes to physiological functions are executed. Altered functions lead to problems such as: hypothalamic amenorrhea, suppressed thyroid function, cold intolerance, and bradycardia. Leptin is an adipocyte hormone secreted in proportion to body adiposity to maintain energy homeostasis by suppressing feeding and promoting energy expenditure. Leptin binds to the signaling form of its receptor, LepRb, to control feeding and other physiological processes such as growth and reproduction. Populations of LepRb neurons are dispersed throughout the brain and in several hypothalamic nuclei. While the connectivity and functions of the arcuate nucleus (ARC) is well-characterized, much less is known about the neurocircuitry of other LepRb-containing loci, which has deterred understanding of their contribution to physiology. In this study, our objective was to examine specific LepRb-expressing populations and identify their regulation of distinct neural targets to control unique

physiological processes. Thus, we developed novel tract tracing tools to selectively study LepRb circuitry. We first examined a population of LepRb neurons in the lateral hypothalamic area (LHA) that directly innervated DA-containing neurons of the ventral tegmental area (VTA)- a core component of the mesolimbic dopamine (DA) system. LHA LepRb populations also synapsed with LHA neurons that express the appetite-inducing neuropeptide orexin (OX), which are also crucial for controlling hedonic food reward via the mesolimbic DA system. In addition, leptin action on LHA LepRb neurons induced activation of neurons in the VTA and significantly upregulated OX expression in the LHA. We additionally investigated the LepRb pathways involved with the control of reproduction, focusing on two populations of reproductive neurons in the hypothalamus, namely those that express gonadotropin releasing hormone (GnRH) and those that express kisspeptin (also known as Kiss1). We determined that while LepRb neurons synapse with Kiss1 populations in both the arcuate (ARC) and anteroventral periventricular (AVPV) nuclei, only the ARC population projects to GnRH neurons and leptin regulates ARC Kiss1 expression, suggesting that ARC-Kiss1 neurons are the main conduit for the leptin-regulation of the hypothalamic reproductive system in the mouse. Furthermore, LepRb neurons in the ventral premammilary nucleus (PMv) directly synapse with GnRH neurons to presumably control reproduction. Taken together, understanding the regulation of these distinct LepRb neuronal pathways by

leptin leads to the overall understanding of how changes in energy homeostasis may influence whole body physiology.

CHAPTER ONE

INTRODUCTION

CENTRAL LEPTIN ACTION IN ENERGY HOMEOSTASIS AND OTHER NEUROENDOCRINE FUNCTIONS

A. Leptin as a regulator of energy homeostasis

1. Leptin and mechanisms of leptin action

In the absence of continuous feeding, body energy stores (primarily fat) are essential to provide energy for physiological functions. In states of negative energy balance, when body fat levels are extremely low (as in starvation, fasting, and a number of disorders of fat cell biology) a variety of behaviors are altered to execute a program of energy-sparing changes to the endocrine and nervous systems. Even in the absence of fasting, decreased energy stores produce a dramatic physiologic phenotype: such is the case in lipodystrophic patients who present with diabetes mellitus, insulin resistance, hypertriglyceridemia, hypothalamic amenhorrea and hepatic steatosis (Shimomura et al., 1999; Oral et al., 2002; Javor et al., 2005). Similarly, individuals with low body fat (e.g. those with anorexia nervosa or extreme

endurance athletes) present features such as hypothalamic amenorrhea, suppressed thyroid function, cold intolerance, and bradycardia (Ballauff et al., 1999; Ahima, 2004; Welt et al., 2004). These observations suggest the presence of signals that relay information about body energy status to other brain centers to control overall physiological function.

A major advancement in understanding the humoral connection between energy imbalance and disrupted physiology took place with the identification of two spontaneously arising recessive alleles causing monogenic obesity and diabetes in mice- *obese* (*ob*) and *diabetes* (*db*) (Coleman, 1978; Flier and Elmquist, 1997; Friedman, 1998). The *ob/ob* and *db/db* animals exhibit similar syndromes of hyperphagic obesity with prominent diabetes and endocrine dysregulation reminiscent of the fasting response (hypothalamic hypogonadism, decreased growth, decreased thyroid function, increased glucocorticoids, etc.). An important clue to the biology of the genes underlying these two animal models came from a series of parabiosis experiments conducted by Coleman in the early 1970's (Coleman, 1973). Linking the circulation of *ob/ob* mice with either wild-type or *db/db* mice promoted weight loss in the *ob/ob* animals, while parabiosis of *db/db* mice with either *ob/ob* or wild-type animals had no effect on the *db/db* animals- suggesting that *ob/ob* mice lacked a circulating factor, while *db/db* mice lacked the receptor for this factor.

This conclusion was later confirmed by the positional cloning of the *ob* gene, which encoded an adipocyte (fat cell)-produced peptide (termed "leptin";

Greek- *leptos*=thin) that promoted weight loss in *ob/ob* mice (Zhang et al., 1994). Leptin is a 16kDa, interleukin-type-6 cytokine that is secreted primarily by adipose tissue (Zhang et al., 1994; Halaas et al., 1995; Friedman, 1998). The leptin receptor (LepR)-encoding gene (*Lepr*) was cloned soon after, revealing that LepR mutation underlies the defects in *db/db* mice (Tartaglia et al., 1995; Chen et al., 1996; Chua et al., 1996).

The physiologic regulation of leptin secretion

Although leptin is produced by other tissues in smaller quantities, it is produced primarily by adipocytes (Zhang et al., 1994; Flier and Elmquist, 1997). While conditionally null animals have not been generated to probe the physiologic relevance of leptin production by specific tissues, the phenotype of human patients and rodent models virtually devoid of adipose tissue (lipodystrophy) is remarkably similar to the phenotype of leptin deficiency (with the exception of adipose mass), including low circulating leptin levels (Shimomura et al., 1999; Oral et al., 2002). Indeed, the hyperphagia and many of the metabolic abnormalities of lipodystrophy are partially or completely remedied by treatment with exogenous leptin (Shimomura et al., 1999; Oral et al., 2002), suggesting that adipose tissue is the main functional source of circulating leptin.

In adipocytes, leptin production is regulated by nutritional status: generally speaking, more stored fat results in greater production of leptin (Figure 1.1), while less stored triglycerides results in decreased production of

leptin (Flier and Elmquist, 1997; Coleman and Herrmann, 1999). Leptin secretion is constitutive: leptin is synthesized and extruded into the secretory pathway for release by mass action (as opposed to being packaged into specialized vesicles for regulated release in response to an acute stimulus) (Bradley and Cheatham, 1999; Coleman and Herrmann, 1999; Lee et al., 2007). Thus, although there is modest diurnal variation in circulating leptin levels, leptin secretion and circulating leptin levels depend primarily upon the rates of transcription and translation of the leptin mRNA, and circulating leptin levels vary little on a minute-to-minute or hour-to-hour basis (Ahima et al., 1998). The regulation of circulating leptin levels by adipose mass positions leptin well to function as an indicator of body energy stores. Since prolonged (many hours to days) changes in nutrition are required to alter leptin levels, leptin presumably signals the long-term status of energy stores, as opposed to acute fluctuations in nutrition.

Leptin action in vivo

Due to the early discovery of *ob/ob* and *db/db* mice, there exists a long-standing literature regarding the phenotype of mice with loss of function mutations for leptin or LepR (Coleman, 1978; Flier and Elmquist, 1997; Friedman, 1998). These mice have virtually indistinguishable phenotypes of hyperphagia, low metabolic rate, and consequently obesity. Additionally, *ob/ob* and *db/db* mice display characteristics reminiscent of the stereotypical starvation response (even in the face of nutrient excess and obesity), with

hypothyroidism, hypothalamic infertility, decreased growth, and decreased immune function (Ahima et al., 1996; Ahima and Flier, 2000). Similar phenotypes have been observed in rare human patients with null mutations for leptin or LepR and in cases of low leptin levels due to lipodystrophy (Oral et al., 2002; Farooqi and O'Rahilly, 2004); these phenotypes are reversed by leptin therapy in leptin-deficient and lipodystrophic patients (but not in those mutant for LepR) (Oral et al., 2002). Thus, leptin deficiency results in increased drive to feed and initiates a series of energy-sparing events.

Leptin thus fits the criteria for a feedback signal from body energy stores to the brain: 1) leptin levels drop during starvation, when fat depots are depleted to support the organism's basic energy needs; and 2) leptin levels rise during re-feeding when fat depots are replenished. Indeed, many of the physiological adaptations triggered by prolonged fasting can be prevented by exogenous leptin during the fast, which falsely signals to the brain that energy stores are replete (Ahima et al., 1996; Lord et al., 1998; Bates et al., 2003). Indeed, leptin replacement therapy in lipodystrophic patients improved glycemic control, decreased triglyceride levels and restored menses. Leptin also contributed to the improvement of insulin resistance and other metabolic abnormalities associated with severe lipodystrophy (Oral et al., 2002; Javor et al., 2005). Some of the symptoms presented in patients with anorexia nervosa could be improved with leptin treatment, for example, the reproductive, thyroid and growth hormone dysfunctions (Welt et al., 2004). Moreover, studies in leptin-deficient humans with morbid obesity who received leptin treatment

resulted in dramatic weight loss, increased physical activity, and resolution of diabetes (Farooqi et al., 1999). Overall, adequate leptin levels suppress feeding, permit energy expenditure on physiological processes such as reproduction and growth, and similarly regulate the autonomic nervous system, other elements of the endocrine system and the immune system (Figure 1.1). Conversely, inadequate leptin increases appetite and inhibits energy expenditure on these events (Ahima et al., 1996; Lord et al., 1998; Bates et al., 2003).

Leptin and LepRb signaling

The leptin receptor (LepR) is a member of the type I cytokine receptor family (Tartaglia, 1997); the gene contains 17 common exons and several alternatively spliced exons. Alternative splicing of transcripts from a single *Lepr* gene generates multiple LepR isoforms (Chen et al., 1996; Chua et al., 1996; Lee et al., 1996; Chua et al., 1997). With the exception of a truncated, non-membrane-bound form (LepRe) that binds and stabilizes circulating leptin (Yang et al., 2004), each of the LepR isoforms contains identical extracellular and transmembrane domains, diverging only in their intracellular sequences. The short forms (a, c, d) are found dispersed throughout peripheral tissues and may play a role in leptin transport across the blood-brain barrier, although their function is uncertain (Banks et al., 1996; Hileman et al., 2002). A number of lines of evidence suggest a crucial role for the “long” LepR form, LepRb, in leptin action. Not only is LepRb the only LepR isoform altered in *db/db* mice,

but transgenic expression of LepRb rescues many of the abnormalities in mice homozygous for the *db*^{3j} mutation (which are devoid of all LepR isoforms) (Chen et al., 1996; Chua et al., 1996; Lee et al., 1996; Chua et al., 2004). Furthermore, of all the LepR isoforms, only the b form contains the functional domains required to modulate the known intracellular signaling effectors of leptin action (Baumann et al., 1996; Kloek et al., 2002).

LepRb has no intrinsic enzymatic activity of its own (Tartaglia, 1997; Kloek et al., 2002); leptin binding to LepRb activates the receptor-associated tyrosine kinase, Janus kinase-2 (Jak2; Figure 1.2). Jak2 stimulates the phosphorylation of several tyrosine residues on the intracellular domain of LepRb: Tyr^{985}, Tyr^{1077}, Tyr^{1138}, as well as the autophosphorylation of Jak2 (Gong et al., 2007). Each of these phosphorylated tyrosine residues then recruits a distinct set of downstream signaling molecules. Phosphorylated Tyr^{985} recruits the SH2-containing tyrosine phosphatase-2 (SHP-2) to initiate the activation of the extracellular signal-regulated kinase (ERK) pathway (Banks et al., 2000; Bjorbaek et al., 2001; Gong et al., 2007). Phosphorylated Tyr^{985} also binds the suppressor of cytokine signaling-2 (SOCS3), which serves as a negative regulator of LepRb signaling (Bjorbaek et al., 1998; Bjorbaek et al., 1999). Phosphorylation of another tyrosine residue, Tyr^{1138}, recruits the signal transducer and activator of transcription-3 (STAT3), a latent transcription factor that is phosphorylated and translocated to the nucleus to mediate transcriptional events (White et al., 1997; Banks et al., 2000). STAT3 signaling promotes the expression of SOCS3, which is the feedback loop that

inhibits LepRb signaling (Bjorbaek et al., 1998; Bjorbaek et al., 1999; Banks et al., 2000). Importantly, the detection of pSTAT3 by immunohistochemical techniques is widely used as a marker for functional LepRb in the CNS (Hosoi et al., 2002; Munzberg et al., 2004). Finally, the phosphorylation of Tyr^{1077} mediates the recruitment and activation of STAT5, presumably contributing to the transcriptional response to leptin. Tyr^{1138} may also play a minor role in the regulation of STAT5 phosphorylation (Hekerman et al., 2005; Gong et al., 2007). The net effects of these multiple signaling pathways mediate physiological leptin action.

The regulation of specific physiological functions by unique Tyr sites has begun to be deciphered by utilizing different knock-in mouse models expressing LepRb molecular mutants for each Tyr site, thus rendering specific residues non-functional while the remaining LepRb signals normally (Bates et al., 2004; Bjornholm et al., 2007). LepR Tyr^{1138} mutant mice (*s/s* mice) are hyperphagic and obese, revealing a crucial role for Tyr^{1138} in the control of feeding, energy expenditure and glucose homeostasis. However, Tyr^{1138} is not required for reproduction or immune function (Bates et al., 2003; Bates et al., 2004; Bates et al., 2005; Dunn et al., 2005). On the contrary, mice with a mutation of Tyr^{985} (*l/l* mice) are lean with exaggerated leptin sensitivity and normal fertility. Thus, the role of Tyr^{985} seems to be in the inhibition of LepRb signaling, presumably via the SOCS3-mediated feedback loop (Bjornholm et al., 2007). Mice containing only the intracellular Jak2 binding site of LepRb (Δ/Δ) are phenotypically similar to *db/db* mice (Robertson et al., 2010)

suggesting that leptin signaling is principally controlled by cascade events stemming from the phosphorylation of LepRb tyrosine residues. Recent data from the Myers lab suggests a modest role of Tyr^{1077} in energy homeostasis, but a larger role related to reproductive function. Female mice with a mutation in Tyr^{1077}(*f/f* mice), while similar to wild type mice in terms of body weight, displayed impaired estrous cycling [Villanueva, unpublished]. Thus, a variety of data have revealed functions of each tyrosine signal on LepRb, suggesting roles for individual LepRb signals in specific leptin actions. Furthermore, we propose that each set of LepRb neurons subserve specific neural and physiological functions.

2. The neural basis of leptin action

LepRb action in the CNS is crucial for overall leptin action, including its effects upon feeding, energy balance, reproduction, and other neuroendocrine functions (Cohen et al., 2001; de Luca et al., 2005; McMinn et al., 2005). Intracerebroventricular (ICV) injection of leptin restores energy homeostasis and neuroendocrine function in fasted or leptin-deficient *ob/ob* animals (Ahima and Flier, 2000). Furthermore, transgenic expression of LepRb in the CNS restores normal energy balance and neuroendocrine function to *db/db* animals (Chua et al., 2004; de Luca et al., 2005). Thus, while it is possible that leptin mediates some effects by direct action on peripheral tissues, CNS leptin action is necessary and sufficient for all leptin actions that have been studied to date (with the exception of immune and other cells of hematopoeitic origin, which

are regulated directly by leptin) (Lord et al., 1998; Farooqi et al., 2002).

Numerous populations of LepRb-expressing neurons exist in the brain (Elmquist et al., 1998; Leshan et al., 2006; Myers et al., 2009) (Figure 1.3A-D), including several brain areas important for energy balance, such as the "satiety centers" in the mediobasal hypothalamus (e.g. the arcuate (ARC) and ventromedial hypothalamic (VMH) nuclei) and the brainstem (Figure 1.3C). Many LepRb-expressing neurons also lie in brain centers that regulate the motivation to feed (e.g. the lateral hypothalamic (LHA) and ventral tegmental (VTA) areas) (Figure 1.3D), as well as other hypothalamic control centers, such as the ventral premammilary (PMv) nucleus.

ARC neurons and the regulation of satiety

Within the ARC, the best-characterized site of leptin action, at least two distinct neuronal populations express LepRb: (1) neurons that co-express neuropeptide Y (NPY) and agouti related peptide (AgRP) and (2) neurons that express pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (Elias et al., 1999; Schwartz et al., 2000) (Figure 1.4). In the ARC POMC neurons, leptin stimulates the synthesis and release of POMC-derived peptides, which mediate a powerful anorectic (appetite-suppressing) satiety signal via activation of CNS melanocortin receptors (MC3R and MC4R), as well as opioid receptors, on target neurons (Elias et al., 1999; Elmquist et al., 1999; Schwartz et al., 2000; Cowley et al., 2001; Appleyard et al., 2003). AgRP inhibits MC3R and MC4R signaling, and NPY

is itself an orexigenic (appetite-stimulating) peptide (Erickson et al., 1996; Seeley et al., 1997; Bates et al., 2003; Fan et al., 2008). Leptin inactivates NPY/AgRP neurons and suppresses expression of these neuropeptides (Korner et al., 2001; Breen et al., 2005; Elmquist et al., 2005). These ARC LepRb neurons densely innervate the parvocellular portion of the LHA, paraventricular (PVH) and dorsomedial (DMH) hypothalamic nuclei, which contain outputs to regulate feeding and energy expenditure (Butler and Cone, 2002; Kishi et al., 2003; Elmquist et al., 2005)(Figure 1.4). ARC LepRb neurons also project to the brainstem to regulate satiety (Grill, 2006; Morton et al., 2006). Thus, leptin signaling to the ARC stimulates the production of anorectic neuropeptides and suppresses orexigenic peptides to promote satiety and permit energy utilization; inadequate leptin action mediates opposite effects (Elmquist et al., 1999; Schwartz et al., 2000).

Leptin action outside of the ARC

While it is clear that LepRb-expressing ARC neurons are important for the response to leptin, it is also evident that they cannot account for all, or even the majority of, leptin action *in vivo*. Not only do non-ARC LepRb-expressing neurons, such as SF1-expressing LepRb neurons in the VMH (Dhillon et al., 2006) and LepRb-expressing neurons in the VTA (Fulton et al., 2006; Hommel et al., 2006), participate in the regulation of feeding and energy balance, but unilateral reactivation of LepRb in the ARC of mice lacking functional LepRb only modestly alters body weight (although dramatically

improving glucose homeostasis and activity) (Coppari et al., 2005). Furthermore, while lack of LepRb→STAT3 signaling in mice with mutant LepRb defective for this signaling pathway results in dramatically increased feeding and obesity similar to *db/db* mice (Bates et al., 2003; Bates et al., 2004; Bates et al., 2005), deletion of STAT3 in ARC POMC or AgRP neurons minimally affects body weight (Kaelin et al., 2006; Xu et al., 2007). Similarly, deletion of the leptin receptor gene (*Lepr*) from ARC POMC and/or AgRP neurons results in modest obesity compared to that of db/db animals (Balthasar et al., 2004; Coppari et al., 2005; van de Wall et al., 2008).

Hence, while LepRb-expressing ARC neurons mediate important leptin effects, they account for only a portion of overall leptin action; thus, other CNS sites must mediate crucial aspects of leptin function. Indeed, ARC LepRb neurons comprise only ~20% of the total CNS LepRb neurons (Elmquist et al., 1998; Leshan et al., 2006; Myers et al., 2009). Furthermore, the brain systems that regulate satiety (e.g. the brainstem) are densely innervated by the ARC, but the ARC does not directly innervate the VTA, an important gateway to the mesolimbic dopamine (DA) system (which regulates motivational aspects of feeding and reproduction). Therefore,we need to examine the potential neural system by which leptin controls the mesolimbic DA system and reproduction.

B. Regulation of the mesolimbic DA system by leptin.

Satiety is the state of being full or gratified to capacity, which promotes the termination of food intake. Leptin controls satiety (and consequently, feeding) via a neural network that originates from the nucleus of the solitary tract (NTS) in the brainstem (Grill, 2006; Morton et al., 2006). The NTS relays information from the vagal nerve and gastrointestinal peptides such as cholecystokinin (CCK) or glucagon-like peptide 1(GLP-1) to act in tandem with leptin to control satiety (Morton et al., 2005; Williams et al., 2006; Huo et al., 2007). Interestingly, the NTS contains a population of LepRb neurons (Elmquist et al., 1998; Leshan et al., 2006; Myers et al., 2009) that may contribute to the control of hindbrain satiety circuits; however the underlying mechanism remains unclear.

Food consumption, however, is not only driven by satiety and physiological energy demands, but also by the value of eating tasty, pleasurable food. For example, we eat a meal for nourishment and when full, we stop eating (in other words, we are physiologically satiated). However, when presented with dessert, we continue eating because the dessert is rewarding and delicious (which describes hedonic food intake). This incentive value of food is encoded by the mesolimbic dopamine (DA) system, thus understanding the neural pathways that regulate hedonic food intake could be important to understanding continued feeding and energy balance.

The incentive value of hedonic stimuli, such as alcohol, drugs of abuse, sex or food, is mediated partly by the mesolimbic DA system (Wise, 2002) which typically consists of gamma-aminobutyric acid (GABA)ergic or DAergic

neurons in the VTA that project to the striatum (including the nucleus accumbens (NAc) to control motivation and reward (van Furth and van Ree, 1996; Kelley and Berridge, 2002; DiLeone et al., 2003; Georgescu et al., 2005) (Figure 1.5). DA dysfunction in patients with anorexia has been suggested to predispose them to anhedonia (lack of pleasure for food) by disturbing their central reward-related systems (Rask-Andersen et al., 2010). Unsurprisingly, leptin and other signals of energy status that might be involved with the motivation to feed, have been implicated in the regulation of the mesolimbic DA system. Leptin regulates the activity and gene expression of DA neurons in the VTA, and leptin-mediated regulation of the mesolimbic DA system modulates hedonic feeding and body weight (Figlewicz et al., 2006; Fulton et al., 2006; Roseberry et al., 2007). Thus, it is clear that leptin regulates the VTA and the mesolimbic DA system, which is an integral part of metabolic regulation. Indeed, a subpopulation of VTA DA neurons express LepRb and leptin modulates food reward (Figlewicz et al., 2001; Figlewicz et al., 2004; Fulton et al., 2006; Hommel et al., 2006; Leshan et al., 2010). Interestingly, however, our recent data reveal that VTA LepRb neurons project to and activate neurons in the central amygdala (CeA; presumably CART-expressing neurons related to anxiety behaviors), but not in the NAc (Leshan et al., 2010) (Figure 1.5), suggesting that VTA LepRb neurons may primarily control behaviors other than those involved with food reward (such as anxiety) and that the control of food reward by leptin is mediated by another population of LepRb neurons.

The LHA is a likely site for regulating the mesolimbic DA system, due to its extensive connectivity with the VTA and the NAc (Wise, 2002). The LHA contains distinct populations of neurons that express orexin (OX) or melanin-concentrating hormone (MCH), each of which participate in the control of feeding and measures of reward (Mieda and Yanagisawa, 2002; DiLeone et al., 2003; Leinninger et al., 2009). MCH neurons send projections directly to the NAc and other areas, and OX neurons densely project to the VTA to regulate motivated behaviors, including feeding and drug-taking (Marcus et al., 2001; Georgescu et al., 2003; Zheng et al., 2007)(Figure 1.5). Thus, these neurons directly modulate the mesolimbic system and influence feeding. The LHA also contains a large population of LepRb-expressing neurons and is known as a "feeding center" in energy balance (Leinninger et al., 2009; Caron et al., 2010). Lesioning of the LHA abolishes food intake; animals exhibit no drive to eat and practically starve themselves (Morrison and Mayer, 1957). Since the LHA is a critical regulator of feeding and the mesolimbic DA system (DiLeone et al., 2003; Ahmed et al., 2005; Boutrel et al., 2005; Harris et al., 2005), LepRb-expressing neurons in the LHA represent potential contributors to the regulation of the mesolimbic DA system by leptin. Indeed, we have recently shown that LHA LepRb neurons send axonal projections to the VTA and modulate the expression of tyrosine hydroxylase (TH- the rate-limiting enzyme for DA synthesis) in the VTA (Leinninger et al., 2009) (Figure 1.5).

While LepRb is not co-expressed in either OX or MCH neurons, leptin modulates the function of both of these LHA neuronal populations. Leptin

inhibits OX neuronal activity while increasing OX expression (Yamanaka et al., 2003). Leptin also inhibits MCH expression, while having no effect on activity, suggesting that leptin regulates these two populations through separate pathways or through distinct populations of LepRb neurons (Segal-Lieberman et al., 2003; Yamanaka et al., 2003). Leptin may regulate OX neurons differently than MCH neurons to balance metabolic need and arousal (since OX neurons also regulate arousal in addition to food intake). Indeed, MCH deficient mice are lean while OX deficient mice are obese and hyperphagic, showing that these neuronal populations play different roles in the physiology of energy balance. Thus, the precise neural mechanisms by which leptin controls OX and MCH neurons remains unclear, and identifying the LepRb neurons that innervate and regulate each set of LHA neurons will be essential for understanding the regulation of the mesolimbic DA system and feeding.

C. Leptin as a permissive signal for reproduction

The energetic costs for reproduction are primarily behavioral in male rodents in order to physically dominate over other males and to attract female mates. Meanwhile, the physical demands of pregnancy, partuition and lactation in females in highly energetically demanding. Thus, there lies a fundamental difference between genders with different strategies for balancing the energetic demands for successful reproduction. The energetic demands of reproduction in females are high due to the needs in pregnancy, lactation, and rearing young. Thus, adequate energy stores are required to undertake

these physiological functions. The “critical weight (fat) hypothesis” postulates that advancement to puberty is promoted in part by the achievement of a critical percentage body fat and that the maintenance of reproductive ability requires the persistence of a minimal level of adipose mass (Frisch and Revelle, 1970; Frisch and McArthur, 1974). Severe metabolic stress leading to substantially diminished energy reserves (i.e. abnormally low body fat percentage, thus resembling states of nutritional deprivation), such as in patients with anorexia nervosa or in extremely active elite athletes, are commonly linked to perturbations in reproductive physiology. Reports from patients with anorexia have revealed a strong association of decreased body fat with infertility (Stewart et al., 1990; Sullivan et al., 1998). In addition, it is estimated that 24% of adolescent athletes (in particular endurance athletes partaking in swimming, gymnastics, track, or ballet) have amenorrhea correlated with decreased body fat and energy insufficiency (Shangold et al., 1990; Otis, 1992; Loucks, 2001).

In females with normal cycles there is an age appropriate secretory pattern of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the adenohypophysis in response to pulsatile gonadotropin releasing hormone (GnRH) from the hypothalamus. Negative energy balance-induced amenorrhea is directly linked to decreased GnRH and gonadotropin levels (Loucks et al., 1989; Burke et al., 2006) which can be prevented or reversed by dietary supplementation (Loucks, 2001). However, the neuroendocrine mechanisms responsible for the tight coupling between

energy homeostasis and reproductive functioning have only recently begun to be deciphered.

Leptin induced changes in reproductive milieu.

Leptin-deficient patients and rodents are anovulatory, and leptin treatment results not only in significant weight loss and increased physical activity, but also normalization of menstrual cycles (Licinio et al., 2004). Similarly, extreme athletes and anorexic patients (states which correlate with low body fat and leptin levels (Ballauff et al., 1999; Holtkamp et al., 2003)) present with hypothalamic amenorrhea that is reversed by leptin administration (Laughlin and Yen, 1997; Thong et al., 2000; Welt et al., 2004; Christo et al., 2008). Similarly, in lipodystrophic patients who are hypoleptinemic, treatment with recombinant leptin restored normal menses by restoring LH pulsatility (Musso et al., 2005). Ovarian function was restored when ovaries from leptin-deficient *ob/ob* mice were transplanted into wild-type hosts (Hummel, 1957) or when leptin was centrally administered to *ob/ob* mice (Campfield et al., 1995; Watanobe, 2002). Leptin administration restores gonadotropin secretion in a pulsatile manner (Ahima et al., 1996; Chehab et al., 1996; Nagatani et al., 1998) and reactivation of LepRb in the CNS of *db/db* mice restores reproductive functioning (Coppari et al., 2005). Thus, leptin represents a key hormone linking energy stores to the control of reproductive functioning mainly at the level of the CNS.

Leptin in the central regulation of reproduction

The hypothalamic-pituitary-gonadal (HPG) axis includes GnRH neurons, which induce pulsatile gonadotropin secretion by the pituitary. GnRH neurons represent the final common neural pathway leading to normal reproductive function. Numerous lines of evidence *in vitro* and *in vivo* show that leptin mediates gonadotropin secretion via the (direct or indirect) regulation of hypothalamic GnRH neurons (Chehab et al., 1996; Yu et al., 1997; Finn et al., 1998; Magni et al., 1999; Watanobe, 2002). In particular, leptin acts directly on the hypothalamus to regulate GnRH pulse amplitude (Parent et al., 2000) and GnRH/LH pulse frequency (Finn et al., 1998; Nagatani et al., 1998; Watanobe, 2002; Quennell et al., 2009). Decreased GnRH neuronal activity is correlated with decreased reproductive function and upregulation of food intake (Gomez et al., 2005). Also, in fasted states, GnRH/LH pulsatile secretion is suppressed concomitant with a suppression of leptin secretion (Cameron and Nosbisch, 1991; Nagatani et al., 1998; I'Anson et al., 2000). Since the GnRH neurons that lie in the preoptic area (POA; location of the majority of GnRH neurons in rodents) of the hypothalamus do not express LepRb, but are leptin responsive (Hakansson and Meister, 1998; Nagatani et al., 1998; Cunningham et al., 1999; Quennell et al., 2009), leptin must mediate the hypothalamic control of reproduction indirectly, by acting on upstream neurons (Figure 1.6).

When studying the role of leptin in controlling reproductive physiology, it is also crucial to consider the neural pathways controlling GnRH neurons.

One potential regulator of GnRH neurons is kisspeptin (also called Kiss1), which is encoded by the *Kiss1* gene. Kiss1 neurons in the forebrain act as mediators for the regulation of the HPG axis by several external stimuli (Roa et al., 2008). Thus, Kiss1 represents a well-established gatekeeper of puberty. Reports show that the majority of Kiss1 neurons in the ARC co-express tachykinin 2 (Tac2, also known as neurokinin B (NKB)) and Dynorphin A (hence, "KNDy" neurons) and vice-versa (Goodman et al., 2007; Navarro et al., 2009). GnRH neurons express the receptor for Kiss1 (Ramaswamy et al., 2008; Herbison et al., 2010), and therefore serve as potential targets for Kiss1 action. Indeed, GnRH release is regulated by Kiss1-expressing neurons in the hypothalamus (d'Anglemont de Tassigny et al., 2008; Ramaswamy et al., 2008; Li et al., 2009). Overall, Kiss1 and GnRH neurons are good potential downstream targets for leptin on the HPG axis.

How might LepRb neurons regulate Kiss1/GnRH neurons?

While LepRb-expressing POMC and NPY/AgRP ARC neurons are important for the anorectic response to leptin (Coppari et al., 2005; Fulton et al., 2006; Hommel et al., 2006; Xu et al., 2007), these two populations of ARC neurons appear to mediate little of leptin's effect upon reproduction. CNS melanocortin signaling from the ARC regulates feeding and energy expenditure, but does not alter reproductive function, as animals overexpressing melanocortin antagonists or lacking MC3/4R display normal fertility (Hohmann et al., 2000; Oosterom et al., 2001; Butler and Cone, 2002;

Balthasar et al., 2004). Furthermore, mice lacking LepRb specifically in POMC neurons were reproductively viable, indicating that the ARC POMC neurons are not crucial for reproductive function (Shi et al., 2010).

There is some evidence for a role of NPY in the regulation of reproduction: central administration of NPY suppresses the gonadal axis and NPY projections lie in close proximity to GnRH neurons in the POA (Clark et al., 1985; Smith and Grove, 2002). While genetic deletion of NPY modestly improves reproduction in ob/ob mice (Erickson et al., 1996), this effect may not be leptin-specific, as disruption of leptin action in the ARC POMC and AgRP neurons does not impair fertility (Balthasar et al., 2004; Shi et al., 2010). Hence, other populations of LepRb neurons besides these two well-known ARC LepRb populations must mediate crucial components of leptin action, including important aspects of the regulation of reproduction.

Candidate populations of LepRb neurons likely to participate in the control of the reproductive axis include poorly characterized populations of non-POMC, non-NPY neurons in the ARC (e.g. Kiss1-expressing ARC neurons) and LepRb-expressing neurons in the PMv (Elias et al., 2000). Some leptin action on the reproductive axis may be mediated by the direct or indirect regulation of Kiss1-expressing neurons in the ARC or AVPV that play a major role in integrating numerous signals to control the overall stimulatory tone onto the GnRH neurons (Figure 1.6) (Smith et al., 2006a). A large population of LepRb-expressing neurons is located in the PMv (Donato et al., 2009; Leshan et al., 2009) and may serve as a mechanism linking energy

balance to reproduction. PMv neurons project to the preoptic area (POA) and other nearby regions, where elements of the reproductive axis (e.g. GnRH or Kiss1 neurons) reside (Canteras et al., 1992; Leshan et al., 2009). Moreover, neurons in the PMv regulate reproductive behavior in males and coordinate GnRH release and ovulation in response to seasonal and other inputs in females (Canteras et al., 1992; Simerly, 1998; Rondini et al., 2004; Sliwowska et al., 2004; Hahn and Coen, 2006; Leshan et al., 2009). PMv LepRb neurons therefore represent a major population of LepRb-expressing/leptin-responsive neurons in the brain that contribute to reproductive function (Elias et al., 2000; Leshan et al., 2009). However, there is more to be elucidated about leptin's central mechanism in the regulation of reproduction.

Other neuroendocrine functions of leptin

In addition to reproduction, leptin also promotes normal thyroid function, growth, and the activity of the sympathetic nervous system (SNS), as well as suppressing the release of glucocorticoids. Clearly, while some thyrotropin-releasing hormone (TRH) neurons in the PVN are regulated via projections from ARC LepRb neurons, a separate population of TRH-expressing periventricular nucleus (PVH) neurons expresses LepRb and is regulated by direct leptin action (Perello et al., 2006). These alternately regulated populations of TRH-expressing neurons may subserve different functions, with those that are indirectly regulated by the ARC LepRb neurons most prominently involved in the regulation of thyroid function, per se. Leptin-

deficient *ob/ob* mice, which have stunted growth, have decreased circulating growth hormone, which is reversed by leptin treatment (Luque et al., 2007). The mechanism(s) by which leptin regulates the growth axis are less clear; the regulation of growth hormone, GHRH, and somatostatin secretion by leptin has not been fully explored, and at least some of the growth effects of leptin may be secondary to the effect of leptin on bone formation. *Ob/ob* mice have elevated glucocorticoid levels, which are also reversed by leptin (Slieker et al., 1996). The regulation of glucocorticoid secretion is likely to be mediated by numerous pathways, but could involve the ARC→PVH circuit, as the PVH contains neurons which secrete corticotropin-releasing hormone (CRH), thus is an important site for the integration of the adrenal axis. Leptin also regulates lactation, although the potential roles for prolactin secretion, oxytocin, and mammary development have not been thoroughly investigated.

Hypothesis tested by this dissertation research. Leptin acts via a distributed network of LepRb-expressing neurons in the brain and each population presumably contains a different complement of neurotransmitters and innervates different target neurons to mediate specific aspects of leptin action, such as hedonic feeding or reproductive function. Therefore, my **overall hypothesis is that specific populations of LepRb-expressing neurons innervate and regulate distinct neural targets to control different physiological processes**. To truly understand the neural mechanisms of leptin action, we must study the circuitry at specific and detailed levels. While

a great deal is known about the ARC LepRb circuitry, less is known about how leptin/LepRb may modulate the LHA/mesolimbic DA system and the neuroendocrine reproductive system (among others). Defining the circuitry by which leptin may act on specific systems will require LepRb-specific tract tracing tools. Thus, **I developed and tested such tracing tools that utilize the trans-synaptic tracer, wheat germ agglutinin (WGA) to study LepRb circuitry.** Furthermore, I utilized these new sets of tools to explore two hypotheses about leptin action in the brain: **A) LHA LepRb-expressing neurons innervate local LHA neurons involved in the regulation of the mesolimbic system (tested in Chapter 2), B) Specific subpopulations of LepRb neurons contribute to the control of reproduction by innervating GnRH and Kiss1 neurons (tested in Chapter 3).** We first utilized transgenic mice that express WGA in LepRb-expressing neurons to visualize LepRb neurons and their target neurons in the LHA. We also used a WGA adenoviral system to explicitly study LepRb neurons in the LHA then tested this functional circuit. For our second hypothesis, we again utilized the WGA transgenic mouse to trace LepRb neurons to GnRH neurons or Kiss1 neurons in the ARC or AVPV, neurons that modulate the hypothalamic reproductive system. We also employed other transgenic mouse models to identify neurons that directly project to GnRH neurons and determined whether leptin functionally regulated the LepRb → Kiss1 circuit. Defining the unique neural pathways by which leptin regulates different physiological functions is crucial for our

understanding of the totality of leptin action in energy homeostasis and whole body physiology.

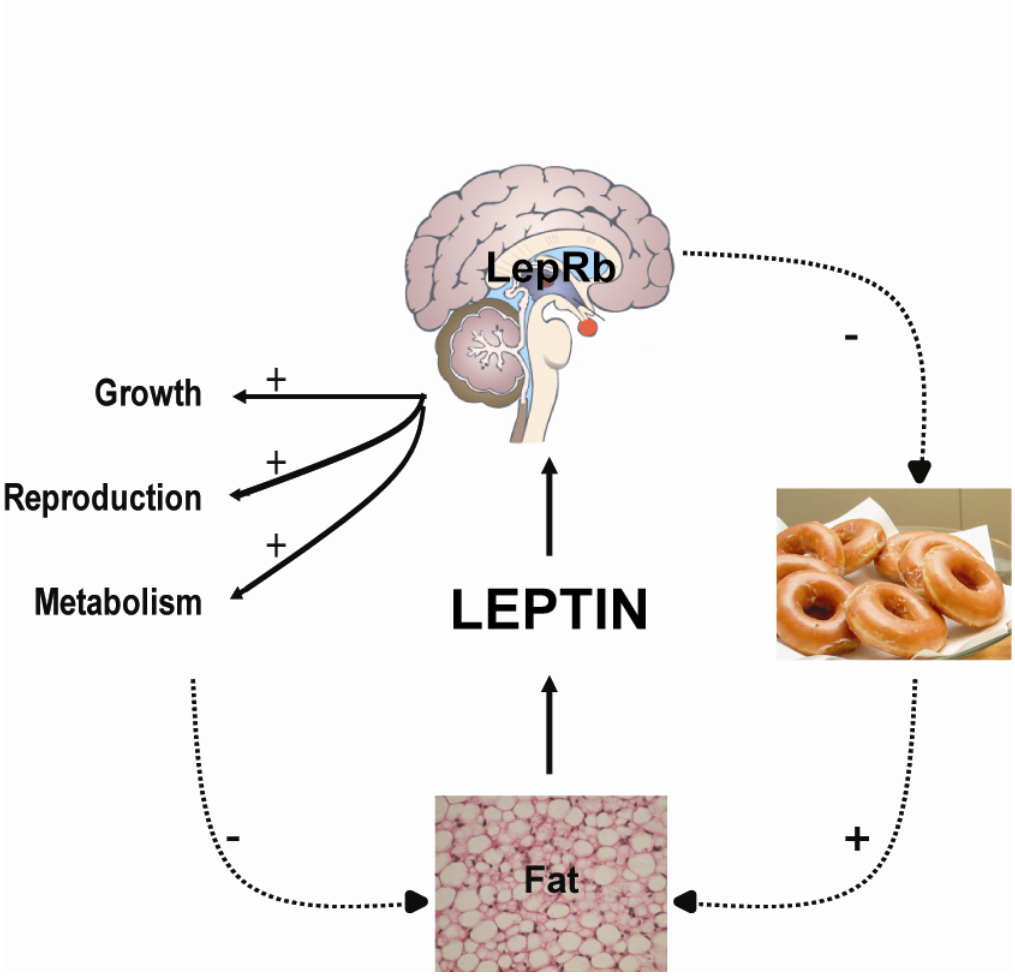

Figure 1.1 - Leptin signals body energy status information to the CNS to regulate physiology. Leptin production and release by adipocytes (fat) is regulated by nutritional status as well as by fat content. With adequate energy stores, leptin activates the long form of its receptor (LepRb) in the brain to permit energy expenditure on processes such as reproduction or growth, while suppressing feeding. (Adapted from Myers, Recent Prog Horm Res, 2004).

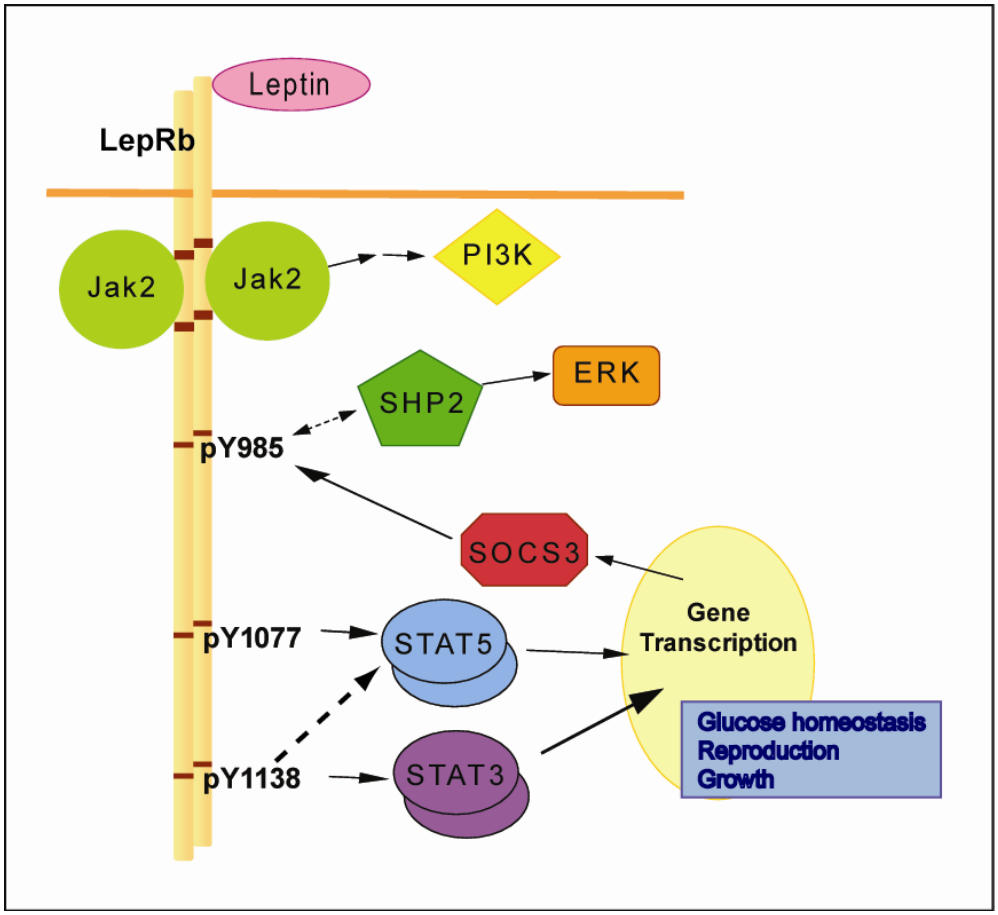

Figure 1.2 Intracellular LepRb signaling pathways. Leptin binding to LepRb activates the associated Janus kinase-2 (Jak2), which auto-phosphorylates and activates downstream tyrosine residues on the intra-cellular domain of LepRb (Tyr^{985}, Tyr^{1077}, Tyr^{1138}). Phosphorylated Tyr^{985} recruits SHP-2 to activate the ERK pathway. Phosphorylated Tyr^{1138} recruits the signal transducer and activator of transcription-3 (STAT3) and its nuclear translocation mediates transcription of several genes including SOCS3. SOCS3 binds to Tyr^{985} and promotes feedback inhibition of LepRb signaling. STAT5 is predominantly recruited by phosphorylated Tyr^{1077}, and to a lesser extent by Tyr^{1138}. Thus, these signaling pathways mediate leptin signaling to control physiological responses such as glucose homeostasis, reproduction or growth.

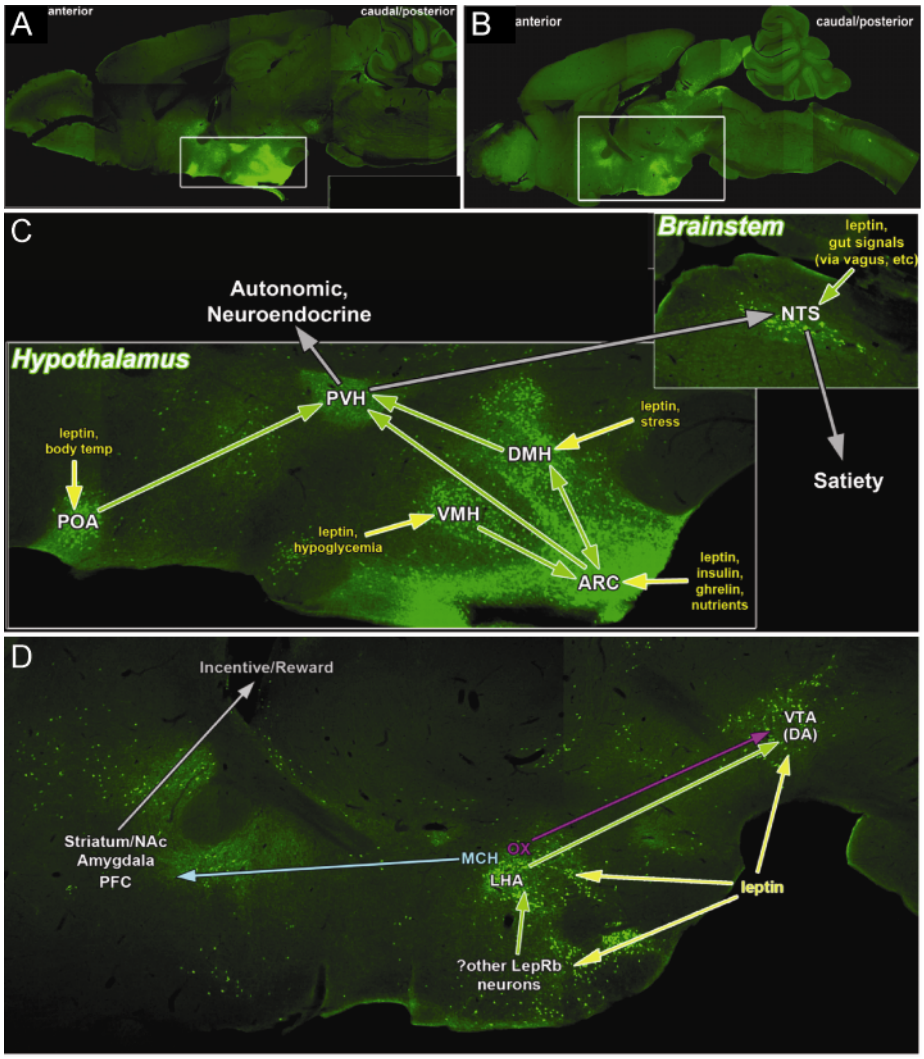

Figure 1.3 Multiple populations of LepRb-expressing neurons in the CNS integrate several external signals. Saggital sections from LepRb^{EGFP} mice show neuroanatomical distribution of LepRb-expressing neurons (green) in the CNS and include the medial hypothalamus and brainstem (A&C) and the lateral portion of the hypothalmus (B&D). Nutrient-sensing ARC and VMH LepRb populations signal through the PVH and the brainstem to regulate feeding and neuroendocrine energy expenditure thus are "satiety centers" of energy balance (C). The LHA and VTA LepRb neurons, as well as LHA MCH and OX neurons, are areas involved in the incentive to feed (D). Panels C&D are magnified views of boxed regions in A&B, respectively. (Images from Myers et al., Cell Metabolism Review, 2009).

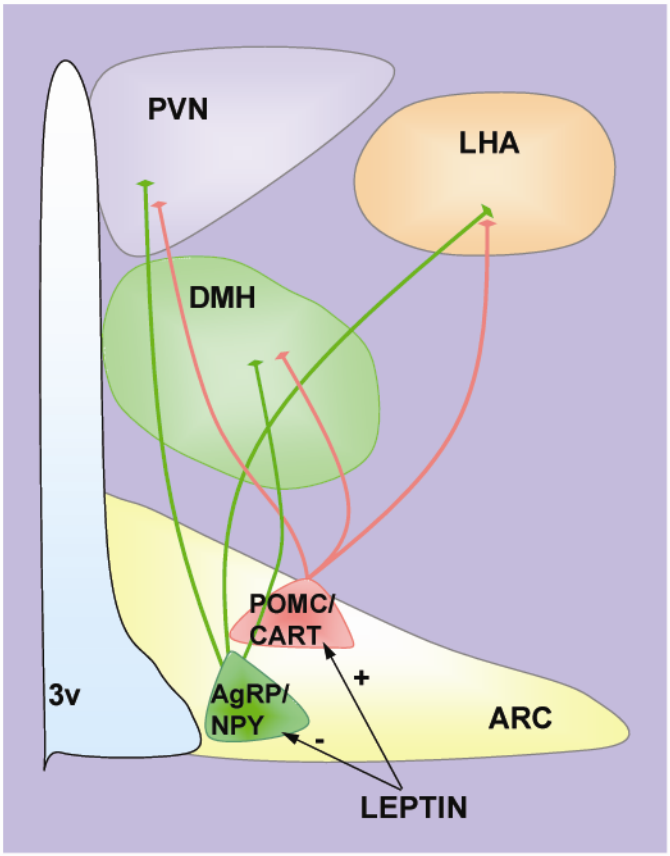

Figure 1.4 LepRb neuronal circuits in the ARC hypothalamus. Leptin stimulates anorexigenic neurons that co-express POMC and CART and inactivates orexigenic AgRP/NPY neurons in the ARC to control feeding and energy homeostasis. These neurons densely innervate the parvocellular neurons in the PVH as well as neurons in the DMH and LHA to regulate feeding and neuroendocrine energy expenditure. (Adapted from Schwartz et al., Nature 2000).

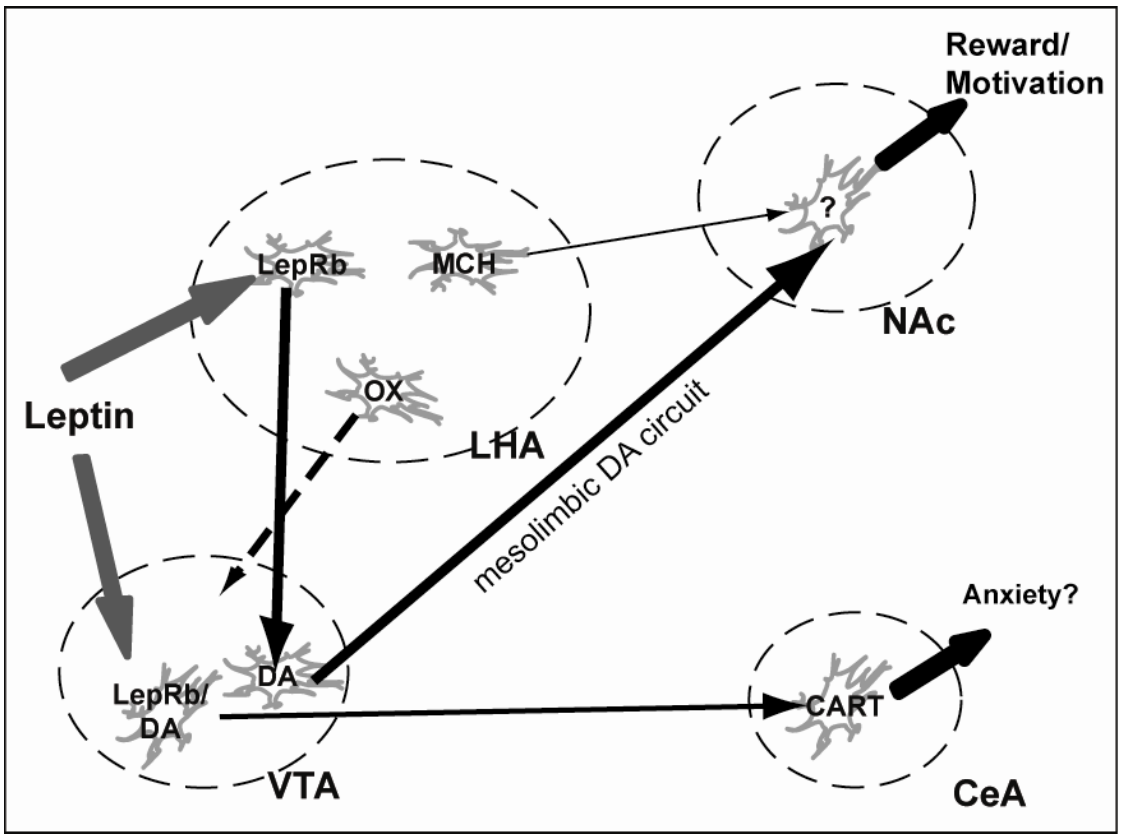

Figure 1.5 LepRb neuronal circuits of the LHA to control neurons of the mesolimbic DA system. LepRb neurons in the LHA project to the DAergic neurons in the VTA to modulate the mesolimbic dopamine reward system. OX neurons in the LHA also project to the VTA and are involved with motivation and reward (dashed lines). LHA MCH neurons project to neurons in the mesolimbic DA system. Meanwhile, DA-LepRb neurons in the VTA project to the CART neurons in the CeA to regulate anxiety-like behaviors. Overall, this complex circuitry pattern modulates the mesolimbic DA system to control motivation and reward relevant to feeding.

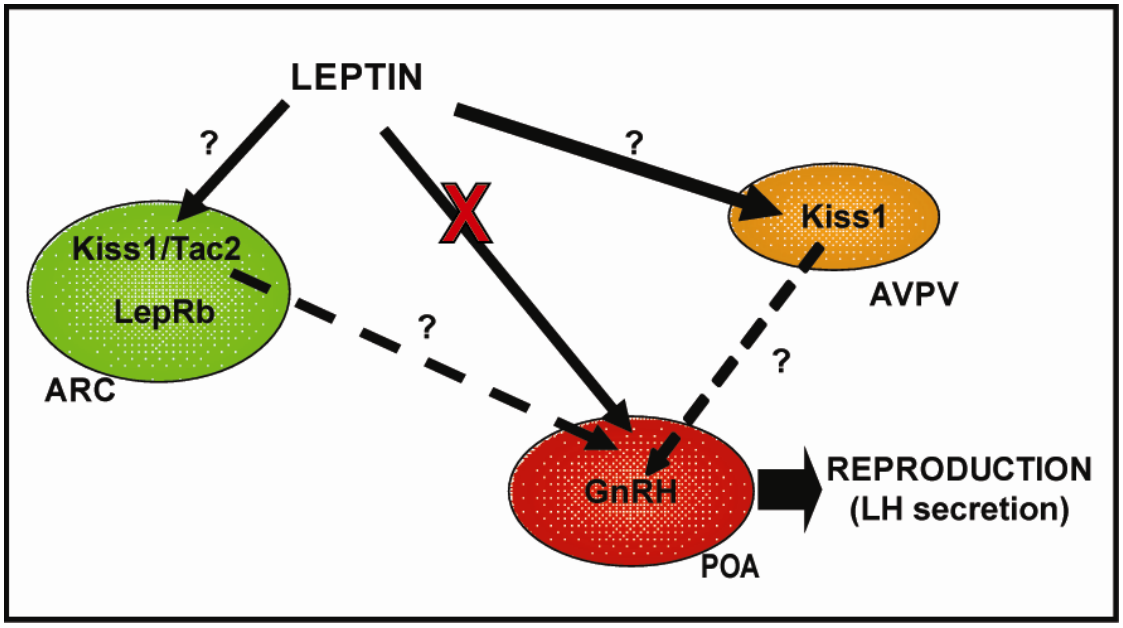

Figure 1.6 Potential targets for leptin in the regulation of the central reproductive axis. Kiss1 and GnRH neurons in the hypothalamus are known to be involved in the regulation of LH secretion, however leptin does not directly control GnRH neurons (as indicated by the red X). Leptin may potentially control Kiss1 neurons in the ARC or AVPV to modulate pulsatile GnRH/LH secretion. Our goals are to determine whether leptin is regulating these Kiss1 neuronal populations (solid arrows) and which populations of LepRb populations are innervating GnRH or Kiss1 neurons (not shown). Additionally, we will determine whether Kiss1 populations directly innervate GnRH neurons (dashed arrows).

CHAPTER TWO

DIRECT INNERVATION AND CONTROL OF OREXIN NEURONS BY LATERAL HYPOTHALAMIC LEPR-B NEURONS

Summary

Leptin, the adipose-derived hormonal signal of body energy stores, acts via the leptin receptor (LepRb) on neurons in multiple brain regions. There exist LepRb neurons in the lateral hypothalamic area (LHA) that are distinct from neighboring leptin-regulated melanin concentrating hormone (MCH)- or orexin (OX)-expressing cells. Neither the direct synaptic targets of LHA LepRb neurons nor their potential role in the regulation of other LHA neurons have been determined, however. We thus generated several adenoviral and transgenic systems in which cre recombinase promotes the expression of the tracer, wheat germ agglutinin (WGA), and utilized these in combination with $LepRb^{cre}$ mice to determine the neuronal targets of LHA LepRb neurons. Here, we reveal that some LHA LepRb neurons project to dopamine neurons in the ventral tegmental area (VTA). LHA LepRb neurons also densely innervate the LHA where they directly synapse with OX, but not MCH, neurons. Indeed, few other LepRb neurons in the brain project to the OX-containing region of the mouse LHA, and direct leptin action on LHA LepRb

neurons increases gene expression in OX neurons and activation of neurons in the VTA. These findings thus reveal a major role for LHA leptin action in the control of OX neurons, suggesting the importance of LHA LepRb neurons in the regulation of OX signaling that is crucial to leptin action and metabolic regulation.

Introduction

Leptin, a polypeptide hormone that is produced by adipocytes in proportion to fat content, signals the repletion of body energy stores to modulate neural processes linked to energy balance (Friedman, 2002; Elmquist et al., 2005; Morton et al., 2006; Myers et al., 2009). Circulating leptin decreases feeding and permits energy expenditure by activating the long-form of its receptor (LepRb) in the brain. Numerous brain regions contain LepRb-expressing, leptin-responsive neurons- including the hypothalamus, where large numbers of LepRb neurons reside in the arcuate (ARC), ventromedial (VMH), and ventral premammillary (PMv) nuclei, as well as the preoptic (POA) and lateral hypothalamic (LHA) areas (Elmquist et al., 1998; Dhillon et al., 2006; Donato et al., 2009; Leinninger et al., 2009; Leshan et al., 2009; Myers et al., 2009; Scott et al., 2009). LepRb neurons are also found in midbrain sites including the ventral tegmental area (VTA) and dorsal raphe (DR), and brainstem nuclei, such as the nucleus of the solitary tract (NTS) (Figlewicz et al., 2006; Fulton et al., 2006; Grill, 2006; Hommel et al., 2006; Myers et al., 2009; Scott et al., 2009; Leshan et al., 2010). While LepRb

neurons in the well-studied ARC, including POMC- and AgRP-expressing cells, contribute importantly to energy balance, these neurons mediate only a fraction of overall leptin action (Balthasar et al., 2004; Dhillon et al., 2006; Clarkson et al., 2009; Myers et al., 2009), suggesting the importance of the many non-ARC LepRb neurons throughout the brain.

The medial basal hypothalamus (including the ARC) modulates satiety, but a variety of data suggest roles for the LHA in controlling the incentive to feed, including by regulating the mesolimbic dopamine (DA) reward system (Georgescu et al., 2003; Georgescu et al., 2005; Harris et al., 2005; Kelley et al., 2005). Indeed, several populations of LHA neurons have been implicated in the regulation of feeding and the mesolimbic DA system. In addition to GABAergic LHA LepRb neurons, which modulate the expression of tyrosine hydroxylase (*Th*; the enzyme that catalyzes the rate-limiting step in DA production) in the mesolimbic DA system in response to leptin (Leinninger et al., 2009), the LHA contains populations of widely-projecting neurons that express the neuropeptides orexin (OX) or melanin concentrating hormone (MCH). Among their other roles, LHA OX neurons project to the VTA, where OX controls drug and food reward; MCH neurons project to the striatum to modulate similar parameters (DiLeone et al., 2003; Harris et al., 2005; Sharf et al., 2009). Leptin decreases MCH action by inhibiting *Mch* expression and blunting endocannabinoid-mediated depolarization-induced suppression of inhibition on MCH neurons (Elias et al., 1998; Jo et al., 2005). The modulation of OX neurons by leptin appears more complex: On one hand, leptin inhibits

the firing of OX neurons in slice preparations and blocks the fasting-induced activation of OX neurons detected by c-fos immunostaining *in vivo* (Mieda and Yanagisawa, 2002; Yamanaka et al., 2003; Funato et al., 2009). Conversely, leptin promotes *Ox* mRNA expression, and OX signaling via the OX2R contributes to leptin action on energy balance (Tritos et al., 2001; Mieda and Yanagisawa, 2002; Yamanaka et al., 2003; Funato et al., 2009). Thus, while leptin inhibits the activity of OX neurons, leptin also promotes *Ox* expression and chronic OX signaling functions in concert with leptin action.

The neural mechanisms by which leptin modulates LHA neurons, such as those expressing OX and MCH, and the potential interaction of LHA LepRb neurons with these neurons (as well as neurons in the VTA and elsewhere) remain unclear. To probe these issues, we generated and utilized several genetic tools; one that expresses a farnesylated EGFP in LepRb neurons to trace long axonal architecture and some that employ the trans-synaptic neuronal tracer wheat germ agglutinin (WGA), to specifically examine LepRb neurons and their synaptic contacts. We show that, in addition to projecting onto VTA DA neurons, LHA LepRb neurons directly innervate local OX, but not MCH neurons. Furthermore, leptin action directly in the LHA regulates OX neuron gene expression.

Materials and Methods

Materials. Leptin was the generous gift of Amylin Pharmaceuticals, Inc. (San Diego, CA).

Experimental animals. The generation of *Lepr*$^{cre/cre}$ (LepRb^{Cre}) mice has been described previously (Leshan et al., 2009); these animals were produced by intercrossing homozygous animals within our facility. *Lepr*$^{cre/cre}$ mice were bred with Gt(*ROSA)26-Sor*^{tm2Sho} mice purchased from Jackson Laboratory to generate double homozygous *Lepr*$^{cre/cre}$*;Gt(ROSA)26-Sor*$^{tm2Sho/tm2Sho}$ (LepRb^{EGFP}) mice, which were propagated by intercrossing. Male C57Bl/6 and *Lep*$^{ob/ob}$ animals 8-10 wk of age were purchased from Jackson Laboratory. For the generation of *iZ/WAP* mice, the coding region for wheat germ agglutinin (WGA) was PCR-amplified from pBluescript II SK-WGA (the generous gift of Dr. Yoshihiro Yoshihara, RIKEN Brain Science Institute, Japan (Sullivan et al., 2003)) and inserted into the pCALL2-IRES-hAP/cg vector (iZ/AP) (the generous gift of Dr. Corrine Lobe, Toronto, ON (Allen et al., 2006)) downstream of the CMV promoter-driven floxed β-geo cassette and upstream of an IRES-alkaline phosphatase (AP) sequence. The resulting pCALL2-WGA/AP (*iZ/WAP*) plasmid was submitted to University of Michigan transgenic core for production of transgenic embryonic stem cell clones. Four hundred and eighty clones were screened for single copy number by qPCR for neo sequences and also screened for β-gal expression via immunocytochemical staining (Roche). Five ES clones were expanded and rescreened, and three positive ES clones were injected into blastocysts and implanted into foster mothers. The resulting chimeric male progeny were bred to C57/Bl6 females for the determination of germline transmission (by brown coat color) and confirmed via Southern blotting for the Neo cassette. Several

F1 generation *iZ/WAP* mice from each ES clone were perfused and screened for CNS β-gal expression by immunofluorescent staining using antibodies against β–gal, as detailed below. One *iZ/WAP* line was determined to express the transgene ubiquitously in the CNS, and was chosen for further study. While similar in genesis to previously reported transgenic animals (Braz et al., 2002), this new line demonstrates broader transgene expression in the brain- including the hypothalamus. Subsequent *iZ/WAP* litters were genotyped by conventional PCR utilizing oligos derived from the original WGA sequence (Forward: AATGAGAAAGATGATGAGCACC; Reverse: AGGTTGTTCGGGCATAGCTT). *iZ/WAP* animals were bred with LepRb^{EGFP} mice (described above) to generate *Lepr$^{cre/cre}$;ROSA26$^{EGFP/EGFP}$;iZ/WAP* (LepRb$^{WGA/EGFP}$) animals for study. In order to determine the optimal time point for trans-synaptic tracing by WGA, we analyzed LepRb$^{WGA/EGFP}$ mice at different ages. LepRb$^{WGA/GFP}$ mice analyzed as early as 4 weeks of age contain much fewer WGA-labeled connections than adult mice. Therefore, we utilized post-pubertal adult male mice for WGA tracing studies.

Animals were housed in our colony in 12h light/dark cycles and given *ad libitum* access to food and water. All care and procedures for mice were according to guidelines approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA).

Perfusion, immunohistochemistry and immunofluorescence. Perfusion and immunohistochemistry were performed essentially as previously described (Munzberg et al., 2007). Briefly, mice were deeply anesthetized

with a lethal dose of intraperitoneal (i.p.) pentobarbital (150 mg/kg) and transcardially perfused with sterile PBS then 10% neutral buffered formalin. Brains were removed, postfixed overnight and dehydrated in a 30% sucrose solution. Then brains were sectioned into 30 μm coronal slices, collected in four consecutive series and stored at –20 °C in cryoprotectant until further use.

Brain sections were incubated in primary antibodies [goat anti-βgal (1:3000, Biogenesis), chicken anti-GFP (1:1000, Abcam), rabbit anti-MCH (1:1000, Phoenix Pharmaceuticals), goat or rabbit anti-OX (1:1000, Santa Cruz/Calbiochem, respectively), goat anti-WGA (1:1000, Vector), mouse anti-TH (1:200, Chemicon)] overnight at 4°C, and then visualized by immunofluorescent secondary detection using species-specific Alexa 488 or 568 antibodies (1:200, Invitrogen). Sections were mounted on slides and coverslipped with Prolong antifade mounting medium (Invitrogen).

Adenoviral Tracers. The Ad-iZ/EGPFf adenoviral system to trace long axonal processes has been described previously (Leinninger et al., 2009; Leshan et al., 2009). For the generation of Ad-iN/WED, we first deleted the β-gal-encoding region of the β-geo fusion in pShuttle/iZ (Leinninger et al., 2009; Leshan et al., 2009) by PCR using the Quikchange kit (Stratagene), generating pShuttle/iN (thereby decreasing the size of the construct to promote proper viral packaging with larger inserts). The coding region of WGA, as above, along with an IRES element and the coding sequence for farnesylated dsRED (dsRedf), was then subcloned into the MCS of pShuttle/iN

vector to generate pShuttle-iN/WED. Vector DNA was purified, linearized and utilized to generate the cre-inducible WGA adenoviral vector (Ad-iN/WED). Concentrated adenoviral stocks were generated and purified as previously described (Morton et al., 2003; Leshan et al., 2009). Cre-dependent WGA expression from Ad-iN/WED was verified *in vitro* by immunocytochemical staining for WGA in infected HEK293 cells also transfected with a vector encoding cre recombinase.

LHA-specific LepRb tract tracing experiments. For tract tracer experiments, adult male $LepRb^{cre}$ or $LepRb^{EGFP}$ mice were anesthetized using isoflurane and placed in a stereotaxic apparatus. After exposing the skull, a guide cannula with stylet was lowered into LHA coordinates (from bregma) (AP:-1.34; ML:-1.1, DV: -5.1) according to Franklin and Paxinos mouse brain atlas (Franklin and Paxinos, 1997). The stylet was removed and replaced by an injector and either 50 nl of 4% fluorogold-equivalent (Sigma) or 200-500 nl of Ad-iZ/EGPFf or Ad-iN/WED was acutely injected, using a 500nl Hamilton syringe at a rate of 100 nl/min. After 10 minutes to allow for absorption of tracer, the injector and cannula were removed and the skull and the incision was sealed and sutured. Mice received pre- and post-surgical analgesia (150µl of 0.01mg/ml Buprenex) and were individually housed for either 2 days (FG-mediated retrograde tracing) or 5 days (Ad-iZ/EGPFf and Ad-iN/WED-mediated anterograde tracing) before perfusion and processing. We initially analyzed Ad-iN/WED and Ad-iZ/EGFPf tracing in $LepRb^{cre}$ mice at 1, 2, 5 and 11 days post-infection to show minimal tracing at 1 and 2 days, but more

robust tracing at 5 days post-infection. Detection of WGA-IR 11 days post-infection did not differ substantially from 5 days, thus we utilized the 5 day time point for further analysis. The total sample size of mice injected with Ad-iN/WED was 52, with approximately 30% of hits targeted specifically to the dorsal perifornical area of the LHA and considered “good hits”. Of these 15 samples with good hits, most samples showed robust WGA-IR in the LHA injection site and these were subsequently analyzed.

Data Collection and analysis. Slides were analyzed via light or fluorescent microscopy using an Olympus BX-51 microscope with filters for Alexa 488 or Alexa 568 and images taken with software as previously described (Munzberg et al., 2007). Using Adobe Photoshop software (Adobe Systems, San Jose CA) images were overlaid in different RGB channels to reveal single- or double-labeled cells. Confocal images were captured with an Olympus FV-500.

Leptin treatment to assess changes in LHA gene expression. Adult male *Lep*$^{ob/ob}$ mice were obtained to assess for gene expression changes by leptin. The first cohort received systemic (i.p) treatment of sterile PBS (n=10) or leptin (5mg/kg; n=10) and the second cohort (total n=20) underwent surgical implantation of cannulae into the LHA (AP:-1.30; ML:-1.12; DV:-4.15), as described previously (Leinninger et al., 2009). Briefly, cannulae with dummy injectors were stereotaxically inserted and affixed into the LHA site. After surgery, mice were single housed and checked daily for food intake and body weight to monitor their recovery. After 7-10 days of recovery, mice were

treated with 250nl of either sterile PBS (n=10) or leptin (0.001 ng/nl, thus each dose = 0.25ng; n=10). Both cohorts were treated with either PBS or leptin every 12 h for a total of 26 hr with the final dose given 2 h before sacrifice and microdissection of brain tissue, as in according to previous studies (Leinninger et al., 2009).

RNA extraction and analysis of gene expression by qPCR. LHA and ARC were microdissected and snap frozen for later processing and analysis of mRNA expression by quantitative RT-PCR (as previously described) (Leinninger et al., 2009). Briefly, RNA was extracted using TRIzol (Invitrogen) and converted to cDNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). cDNA was analyzed in triplicate via qRT-PCR for *Gapdh* and neuropeptide gene expression (as previously described (Bates et al., 2003)), or with assays supplied by Applied Biosystems using an Applied Biosystems 7500. Gene expression was normalized to *Gapdh* expression and relative mRNA expression was calculated via the $2^{-\Delta\Delta Ct}$ method. For cannulated mice, data from the ipsilateral (cannulated) side were also normalized to the contralateral (unperturbed) side. Statistical significance was determined by Student's t test as computed with Excel.

Leptin treatment to assess changes in downstream neuronal activation. Adult male C57/Bl6 wild-type mice (n=5 per treatment group) were obtained and cannulae implanted into the LHA, as described above. Following recovery, mice were treated acutely with either PBS or leptin (as above) for 15 min before perfusion. Acute treatment results in rapid changes in neuronal

activation, whereas chronic treatment (as in previous experiment) is required to assess longer-term transcriptional gene expression changes. Brain sections were analyzed immunohistochemically for phosphorylated cAMP response element-binding (pCREB) protein (1:100, Cell Signaling) to assess neuronal activation of downstream neurons in the VTA by intra-LHA leptin. Our previous experience reveals that intra-LHA cannulation interferes with the examination of LHA c-fos-IR (data not shown), and that leptin-induced cfos is undetectable in the VTA, thus we focused on the downstream induction of pCREB in response to intra-LHA leptin infusion. CREB is a transcription factor that, when phosphorylated, induces intracellular transcription changes secondary to growth factors and neuronal activation, and therefore serves as a surrogate marker of neuronal activation/response.

Results

*Tools to examine neural mechanisms of leptin action***.** As the identification of LepRb neurons by direct detection of LepRb remains problematic, we previously generated $LepRb^{EGFP}$ reporter mice (Leinninger et al., 2009; Leshan et al., 2009; Myers et al., 2009) (Figure 2.1A-C). While EGFP expression in $LepRb^{EGFP}$ reporter mice revealed that LHA LepRb neurons are distinct from MCH and OX neurons (Leinninger et al., 2009) (Figure 2.1B,C), reports have shown that leptin modulates the activity and gene expression of MCH and OX neurons (Mieda and Yanagisawa, 2002; Yamanaka et al., 2003; Jo et al., 2005; Funato et al., 2009), suggesting that

LepRb neurons synapse with these LHA neurons to regulate them. To permit the examination of projections from LepRb neurons and the identification of their synaptic targets, we thus generated transgenic animals and adenoviral systems to promote the expression of farnesylated EGFP (EGFPf; which is targeted to the membrane and thus more robustly reveals axonal projections than standard cytoplasmic EGFP) or wheat germ agglutinin (WGA; a lectin that passes trans-synaptically to accumulate in target neurons (Braz et al., 2002; Sullivan et al., 2003; Zylka, 2005) (Figure 2.1A).

LepRb$^{EGFP/WGA}$ mice reveal LHA neurons that lie in synaptic contact with LepRb neurons. To examine whether LepRb neurons lie in synaptic contact with MCH and OX neurons in the LHA, we generated the *iZ/WAP* transgenic mouse line, in which cre recombinase promotes the expression of WGA in cre-expressing neurons. We bred *iZ/WAP* to the *Lepr^{cre};ROSA26-EGFP* (LepRb^{EGFP}) background to produce LepRb$^{EGFP/WGA}$ animals in which LepRb neurons express EGFP and WGA, and in which WGA accumulates in non-EGFP-expressing neurons that lie in synaptic contact with LepRb neurons (Figure 2.1A). While an internal ribosome entry site (IRES) and the sequence encoding alkaline phosphatase (AP) was inserted into our construct downstream of WGA, the IRES-mediated expression of AP proved to be insufficient to mediate its detection in the CNS (data not shown), necessitating the use of cre-inducible EGFP to label primary LepRb neurons.

Staining for WGA in LepRb^{EGFP} mice or in *iZ/WAP* mice not containing a cre allele revealed no detectable WGA-immunoreactivity (-IR) (data not shown), but numerous strongly WGA-IR (as well as GFP-IR) neurons were detected in the hypothalamus of LepRb$^{EGFP/WGA}$ mice, including in the LHA (Figure 2.2A-L). Co-staining for GFP/LepRb and WGA revealed neurons within the LHA that contained both GFP- and WGA-IR neurons, representing WGA-expressing LHA LepRb neurons (Figure 2.2A-D, arrows). Additionally, we detected many WGA-IR cells that were devoid of GFP-IR, representing neurons that lie in synaptic contact with LepRb neurons, but which are not themselves LepRb neurons (Figure 2.2A-D, arrowheads). Also, in utilizing these LepRb$^{EGFP/WGA}$ mice, we explored whether LepRb neurons communicate with MCH and/or OX neurons by examining the colocalization of WGA with either MCH-IR or OX-IR neurons (Figure 2.2 E-H, I-L, respectively). This analysis revealed a substantial number of MCH and OX neurons containing strong WGA-IR. Thus, while MCH and OX neurons in the LHA do not express LepRb to directly respond to leptin, they represent synaptic targets of LepRb neurons, consistent with the previously described regulation of OX and MCH neurons by leptin (Elias et al., 1998; Yamanaka et al., 2003). Since LepRb neurons throughout the brain express WGA, the analysis of LepRb$^{EGFP/WGA}$ mice does not identify the specific population of LepRb neurons that project onto MCH or OX neurons, however.

Few extra-LHA LepRb neurons project to the dorsal perifornical LHA. To identify brain regions containing LepRb neurons that might project onto neurons in the LHA, we injected the retrograde tracer, fluorogold (FG), into the LHA of $LepRb^{EGFP}$ mice (Figure 2.3A), and analyzed these brains for regions containing FG- and GFP-IR (Figure 2.3B-F). FG was specifically injected into the dorsal perifornical LHA in the region containing LHA LepRb neurons (Figure 2.3A), which are co-distributed with OX neurons (Figure 2.1C). Several brain regions contained both LHA-projecting (FG-IR) and LepRb (GFP-IR) neurons, including the VTA (Fig 2.3C,D) and the POA (Figure 2.3E,F), although only a few LepRb/GFP neurons in the VTA accumulated FG from the dorsal perifornical LHA. Little or no FG from this region of the LHA accumulated in ARC neurons (Figure 2.3B). Consistently, few neuropeptide-IR projections from ARC POMC (αMSH) and AgRP neurons were detected in the main OX field, but rather densely innervated the surrounding area (Figure 2.4A&B, respectively). These data reveal that few extra-LHA LepRb neurons project to the region of the LHA containing LepRb and OX neurons, thus suggesting that LHA LepRb neurons might themselves represent the primary LepRb neurons that innervate this area and the OX neurons contained therein.

Innervation of the VTA and LHA by LHA LepRb neurons. To more closely examine the potential innervation of the perifornical LHA by LHA LepRb neurons, we first injected the previously described Ad-iZ/EGFPf (Leinninger et al., 2009; Leshan et al., 2009) into $LepRb^{cre}$ mice (Figures 2.1A,

and 2.5), thus mediating the expression of EGFPf specifically in cre-expressing (i.e., LepRb) neurons. Since EGFPf is targeted to the membrane, it robustly reveals the axonal architecture of EGFPf-expressing cells. We thus injected Ad-iZ/EGFPf into the LHA of LepRb^{cre} mice (Figure 2.5A), promoting the expression of EGFPf in LHA LepRb neurons, and analyzed the GFP-IR neurites within the LHA (Figure 2.5B,C). Many of these neurites demonstrated a “beads on a string” appearance consistent with the presence of synapses, similar to appearance of distant LHA LepRb axons in more distant regions, such as the VTA (Figure 2.5D-F). These data thus suggest that LHA LepRb neurons may innervate local, as well as distant, neurons. Furthermore, in analyzing these regions for GFP-IR and OX-IR, we detect EGFP/LepRb fibers in close contact with OX neurons (Figure 2.5G-L), suggesting that LHA LepRb neurons may innvervate local OX neurons.

To reveal specific populations of neurons that lie in synaptic contact with LHA LepRb neurons, we generated the transsynaptic adenoviral tracer, Ad-iN/WED, to mediate the expression of a WGA/EGFP fusion protein in cre-expressing cells (Figure 2.1A). Thus, site-specific injection of Ad-iN/WED to cre-expressing animals is expected to promote the expression of WGA/EGFP in anatomically restricted populations of cre-expressing neurons and WGA/EGFP accumulation in the synaptic targets of these cre-expressing neurons. While Ad-iN/WED was also designed to express dsREDf to reveal the neurites of the cre-expressing cells, IRES-mediated dsRedf expression

was insufficient to enable its detection in *in vitro* tests or *in vivo* (data not shown).

Intra-LHA administration of Ad-iN/WED of LepRb^{cre} mice resulted in WGA-IR in the LHA of LepRb^{cre} mice (Figure 2.6A,B); no WGA-IR was detected following the injection of Ad-iN/WED in non-cre-expressing mice, however (data not shown). We analyzed WGA-IR neurons in LepRb^{cre} mice where the injection sites were restricted to the dorsal perifornical area of the LHA in order to determine the areas innervated by LHA LepRb neurons (Figure 2.6A,B), and initially examined WGA accumulation in the LHA and the major extra-LHA projection site of LHA LepRb neurons, the VTA (Figure 2.6C-F)(Leinninger et al., 2009). While this analysis demonstrated the presence of copious WGA-IR neurons in the LHA, it revealed very few WGA-IR neurons in the VTA. Some of these VTA WGA-IR neurons colocalized with TH, suggesting that at least some LHA LepRb neurons directly innervate VTA DA neurons. Other distant regions, including the ARC, similarly contained few or no WGA-IR neurons (data not shown). While these data are consistent with a direct synaptic connection between some LHA LepRb neurons and VTA DA targets, the paucity of detectable WGA-IR VTA neurons suggests either that the WGA/EGFP transgene expressed by Ad-iN/WED poorly traversed synapses far removed from the primary WGA/EGFP-expressing neurons, or that LHA LepRb neurons directly innervate the VTA less strongly than we previously thought (Leinninger et al., 2009).

LHA leptin action regulates activation of neurons in the VTA. To determine the downstream neuronal activation of VTA neurons in response to leptin in the LHA, we examined the ability of LHA leptin to activate these neurons. Previous data have demonstrated that systemic leptin increases pCREB in the VTA (Leshan et al., 2010), thus indicating that leptin functionally modulates intracellular signaling in VTA neurons. We demonstrate that acute intra-LHA leptin treatment increased the number of VTA neurons expressing pCREB, thus indicating that leptin action via LHA LepRb neurons functionally modulates intracellular signaling in VTA neurons (Figure 2.6 G-I).

LHA LepRb neurons synapse with OX, but not MCH, neurons. To examine the potential innervation of local LHA neurons by LHA LepRb neurons, we examined the accumulation of WGA by OX and MCH neurons in the LHA of $LepRb^{cre}$ mice following the intra-LHA injection of Ad-iN/WED. This analysis revealed the accumulation of WGA-IR in numerous OX-IR neurons (Figure 2.7 E-H), but not MCH-IR neurons (Figure 2.7 A-D). Hence, LHA LepRb neurons lie in synaptic contact with OX neurons, but not MCH neurons. These data thus reveal that non-LHA LepRb neurons must mediate the effects of leptin on MCH neurons, and also suggest a role for LHA LepRb neurons in the control of OX neurons by leptin.

LHA leptin action regulated gene expression in OX neurons. To determine the potential role for LHA LepRb neurons in the control of OX

neurons in response to leptin, we examined the ability of LHA leptin to regulate these neurons. We and others have demonstrated that leptin modulates OX neurons in multiple manners: systemic leptin administration promotes *Ox* mRNA expression (Figure 2.8 A (Yamanaka et al., 2003)), but also inhibits the fasting-stimulated activation of OX neurons, as detected by c-fos-IR (Tritos et al., 2001; Mieda and Yanagisawa, 2002; Yamanaka et al., 2003; Funato et al., 2009). Since our previous experience reveals that intra-LHA cannulation interferes with the examination of LHA c-fos-IR (data not shown), we thus focused on the modulation of OX neuron gene expression in response to intra-LHA leptin infusion. We studied the regulation of ARC and LHA gene expression in response to systemic (5 mg/kg i.p.) or intra-LHA (0.25 ng total) leptin in *Lep*$^{ob/ob}$ animals, in which the absence of endogenous leptin permits the sensitive examination of leptin action. We previously demonstrated the confinement of leptin action to the LHA using this small dose of intra-LHA leptin (Leinninger et al., 2009). Animals were treated with vehicle or leptin for 26 h prior to sacrifice and harvesting of microdissected ARC and LHA tissue and the preparation of RNA for the analysis of gene expression by qPCR. As previously reported, leptin induces *Socs3* expression in a cell-autonomous manner (Banks et al., 2000; Bjorbaek et al., 2001); changes in *Socs3* mRNA thus serve as a marker of direct leptin action in the ARC (Baskin 2000; Elias 1999). Systemic leptin treatment significantly increased the expression of *Pomc* and *Socs3* in the ARC, as well as increasing the expression of *Ox* (by 2-fold) and *Nptx2* (neuronal activity-regulated pentraxin (NPTX2; also called

Narp), which is co-expressed with OX in the LHA (Reti et al., 2002; Blouin et al., 2005; Crocker et al., 2005) by 20% in the LHA (Figure 2.8A). Intra-LHA leptin failed to significantly modulate ARC *Pomc* or *Socs3* gene expression, however, consistent with the predicted site-specificity of intra-LHA leptin action and the lack of projections from LHA LepRb neurons to the ARC (from Figure 2.3B). In contrast, intra-LHA leptin promoted a dramatic increase in LHA *Ox* (approximately 25-fold) and *Nptx2* (approximately 4-fold) mRNA expression (Figure 2.8B). Thus, leptin action via LHA LepRb neurons robustly modulates gene expression in OX neurons.

Discussion

We have examined the innervation of specific populations of LHA neurons by LepRb neurons, revealing that, while both OX and MCH neurons receive synaptic contact from LepRb neurons, local LHA LepRb neurons project onto OX, but not MCH, neurons (Figure 2.9). Consistent with the direct projection of LHA LepRb neurons onto local OX neurons, LHA leptin action robustly modulates gene expression in OX neurons, revealing the functional importance of this local circuit.

The strength with which local LHA leptin promotes *Ox* and *Narp* mRNA expression compared with that observed in response to systemic leptin (along with the paucity of projections to the OX field from other populations of LepRb neurons) suggests that LHA LepRb neurons likely represent the main neural mediators of leptin action on OX neurons. While the technical difficulties

associated with measuring c-fos (a surrogate for neuronal activity) in the region surrounding a cannula prevented us from examining the regulation of OX neuron activity in response to LHA leptin, the lack of significant LepRb projections to this region from elsewhere and the GABAergic nature of the LHA LepRb neurons(Leinninger et al., 2009) prompts us to hypothesize that LHA LepRb neurons might inhibit the activity of OX neurons, as well as controlling their gene expression.

While it is clear from our present observations that MCH neurons lie in synaptic contact with LepRb neurons, the lack of WGA accumulation in MCH neurons following intra-LHA injection of Ad-iN/WED in $LepRb^{cre}$ mice reveals that the LepRb neurons that project onto MCH neurons likely lie outside of the LHA. The population of LepRb neurons that lie upstream of MCH neurons thus remains unclear, although a variety of previously published data suggest a potential role for ARC melanocortin neurons in this regulation (Hanada et al., 2000). Tracing from the larger LHA (not specifically the dorsal perifornical area, as we have done here) revealed a population of leptin-activated ARC neurons projecting to the LHA (Elias et al., 1998). Indeed, *Mch* expression is increased in mice overexpressing the melanocortin antagonist, Agouti, (although *Ox* is not altered); also, melanocortin agonists/antagonists regulate *Mch* expression (Hanada et al., 2000; Tritos et al., 2001; Kim et al., 2005).

Our present data demonstrating the regulation of OX neurons by LHA LepRb neurons suggests important roles for these LHA LepRb neurons in energy balance and in CNS leptin action. While acute injection of OX into the

CNS promotes activity, wakefulness, and hyperphagia, the long-term role of OX is to promote activity and energy expenditure, while decreasing feeding (Tritos et al., 2001; Mieda and Yanagisawa, 2002; Yamanaka et al., 2003; Funato et al., 2009). Indeed, mice (and humans) null for OX are obese, while widespread overexpression of OX promotes leanness (Sakurai et al., 1998; Hara et al., 2001; Funato et al., 2009). Much of this OX action on energy balance depends upon the OX2R, as mice null for this receptor demonstrate increased feeding and become more obese than controls on a high fat diet; systemic treatment with an OX2R agonist prevents diet-induced obesity (Funato et al., 2009). Presumably, therefore, the acute effects of OX may be mimicked by increased activity of OX neurons during food restriction (which is blunted by leptin), while the increased expression of *Ox* promoted by leptin (via LHA LepRb neurons) would be expected to promote the kind of chronic OX effects required for leptin action. Indeed, we previously showed that intra-LHA leptin decreased feeding and body weight over 24 hours in $Lep^{ob/ob}$ animals (Leinninger et al., 2009).

Our previous analysis of LHA LepRb neurons also identified projections from these neurons to the VTA, and revealed that LHA leptin treatment of $Lep^{ob/ob}$ animals promotes VTA *Th* expression and increased DA content in the nucleus accumbens (NAc) (Leinninger et al., 2009). Our present findings that LHA LepRb neurons project onto and regulate OX neurons, which themselves innervate the VTA to modulate the actions of the mesolimbic DA system (Nakamura et al., 2000; Harris et al., 2005; Kelley et al., 2005), suggests that

LHA LepRb neurons may modulate the mesolimbic DA system indirectly, via OX neurons, as well as by direct projection to the VTA (Figure 2.9).

Furthermore, intra-LHA leptin treatment increased neuronal activation (marked by pCREB) in the VTA, suggesting that LHA LepRb neurons functionally regulate VTA neurons. While it is unclear whether this VTA modulation is due to LHA LepRb neurons projecting directly or indirectly (via OX neurons) onto VTA, we speculate that the regulated VTA neurons may be DAergic, given that DA neurons comprise >90% of the VTA neuronal population. LHA LepRb-mediated regulation of DA neurons is consistent with the reported effects of intra-LHA leptin on VTA *Th* (produced in DA neurons), and may represent another means by which LHA LepRb neurons regulate the mesolimbic DA system. However, understanding the exact mechanism(s) of this regulation will require further analysis.

Thus, it is important to dissect the specific functions of different populations of LepRb neurons. These studies reveal that LHA LepRb neurons project to OX neurons to presumably regulate hedonic feeding via the mesolimbic DA system. While outside the scope of this study, it will be important to distinguish the relative contributions of these direct and indirect pathways from LHA LepRb neurons to the mesolimbic DA system and energy balance.

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Notes

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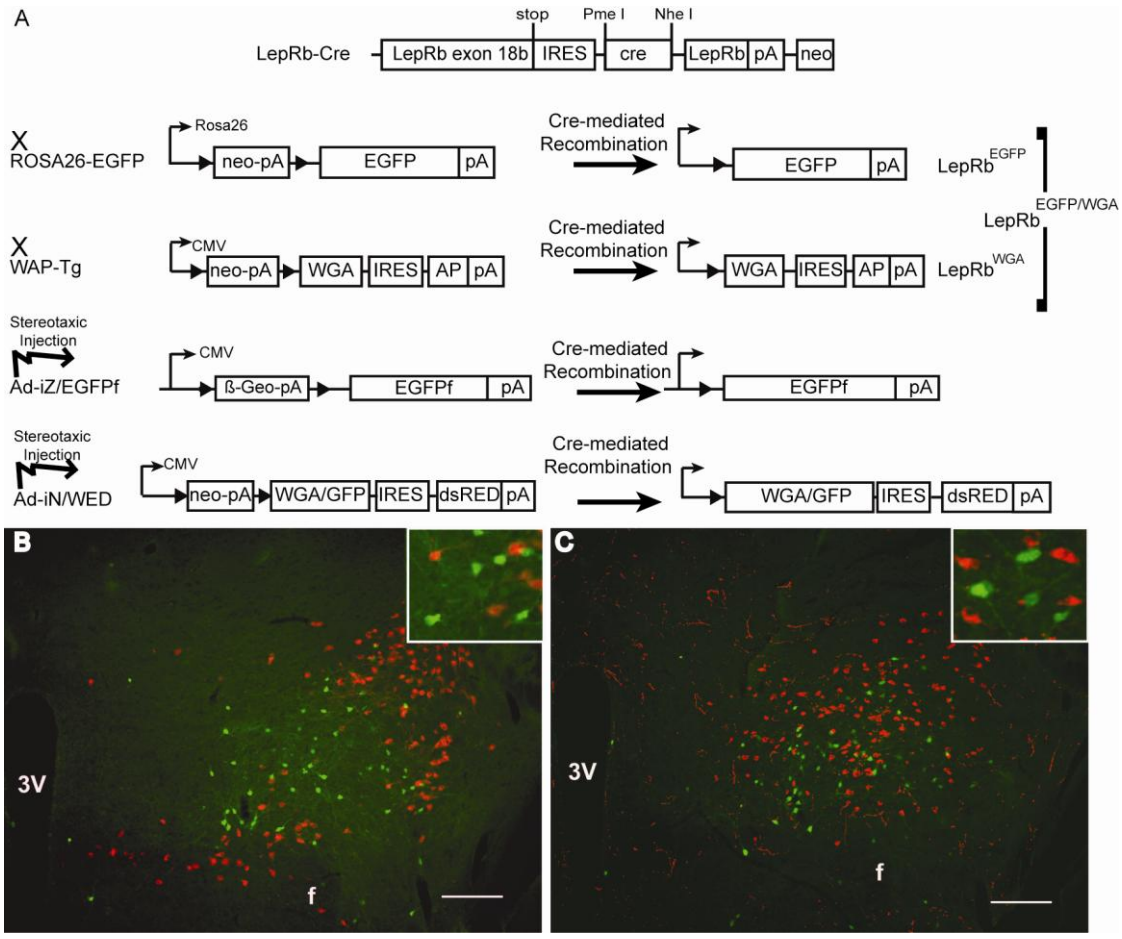

Figure 2.1 LepRb^{cre}-dependent transgenic and adenoviral tracers to study LepRb-expressing cells in the LHA. (A) Schematic diagram showing cre-mediated tracer (EGFP, WGA, or EGFPf) expression in LepRb-expressing cells of LepRb^{EGFP} mice and LepRb$^{EGFP/WGA}$ mice and also upon injection of Ad-iZ/EGFPf and Ad-iN/WED adenoviruses. (B, C) Immunofluorescent detection of EGFP (green) and (B) MCH (red) or (C) orexin (red) in the LHA of LepRb^{EGFP} mice. Results are representative of 4 animals. Insets: represent digitally zoomed images of labeled LHA neurons. Scale bars = 10uM, 3V = third ventricle; f = fornix.

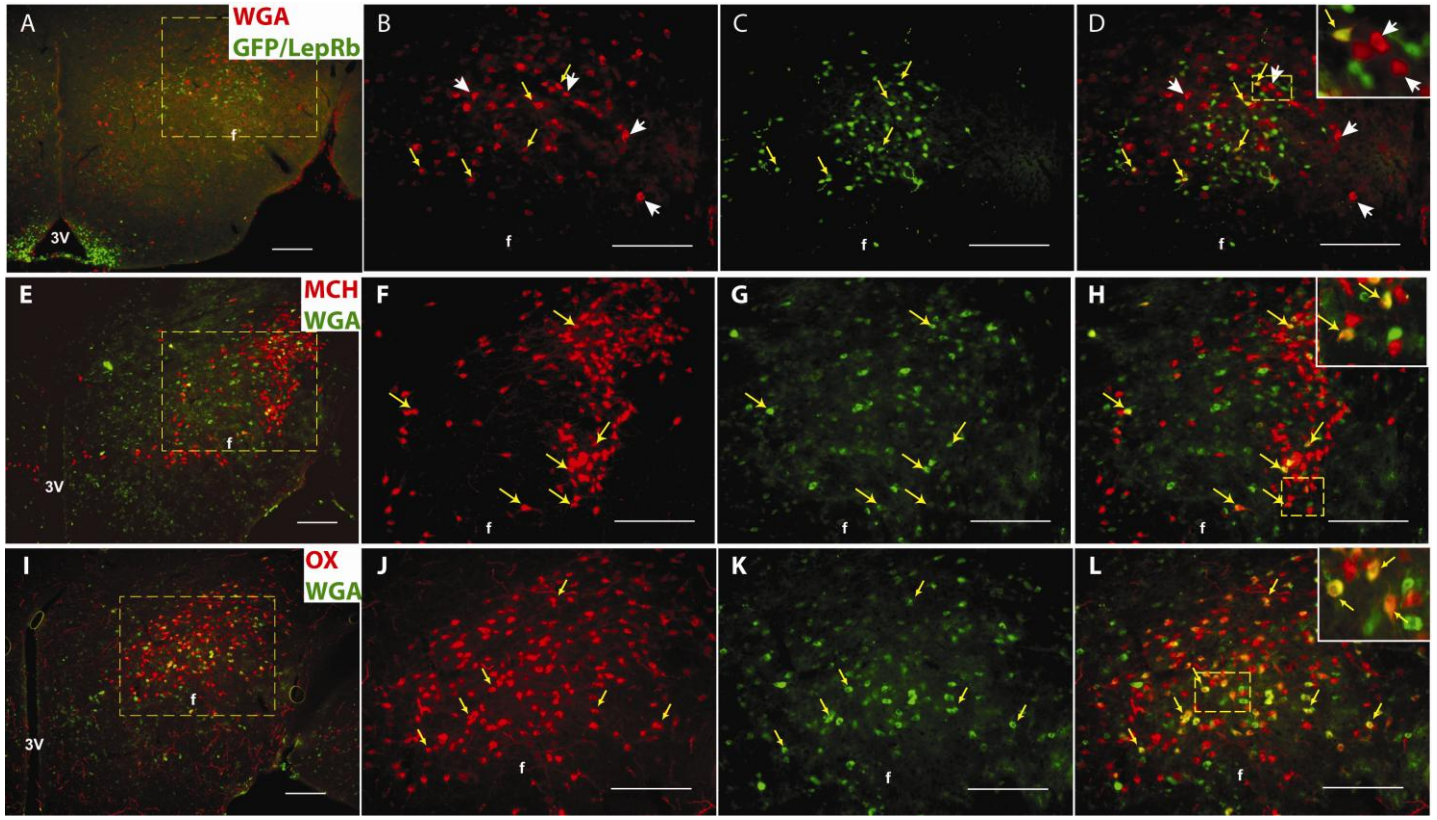

Figure 2.2 LepRb$^{EGFP/WGA}$ mice reveal LepRb-expressing cells and their projection targets in the LHA. (A) Immunofluorescent detection of WGA (red) and GFP/LepRb (green) in the LHA of LepRb$^{EGFP/WGA}$ mice. Immuno-fluorescent detection of (E&I) WGA (green) and (E) MCH (red) and (I) OX (red) in the LHA of LepRb$^{EGFP/WGA}$ mice. Panels B-D, F-H, and J-L show enlarged images of the boxed regions of panels A, E, and I, respectively. Panels B, F, and J show red channel only, C, G, and K show green channel only, and D, H, and L show merged images. Results representative of n=4 animals. Insets: higher magnification view of boxed regions. Yellow arrows= representative of colabeled cells; white arrowheads = single labeled cells. Scale bars = 10uM, 3V = third ventricle; f = fornix.

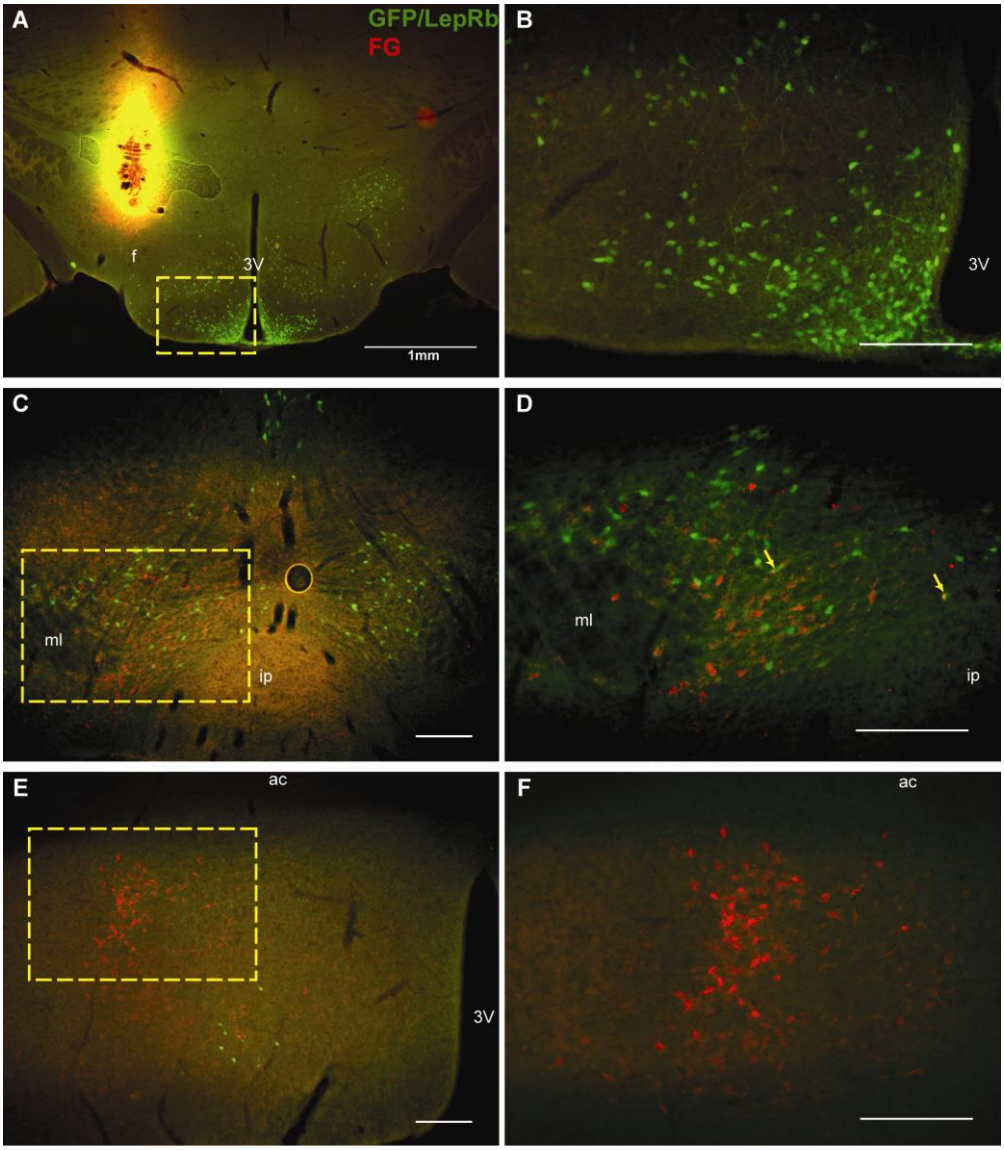

Figure 2.3 Retrograde FG tracing of LHA neurons in LepRb^{EGFP} mice to determine presynaptic LepRb populations. The retrograde tracer, fluorogold, was injected into the dorsal perifornical region of the LHA of LepRb^{EGFP} mice. After perfusion, the brains were processed for the immunofluorescent detection of GFP/LepRb (green) and FG (red). (A) View of the LHA injection site in a representative animal. (B) ARC, (C&D) VTA and (E&F) lateral POA. Panels B,D,F are magnified images of boxed areas in A,C,E, respectively. Results representative of 2 animals with similar injection sites. Arrows= representative of colabeled cells. Scale bars = 10uM, unless otherwise noted. 3V = third ventricle; f = fornix; ip = intrapenduncular nucleus; ml = medial lemiscus; ac = anterior commissure.

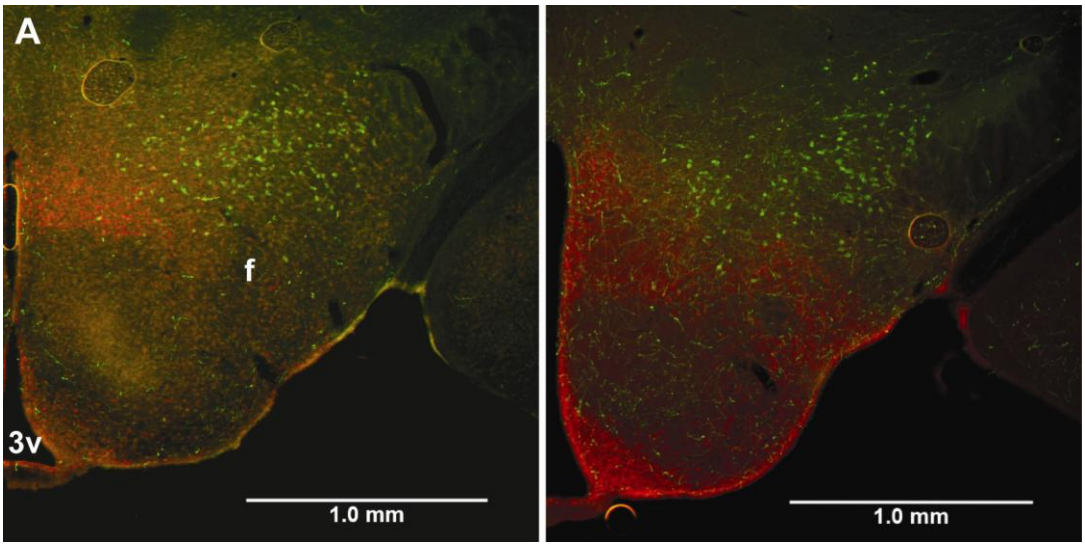

Figure 2.4 Few ARC POMC and AgRP neurons project to the OX field in the LHA. Immunofluorescent detection of OX neurons in the LHA (green; A&B) and α -MSH (red; A) and AgRP (red; B) fibers reveal that few α-MSH- and AgRP–IR projections are detected in the main OX field in the LHA. Results are representative of 3 animals. Scale bars = 1mm. 3V = third ventricle; f = fornix.

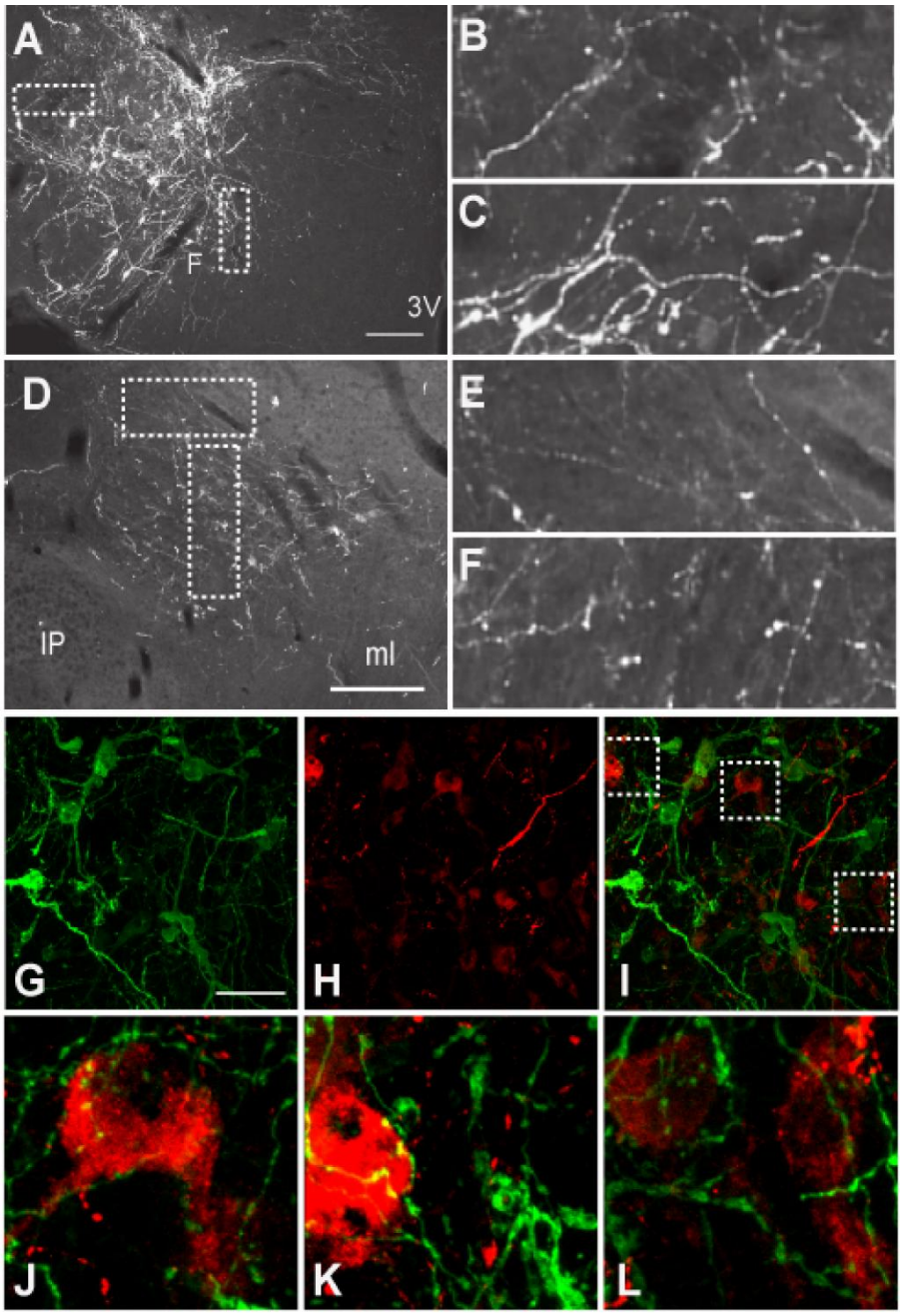

Figure 2.5 Ad-iZ/EGFPf injection to the LHA of LepRb^{cre} mice provides evidence for intra-LHA as well as intra-VTA synapses on projections for LHA LepRb neurons. Immunofluorescent detection of EGFP in the (A-C) LHA and (D-F) in the VTA. Panels B,C and E,F represent digitally zoomed images of synaptic boutons in panels A and D, respectively. Confocal images of (G) EGFP/LepRb (green) and (H) OX (red). Panels I-L represent merged images and J-L are digitally magnified views of synaptic boutons in panel I. Results are representative of 9 animals. Panels A&D scale bars = 10uM, Panels G-L scale bars = 50uM. 3V = third ventricle; f = fornix; ip = intrapenduncular nucleus; ml = medial lemiscus.

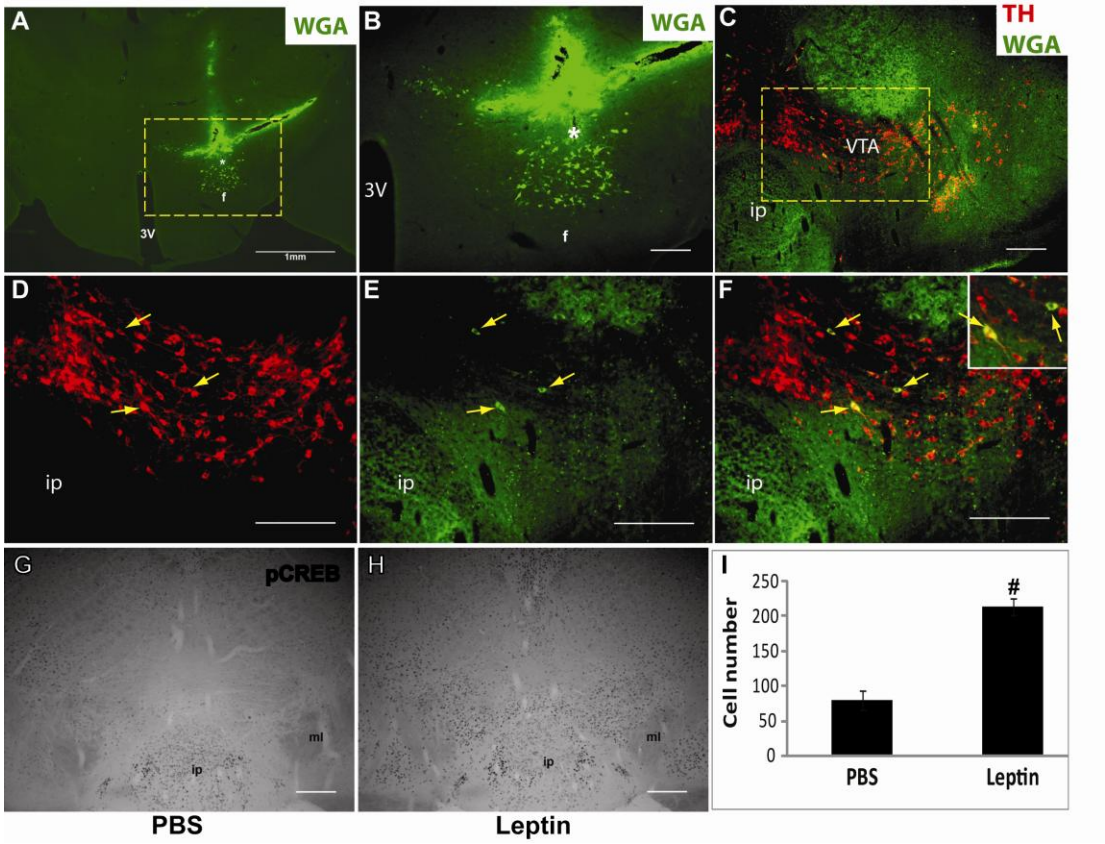

Figure 2.6 Modest accumulation of WGA in VTA following Ad-iN/WED injection to the LHA of LepRb^{cre} mice and activation of neurons in the VTA by intra-LHA leptin. (A) Immuno-fluorescent detection of a robust population of WGA-IR neurons (green) in the LHA of a representative intra-LHA Ad-iN/WED-injected LepRb^{cre} mouse. (B) Enlarged image of the boxed region in A. (C) Detection of WGA (green) and a robust population of TH-IR neurons (DA; red) in the VTA. (D-F) Show single channel and merged enlarged images of the boxed region from C. (G-I) Animals were treated with intra-LHA PBS (G) or leptin (H; 0.25ng total) for 15m then VTA pCREB was detected by immunohistochemistry. (I) pCREB-IR was quantified using ImageJ software. N=5 per condition; data plotted as mean +/- SEM. * indicates injection site; #= $p<0.05$ by Student's t-test; arrows = representative of colabeled cells. Scale bars = 10uM, unless otherwise noted. 3V = third ventricle; f = fornix; ip = intrapenduncular nucleus; ml=medial lemiscus.

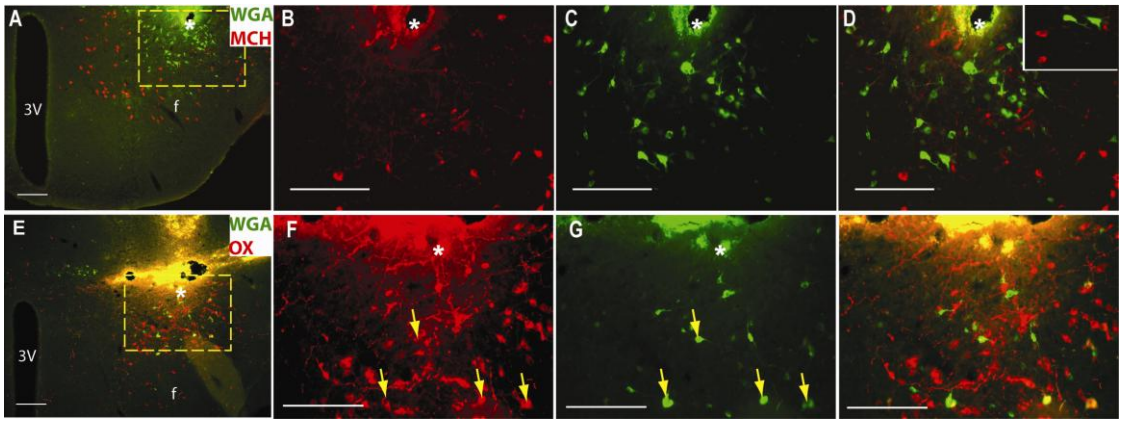

Figure 2.7 Local tracing of LepRb neurons and their synaptic targets in the LHA after Ad-iN/WED injection to the LHA of LepRb^{cre} mice.
Immunofluorescent detection of (A&E) WGA (green) and (A) MCH (red) or (E) OX (red) in the LHA after stereotaxic injection of Ad-iN/WED to the LHA. Panels B-D and F-H show single channel and merged enlarged images of the boxed regions from A and E, respectively. Results are representative of n=5 animals. Insets: higher magnification view of labeled neurons. * indicates injection site; arrows = colabeled cells. Scale bars = 10uM, 3V = third ventricle; f = fornix.

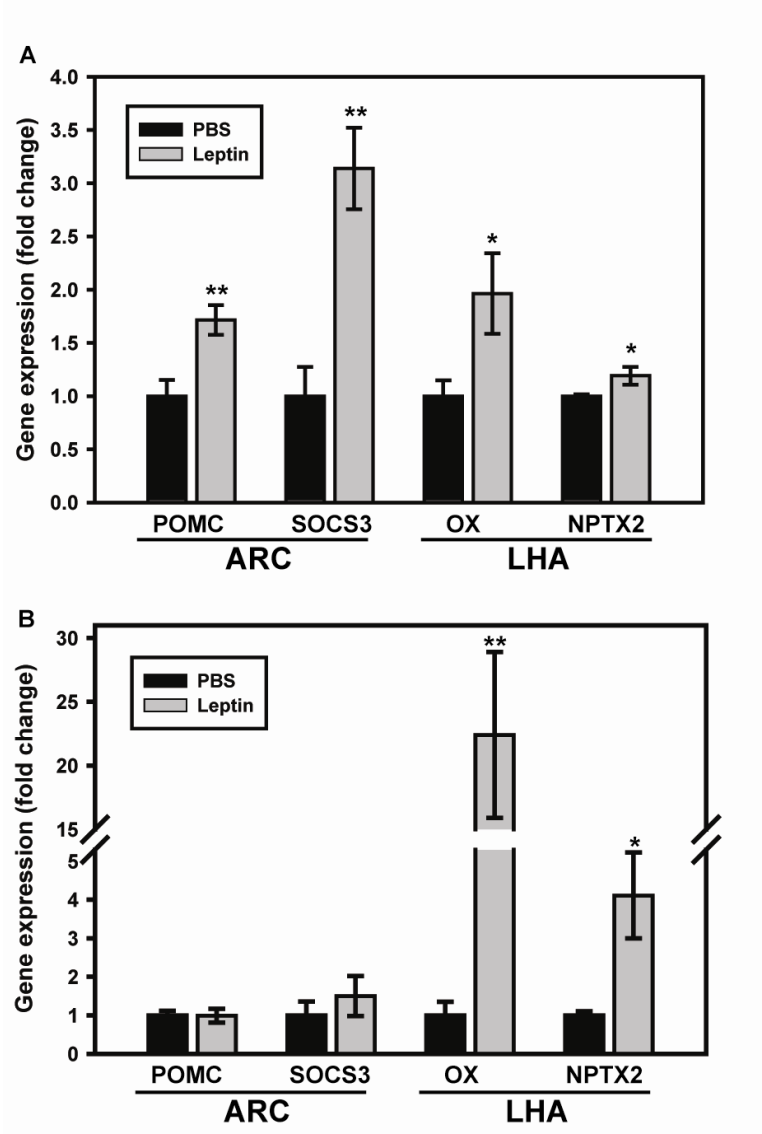

Figure 2.8 Changes in gene expression after systemic and local leptin treatment in *Lep*$^{ob/ob}$ (ob/ob) mice. Changes in gene expression in microdissected tissue of ARC or LHA from *Lep*$^{ob/ob}$ mice following 26h (A) systemic (IP, 5 mg/kg) or (B) intra-LHA (0.25 ng) treatment with PBS (black bars) or leptin (gray bars). Sample size=10 per treatment, per condition. Expression data are plotted relative to *Gapdh* expression (calculated by $2^{-\Delta\Delta Ct}$ method) and normalized to control (PBS-treated) levels and contralateral sides (for intra-LHA group) $\pm$ SEM. *p<0.05, **p<0.01 relative to PBS (by t test).

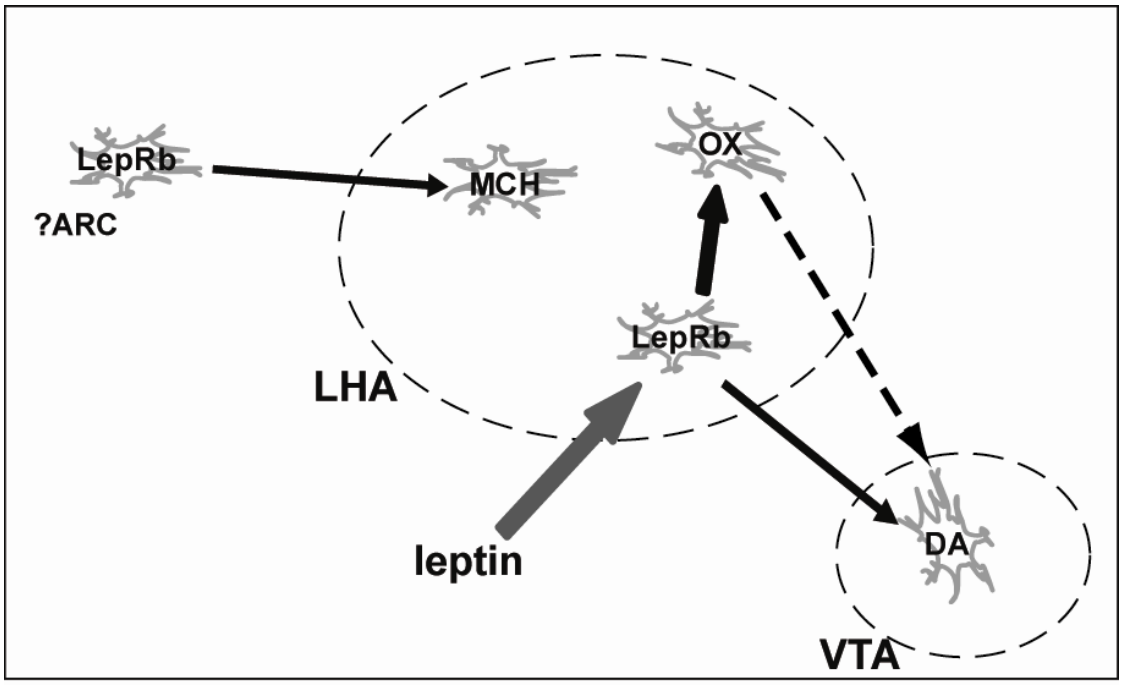

Figure 2.9 Model for regulation of LHA OX and MCH neurons by LepRb neurons, and potential mechanisms of LHA LepRb neuron action.

CHAPTER THREE

MAPPING THE NEURAL PATHWAYS THAT LINK ENERGY BALANCE AND REPRODUCTION

Summary

Metabolic stress, as in individuals with low body fat, causes hypothalamic amenorrhea. Leptin, a hormone produced by adipocytes in proportion to fat/energy stores, signals the sufficiency of body energy by binding to the leptin receptor (LepRb) on neurons in the hypothalamus, thereby permitting the expenditure of energy on processes such as reproduction. Leptin treatment restores the function of the neuroendocrine reproductive axis in animals and humans with low body fat, as well as in leptin-deficient, infertile *ob/ob* mice. The neural mechanism(s) by which leptin regulates the neuroendocrine reproductive axis remain poorly understood. Potential mechanisms include either the co-expression of LepRb or the interaction of LepRb neurons with GnRH or kisspeptin (also called Kiss1) neurons in the ARC and AVPV (which are implicated in the control of GnRH neurons). Here, we utilize numerous genetic mouse models to probe these

issues. We demonstrate the innervation of GnRH neurons by ARC, but not AVPV, Kiss1 neurons. Neither GnRH nor Kiss1 neurons (in the ARC and AVPV) express LepRb, but they receive synaptic input from LepRb neurons. Moreover, leptin modulates gene expression in ARC Kiss1 neurons but not in AVPV neurons. The majority of LepRb neurons projecting to GnRH neurons lie in the ventral premammillary (PMv) nucleus of the hypothalamus. Thus, the PMv LepRb → GnRH neuronal circuit and the LepRb → ARC-Kiss1 → GnRH circuit are best positioned to link changes in nutritional status to alterations in reproductive function.

Introduction

The energetic demands of reproduction (for pregnancy, partuition, lactation and child rearing) are substantial. Consistent with these demands, the "critical weight (fat) hypothesis", postulates that a minimal level of fat is essential for the advancement to puberty and the maintenance of reproductive capability (Frisch and Revelle, 1970; Frisch and McArthur, 1974). The hormone leptin appears to play a central role in the control of reproduction by energy status (Ahima et al., 1996; Chehab et al., 1996). Leptin is an interleukin-6 family cytokine that is secreted by adipose tissue to signal body energy stores to the central nervous system (Zhang et al., 1994; Halaas et al., 1995; Friedman, 1998). As a signal of sufficient energy, ample leptin levels suppress feeding by promoting satiety and permit energy expenditure by regulating neuroendocrine function (Zhang et al., 1994; Halaas et al., 1995;

Ahima et al., 1996; Friedman and Halaas, 1998). Conversely, in fasted states, leptin levels fall and thereby promote feeding and shift energy use towards survival.

Leptin serves as a “permissive” metabolic signal for reproduction and threshold leptin levels are required to achieve puberty and for the maintenance of reproductive capacity. Leptin deficient *ob/ob* mice display characteristics similar to the stereotypical starvation response (even in the face of nutrient excess and obesity), including hypothalamic infertility, which is reversed by leptin administration (Ahima et al., 1996; Barash et al., 1996; Chehab et al., 1996). Leptin reverses the fasting-induced hypogonadotropic hypogonadism and restores puberty and fertility (Frederich et al., 1995; Ahima et al., 1996; Barash et al., 1996; Chehab et al., 1996; Nagatani et al., 1998; I'Anson et al., 2000). Importantly, central leptin action is sufficient to overcome reproductive dysfunction in low leptin states (Campfield et al., 1995; Watanobe, 2002; de Luca et al., 2005). Leptin modulates pulsatile gonadotropin secretion from the anterior pituitary via the regulation of hypothalamic GnRH neurons (Chehab et al., 1996; Finn et al., 1998; Nagatani et al., 1998; Magni et al., 1999; Quennell et al., 2009). These data suggest that leptin may serve as a neuroendocrine signal linking energy homeostasis and reproduction. Since GnRH neurons that lie in the preoptic area (POA) of the hypothalamus (the location of the majority of GnRH neurons in rodents) do not express LepRb (Hakansson and Meister, 1998; Nagatani et al., 1998; Cunningham et al., 1999; Quennell et al.,

2009), leptin must then mediate the hypothalamic control of reproduction indirectly by acting on neurons upstream of GnRH neurons.

Candidate populations of neurons likely to participate in the control of energy balance and the reproductive axis include those expressing the neuropeptide kisspeptin (also called Kiss1, Kiss10 or metastin). Kiss1 protein is encoded by the *Kiss1* gene in rodents and humans, and signals through the GPR54 G-protein coupled receptor (also called *AXOR12* and *hOT7T175)* (Lee et al., 1999; Kotani et al., 2001; Muir et al., 2001). Mutations in the GPR54 receptor hinder normal reproductive function in humans (de Roux et al., 2003; Seminara et al., 2003) and mice (Funes et al., 2003; Messager et al., 2005; Kauffman et al., 2007; Lapatto et al., 2007). Kiss1-deficient mice have defects similar to the GPR54 mutant mice (abnormal pubertal maturation, hypogonadotropic hypogonadism), which are reversed by Kiss1 administration (d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007). The majority of Kiss1 neurons in the ARC co-express tachykinin 2 (Tac2, also known as neurokinin B (NKB)) and Dynorphin (hence, these cells have been termed "KNDy" neurons) (Goodman et al., 2007; Navarro et al., 2009). Conversely, the majority of ARC Tac2 neurons coexpress *Kiss1* mRNA. GPR54 is highly expressed in the hypothalamus (Lee et al., 1999; Kotani et al., 2001; Muir et al., 2001) as well as by the majority of GnRH neurons (Irwig et al., 2004; Han et al., 2005; Messager et al., 2005) and Kiss1 fibers are in close apposition to GnRH neurons (Kinoshita et al., 2005; Clarkson and Herbison, 2006) suggesting that GnRH cells are a direct target for Kiss1. Populations of

neurons that express Kiss1 are found in the ARC and anteroventral periventricular nucleus (AVPV) (Gottsch et al., 2004; Irwig et al., 2004; Kinoshita et al., 2005; Smith et al., 2006c).

Estradiol differentially regulates *Kiss1* expression: estrogen upregulates *Kiss1* mRNA in the AVPV, while it decreases *Kiss1* expression in the ARC (Smith et al., 2005a; Smith et al., 2005b). *Kiss1* mRNA is also regulated by nutritional status. For example, fasting suppresses *Kiss1* and *GPR54* expression in rodents, thus attenuating the reproductive axis (Castellano et al., 2005; Luque et al., 2007). Administration of Kiss1 induces GnRH (Castellano et al., 2005; Tovar et al., 2006) and LH secretion in rodents (Gottsch et al., 2004; Irwig et al., 2004; Navarro et al., 2004; Messager et al., 2005; Castellano et al., 2006), thus restoring reproductive function. Furthermore, *ob/ob* mice lacking leptin have decreased hypothalamic *Kiss1* expression, which is reversed by systemic leptin replacement (Smith et al., 2006b). Furthermore, CNS leptin treatment restored *Kiss1* expression in the ARC of *ob/ob* mice (Castellano et al., 2006).

Thus, it is clear that leptin regulates reproduction at the central level and that nutritional states affect the regulation of reproduction by Kiss1. Based on these data, we therefore hypothesize that leptin controls reproduction by the innervation of hypothalamic Kiss1 and/or GnRH neurons. The mechanisms by which LepRb-expressing neurons interact with GnRH or Kiss1 neurons to influence reproduction remain elusive, however. Here, we map the neural pathways by which LepRb populations may regulate Kiss1 and

GnRH neurons and determine if leptin treatment influences gene expression in Kiss1 neurons.

Materials and Methods

Materials. Leptin was the generous gift of Amylin Pharmaceuticals, Inc. (San Diego, CA).

Experimental animals. The generation of *Lepr*$^{cre/cre}$ (LepRb^{Cre}) mice has been described previously (Leshan et al., 2009). *Lepr*$^{cre/cre}$ mice were bred with Gt(*ROSA)26-Sor*^{tm2Sho} mice purchased from Jackson Laboratory to generate double homozygous *Lepr*$^{cre/cre}$*;Gt(ROSA)26-Sor*$^{tm2Sho/tm2Sho}$ (LepRb^{EGFP}) mice, which were propagated by intercrossing. Female C57Bl/6 wild-type animals 8-10 weeks of age were purchased from Jackson Laboratory. The previously described transgenic BIG mice were obtained from our collaborators (Drs. Linda Buck and Ulrich Boehm) to aid in GnRH neuronal tract tracing and were subsequently propagated in our colony (Boehm et al., 2005). Tacykinin2 (Tac2)-GFP Bac transgenic mice that express GFP in Tac2- expressing neurons were obtained from Gensat MMRRC to aid in the detection of Kiss1-ARC neurons and subsequently propagated in our colony. The generation of *iZ/WAP* (LepRb$^{EGFP/WGA}$) mice was described previously (Leshan et al., 2010). Animals were housed in our colony in 12h light/dark cycles and given *ad libitum* access to food and water. All care and procedures for mice were according to guidelines approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA).

Perfusion, immunohistochemistry and immunofluorescence. Perfusion and immunohistochemistry were performed essentially as described (Munzberg et al., 2007) on adult, post-pubertal female mouse models. Sacrificing of female mice for neuronal activation or LepRb activation studies was performed between 1 and 3 pm and when the mice were in diestrus, as determined by vaginal smears, to standardize for potential diurnal or estrous cycle effects. Briefly, mice were deeply anesthetized with a lethal dose of intraperitoneal (i.p.) pentobarbital (150 mg/kg) and transcardially perfused with sterile PBS then 10% neutral buffered formalin. Brains were removed, postfixed overnight and dehydrated in a 30% sucrose solution. Then brains were sectioned into 30 μm coronal slices, collected in four consecutive series and stored at –20 °C in cryoprotectant until further use.

Brain sections were incubated in primary antibodies overnight at 4°C, and then visualized by immunofluorescent secondary detection using species-specific Alexa 488 or 568 antibodies (1:200, Invitrogen). Sections were mounted on slides and coverslipped with Prolong antifade mounting medium (Invitrogen). Primary antibodies used for these studies included: chicken anti-GFP (1:1000, Abcam), goat anti-WGA (1:1000, Vector), rabbit anti-Kiss1 (1:2000, Chemicon), rabbit anti-GnRH (1:1000; Abcam), rabbit anti-pSTAT3 (1:1000, Cell Signaling), and rabbit anti-cfos (1:40,000, Calbiochem).

Leptin treatment to assess changes in GnRH or Kiss1 neuronal activation. Adult female BIG mice in diestrus were leptin (5mg/kg) or vehicle

treated (i.p) for 4 hr then perfused and analyzed for immunohistochemical analysis of cfos (marker for neuronal activation) in GnRH or Kiss1 neurons.

Data Collection and analysis. Slides were analyzed via light or fluorescent microscopy using an Olympus BX-51 microscope with filters for Alexa 488 or Alexa 568 and images collected with software, as previously described (Munzberg et al., 2007). Using Adobe Photoshop software (Adobe Systems, San Jose CA) images were overlaid in different RGB channels to reveal single- or double-labeled cells. Confocal images were captured with an Olympus FV-500 using sequential laser scanning.

Leptin treatment to assess changes in Kiss1 gene expression. Adult female wild-type mice (n=30 total) were ovarectomized (OVX) to eliminate endogenous ovarian hormone changes and then allowed to recover for 7-10 days before treatment. Upon recovery, mice were divided into three cohorts (n=10 each group): fed *ad libitum* and systemically treated with vehicle (i.p); fasted for 48 h and systemically treated with sterile PBS; or fasted for 48 h and systemically treated with leptin (5mg/kg). Injections were given every 12 h during the total treatment time with a final treatment administered 2 h before sacrificing and microdissection of brain tissue, as previously described (Leinninger et al., 2009). Terminal trunk blood was also collected for analysis of serum LH levels by radioimmunoassay (data not shown). ARC or AVPV tissues were lost during microdissection of two separate mice. Also, two samples with undetectable levels of LH were excluded from gene expression

analysis. Thus, the final sample sizes were 7-9 for each of the three treatment groups.

RNA extraction and analysis of gene expression by qPCR. ARC and AVPV tissue were microdissected and snap frozen for later processing and analysis of mRNA expression by quantitative RT-PCR (as previously described) (Leinninger et al., 2009). Briefly, RNA was extracted using TRIzol (Invitrogen) and converted to cDNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). cDNA was analyzed in triplicate via qRT-PCR for *Gapdh* control gene and neuropeptide gene expression (*Kiss1* or *Tac2* genes) using previously described assays (Bates et al., 2003), or assays supplied by Applied Biosystems; samples were processed using an Applied Biosystems 7500 thermocycler. Gene expression was normalized to *Gapdh* expression and relative mRNA expression was calculated the $2^{-\Delta\Delta Ct}$ method. Statistical significance was determined by one-way ANOVA with Bonferroni's post testing with InSTAT software for Mac.

Results

Kiss1 neurons in the ARC coexpress Tac2. Visualization of Kiss1 neurons in the ARC by immunofluorescent staining methods was not optimal, as staining predominantly identified fibers but very few cell bodies. Reports show that the majority of Kiss1 neurons in the ARC co-express tachykinin 2 (Tac2, also known as neurokinin B (NKB)) and Dynorphin A (hence, "KNDy" neurons) and vice-versa (Goodman et al., 2007; Navarro et al., 2009). Thus,

we obtained Tac2-GFP transgenic mice that express GFP in Tac2-expressing neurons and utilized the detection of GFP-IR in the ARC as a surrogate for KNDy neuron identification. Immunofluorescent analysis of Tac-GFP mice revealed that the detectable Kiss1-IR cell bodies in the ARC colocalized with Tac2-GFP (Figure 3.1A-C). In contrast, Kiss1-IR cells in the AVPV (which do not contain Tac2) do not co-localize with GFP in Tac2-GFP mice (Figure 3.1D-F). Thus, detection of Tac2-GFP-IR will serve as a surrogate for the localization of ARC KNDy neurons and Tac2-GFP neurons will be termed "KNDy neurons".

Projection of KNDy neurons to GnRH neurons. In order to determine the potential role of ARC and AVPV Kiss1 neurons in the regulation of GnRH neurons, we employed transgenic BIG mice, which express barley lectin (BL) (which is related to wheat germ agglutinin (WGA) and mediates anterograde and retrograde trans-synaptic tracing) and IRES-GFP under the GnRH promoter (Figure 3.2A). Thus, BIG mice express both GFP and BL in GnRH neurons and synaptically connected neurons accumulate BL only. Immunofluorescent analysis of the AVPV in the BIG mice (Figure 3.2 B-E) reveals little accumulation of BL-IR in AVPV Kiss1 neurons (Figure 3.2E). Immunofluorescent analysis of Tac2-GFP mice revealed that the majority of ARC KNDy neurons (identified by Tac2/GFP; Figure F-I) colocalized with WGA (Figure 3.2I). Thus, ARC KNDy neurons may represent the main contributors to Kiss1 regulation of GnRH neurons in the mouse.

Neither GnRH neurons nor Kiss1 neurons contain LepRb. As the identification of LepRb neurons by direct immunohistochemistry remains problematic (due to the lack of sensitive antibodies specific for LepRb), we employed the $LepRb^{EGFP}$ reporter mice (in which LepRb neurons express EGFP to facilitate the detection of LepRb neurons). EGFP expression in the POA confirmed LepRb-expressing cells to be distinct from GnRH neurons (Figure 3.3A). We additionally analyzed the potential colocalization of LepRb/EGFP with Kiss1. Immunohistochemical analysis of $LepRb^{EGFP}$ mice reveal that Kiss1 neurons in the AVPV do not colocalize with EGFP (Figure 3.3B). Analysis of Tac2-GFP reporter mice (for leptin-induced pSTAT3, a functional marker of LepRb neurons (Munzberg et al., 2003)) reveals that ARC KNDy neurons do not colocalize with leptin-stimulated pSTAT3-expressing neurons (Figure 3.3C). These data suggest that leptin does not control Kiss1 or GnRH neurons by direct action.

LepRb neurons innervate GnRH and Kiss1 neurons. In order to examine whether LepRb neurons directly innervate GnRH or Kiss1 neurons to modulate reproductive function, $LepRb^{WGA/EGFP}$ mice (as previously described in chapter 2) were analyzed for WGA- and GnRH-IR. In these mice, WGA-IR reveals LepRb neurons and their efferent contacts. Immunofluorescent analysis of $LepRb^{WGA/EGFP}$ mice reveals the accumulation of WGA-IR in GnRH-IR neurons in the POA (Figure 3.4-top row), indicating that GnRH

neurons are synaptically connected with LepRb neurons. Furthermore, Kiss1-IR neurons in the AVPV demonstrate WGA accumulation (Figure 3.4-middle row), and our preliminary analysis of $LepRb^{WGA/EGFP}$ mice (utilizing antibodies for Kiss1) demonstrate accumulation of WGA in the few detectable Kiss1-ARC neurons (Figure 3.4-bottom row). These data suggest the innervation of Kiss1 and GnRH neurons by unidentified groups of LepRb neurons.

Regulation of GnRH and Kiss1 neurons by leptin. In order to determine the ability of leptin to regulate GnRH and Kiss1 neurons, we initially assessed leptin-stimulated cFos expression in these neurons. Immunofluorescent analysis of cFos and GFP-IR in GnRH neurons of BIG mice following leptin treatment revealed little cFos colocalization (Figure 3.5A). Similarly leptin fails to stimulate cFos in GFP-IR ARC KNDy cells (Figure 3.5B). Thus, leptin alone is insufficient to modulate GnRH or KNDy neuronal activity. Unlike GnRH, which is regulated primarily at the level of release (not expression), many physiological parameters regulate *Kiss1* expression. In order to further explore the functional significance of LepRb → Kiss1 circuits, we assessed whether leptin could influence *Kiss1* expression in the ARC or AVPV. We thus analyzed female C57/Bl6 wild-type mice that were OVX, then fasted and treated with either leptin or PBS for 48 h. Quantitative PCR analysis shows that *Kiss1* expression in microdissected ARC tissue significantly decreased with fasting compared to fed controls, which was unchanged by leptin treatment (Figure 3.6A). ARC *Tac2* expression did not change with fasting or

with leptin treatment (Figure 3.6B). We additionally analyzed AVPV tissue from these mice, which revealed that *Kiss1* expression also did not change with fasting or with leptin treatment (Figure 3.6C). Thus, fasting has a strong effect on *Kiss1* expression in the ARC, whereas AVPV *Kiss1* and ARC*Tac2* expression is weakly (if at all) regulated by fasting or leptin.

Identification of LepRb populations that innervate GnRH neurons. In order to identify the population(s) of LepRb neurons that innervate GnRH neurons, we again utilized the BIG mice to trace GnRH afferents from various hypothalamic nuclei. LepRb neurons were identified by immunohistochemical staining for leptin-induced pSTAT3 nuclei and cytoplasmic WGA/BL, which revealed neurons in the PMv in synaptic contact with GnRH neurons, as previously reported (Leshan et al., 2009) (Figure 3.7). In the ARC, the density and intensity of pSTAT3-IR DAB deposition interfered with the detection of WGA-IR. Since ARC WGA-IR in BIG mice localized primarily to KNDy neurons (which do not contain LepRb), ARC LepRb neurons are unlikely to directly innervate GnRH neurons. Additionally, few WGA-IR neurons were colocalized with pSTAT3 in the VMH, or DMH (data not shown). Thus, LepRb neurons in the PMv represent the main direct projection from LepRb to GnRH neurons.

Discussion

We have examined the innervation of hypothalamic neurons that participate in the control of reproduction by LepRb neurons and revealed that ARC and AVPV Kiss1 neurons (as well as GnRH neurons) receive synaptic input from LepRb neurons, but only ARC KNDy neurons directly project to GnRH neurons (Figure 3.8). While the finding that LepRb-expressing neurons did not colocalize with KNDy neurons differs from the conclusions of others (Smith et al., 2006b), previous reports relied on double *in situ* methods to detect mRNA expression (rather than protein), which could be confounded by the high density of ARC LepRb neurons. We have determined the innervation of GnRH and AVPV Kiss1 neurons by LepRb neurons, but the poor detection of ARC KNDy neurons by Kiss1-IR dictates that the thorough analysis of this circuit awaits the generation of $LepRb^{WGA}$; Tac2-GFP mice. Functionally, however, while leptin failed to regulate AVPV *Kiss1*, leptin increased the fasting-induced suppression of *Kiss1* and *Tac2* gene expression in the ARC. Thus, the leptin/LepRb → ARC KNDy neuronal circuit is functionally important as the potential link between the hypothalamic reproductive system with energy balance.

Interestingly, our data indicate that leptin does not activate Kiss1 neurons in the ARC, as assessed by analysis of cfos induction in ARC KNDy neurons. Considering the GABAergic nature of most LepRb neurons in the ARC and elsewhere (Horvath et al., 1997; Cowley et al., 2001), this may indicate that while leptin suppresses the inhibitory input from NPY/GABAergic neurons to ARC Kiss1 neurons, which may be insufficient to activate Kiss1

neurons in the absence of other stimuli. Similarly, while LepRb neurons innervate the majority of GnRH and AVPV-Kiss1 neurons, our preliminary analysis reveal little to no activation of GnRH or AVPV Kiss1 neurons by leptin. Thus, consistent with a variety of physiological data (Ahima et al., 1996; Barash et al., 1996; Chehab et al., 1996), leptin appears permissive for these circuits, but insufficient to activate them on its own.

Our data reveal the strong connection between ARC-KNDy neurons and GnRH neurons but fail to demonstrate similarly strong connections between AVPV Kiss1 and GnRH neurons. The finding that AVPV Kiss1 neurons are not directly connected to GnRH neurons is surprising, as a role for AVPV Kiss1 neurons in regulating the LH surge has been assumed (Han et al., 2005; Smith et al., 2006a). Thus, it is possible that ARC Kiss1 neurons play a more direct role in the regulation of GnRH neurons than do AVPV Kiss1 neurons. Testing this concept directly will require the development of genetic or other tools to independently manipulate the two populations of Kiss1 neurons.

LepRb neurons in the PMv appear to represent the major group of LepRb neurons that directly synapse with GnRH neurons, presumably to directly regulate gonadotropin secretion (Figure 3.8). Repeating the BL/WGA-IR + pSTAT3 colocalization studies using immunofluorescent staining techniques in place of immunohistochemical/ DAB labeling (with possible quenching of WGA fluorescent signal) will be required to confirm this conclusion, however. These technical issues aside, LepRb populations in the

PMv are well poised to coordinate energy homeostasis and reproductive function, as the PMv integrates metabolic and sexual odorant cues to regulate LH secretion (Donato et al., 2009; Leshan et al., 2009). However, the mechanism by which LepRb PMv neurons control reproduction remains unclear and requires additional investigation.

Collectively, these data shed light on a number of central mechanisms by which leptin may control reproductive physiology. Leptin acts transsynaptically on ARC KNDy neurons to modulate gene expression in these neurons that are strongly connected to GnRH neurons. The role of leptin in regulating AVPV Kiss1 neurons, and their potential regulation of GnRH neurons remains less clear. Also unknown is the identity of the LepRb-expressing neurons that innervate each group of Kiss1 neurons. Based upon proximity, projection patterns, and known functions of the regions, PMv and/or ARC LepRb neurons represent reasonable candidates. Together with the PMv neurons that directly contact GnRH neurons, the LepRb neurons that control Kiss1 neurons represent likely modulators of central reproductive function.

Acknowledgements

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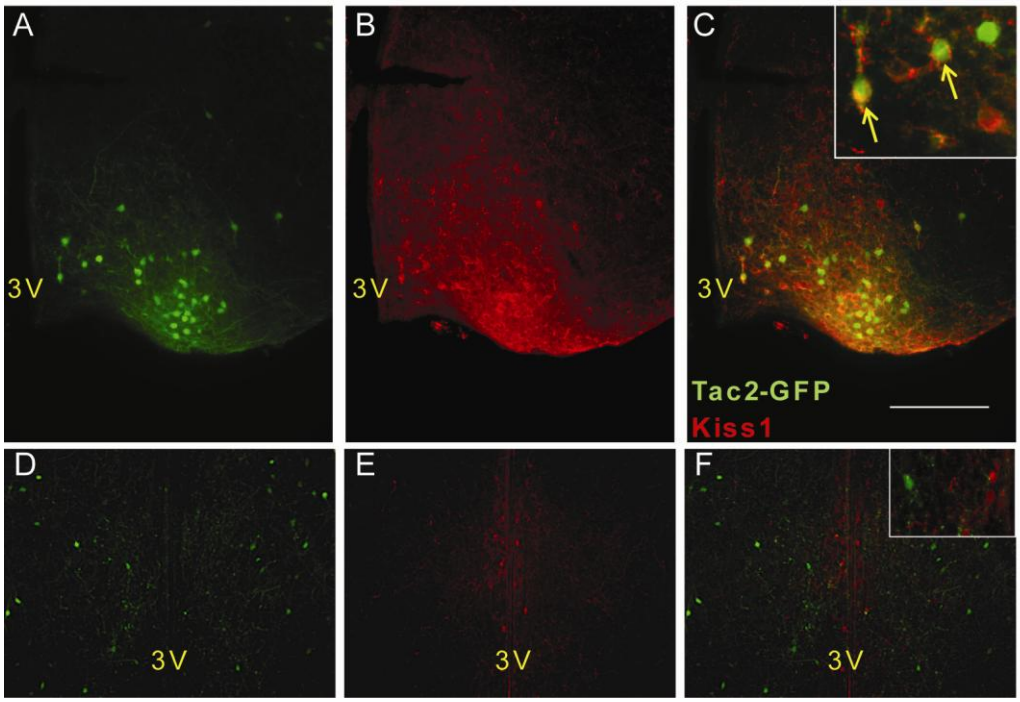

Figure 3.1 Kiss1 neurons in the ARC co-express Tac2. Immunofluorescent analysis of the ARC (A-C) and AVPV (D-F) in Tac2-GFP mice revealed that Tac2 neurons (A&D; green) colocalized with Kiss1 neurons (B&E; red) in the ARC, but not that AVPV. Panels C&F are merged images of A&B and D&E, respectively. Results are representative of 4 animals. Arrows represent colocalized neurons. Insets represent digital zoom of cells. 3V=third ventricle. Scale bar=10uM.

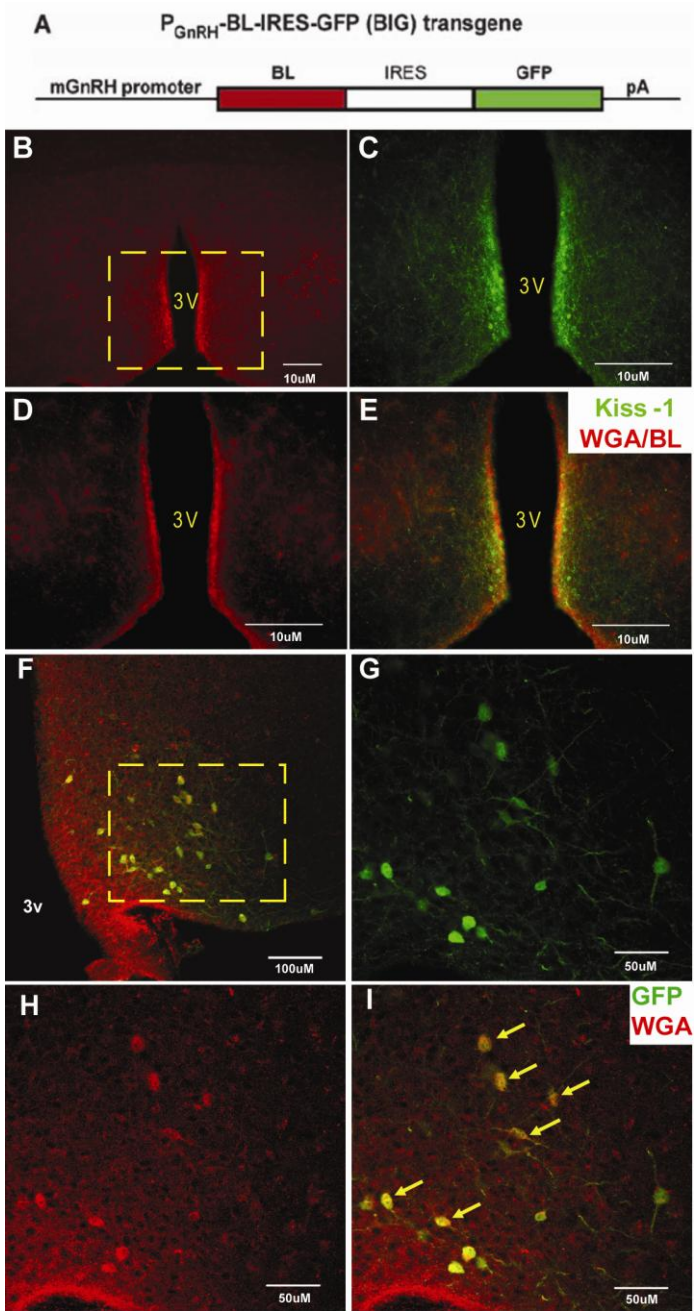

Figure 3.2 Kiss1 populations project onto GnRH neurons. Transgenic "BIG" mice allow the visualization of afferents and efferents of GnRH neurons with WGA/BL tracing (A). Immunofluoresent analysis of the AVPV (B-E) in these mice reveal few Kiss1 cells (C; green) that colocalize with WGA (D; red). Panel E represents the merged image of C&D. Confocal analysis of the ARC (F-I) reveal the majority of ARC KNDy cells (marked by Tac2-GFP; green; G) colocalize with WGA (red; H). Panel I represents the merged ARC images of G&H. Panels C-E and G-I are magnified views of boxed region in B&F, respectively. Results are representative of 3 animals. Arrows= colocalized cells. Scale bars as indicated. (Construct in panel A taken from Boehm 2005).

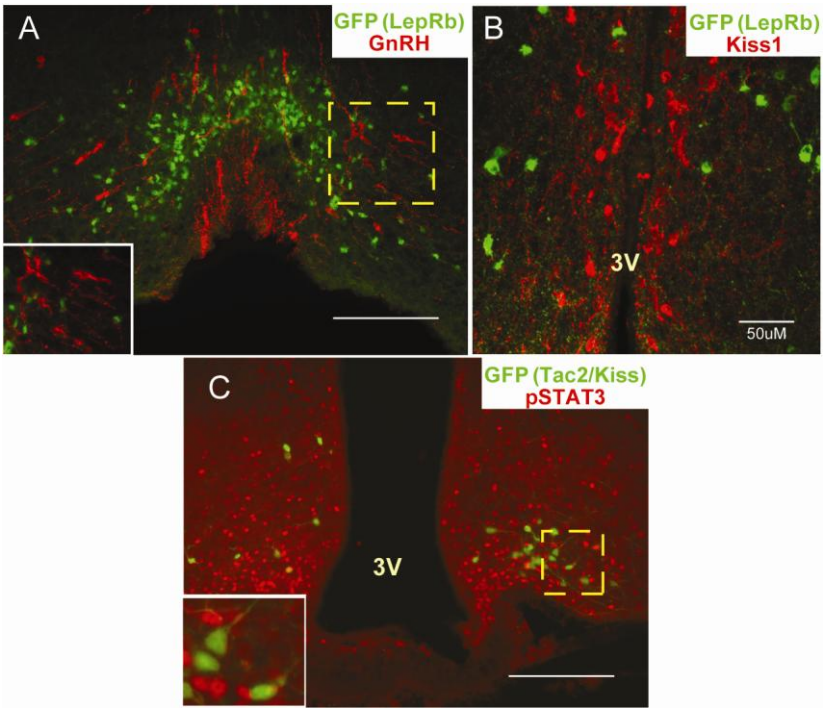

Figure 3.3 LepRb is not expressed in hypothalamic GnRH or Kiss1 neurons. $LepRb^{EGFP}$ mice reveal that GnRH neurons in the POA (A; red) and AVPV-Kiss1 neurons (B; red) do not express LepRb-GFP (A&B; green). Panel C represents KNDy neurons labeled with GFP (green) in the ARC of Tac2-GFP mice that do not express leptin-induced pSTAT3 (red). Results are representative of 3-4 animals. Insets represent zoomed image of boxed region. Scale bars =10um unless otherwise noted. 3V=third ventricle.

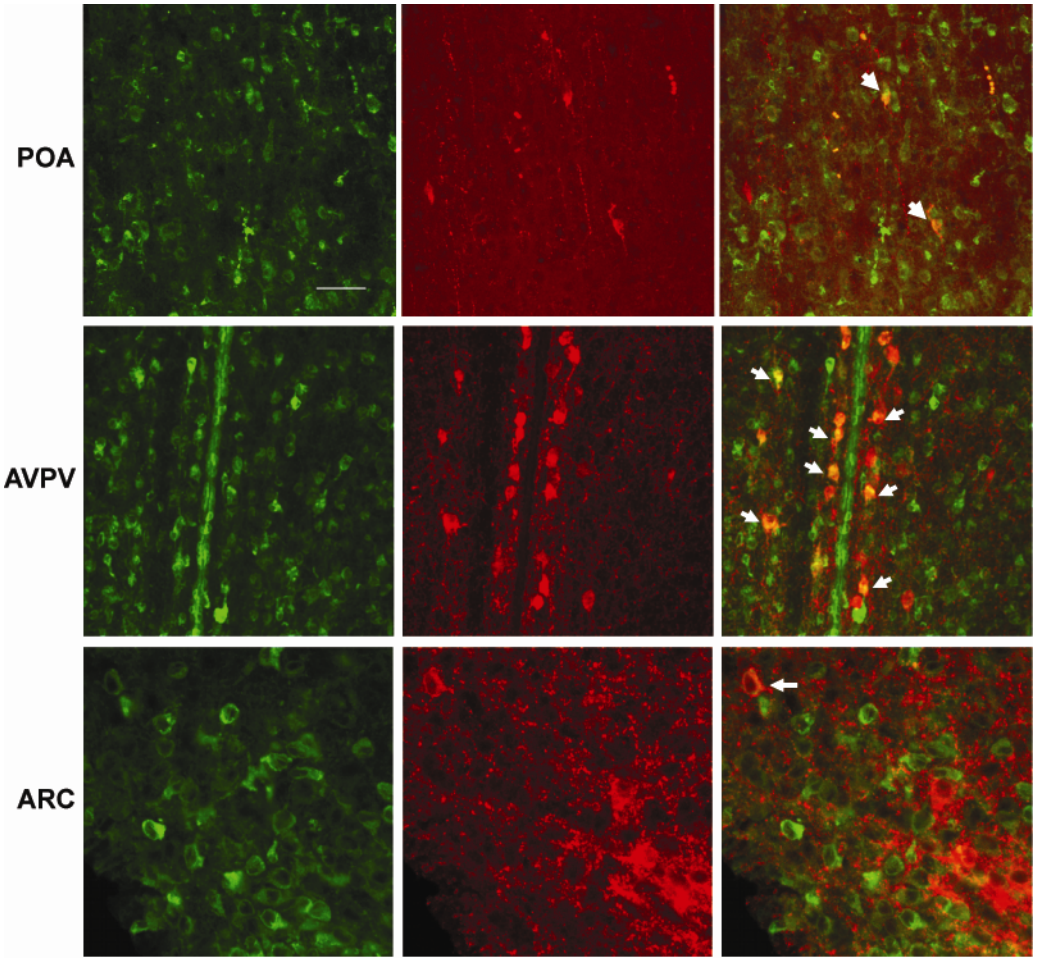

Figure 3.4 LepRb neurons synapse with GnRH and Kiss1 neurons. $LepRb^{EGPF/WAP}$ mice reveal that LepRb neurons (green-all rows) project to GnRH neurons (red; top row), AVPV-Kiss1 neurons (red; middle row), and ARC KNDy neurons (red; bottom row) cells. Results in POA and AVPV are representative of 4 animals. Preliminary results in ARC are from n=1. Arrows represent colocalized cells. Scale bar=50um.

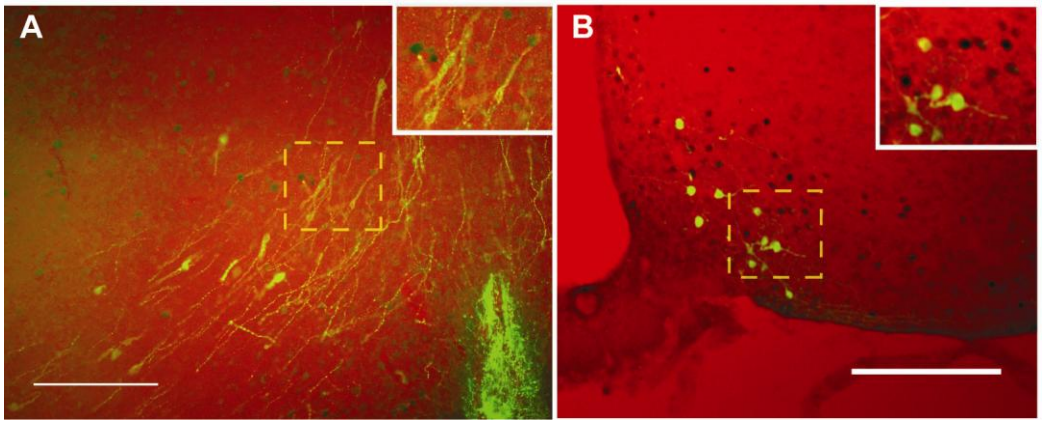

Figure 3.5 Leptin fails to activate GnRH neurons or ARC KNDy neurons. Immunofluorescent analysis of BIG mice reveal that leptin does not induce cFos activation (black; A&B) in GnRH neurons (green; A) in the POA. Analysis of Tac2-GFP mice reveal that KNDy neurons (green; B) in the ARC do not colocalized with cFos (black). Results are representative from 3 animals. Insets= digital zoom of boxed area. Scale bars=10uM.

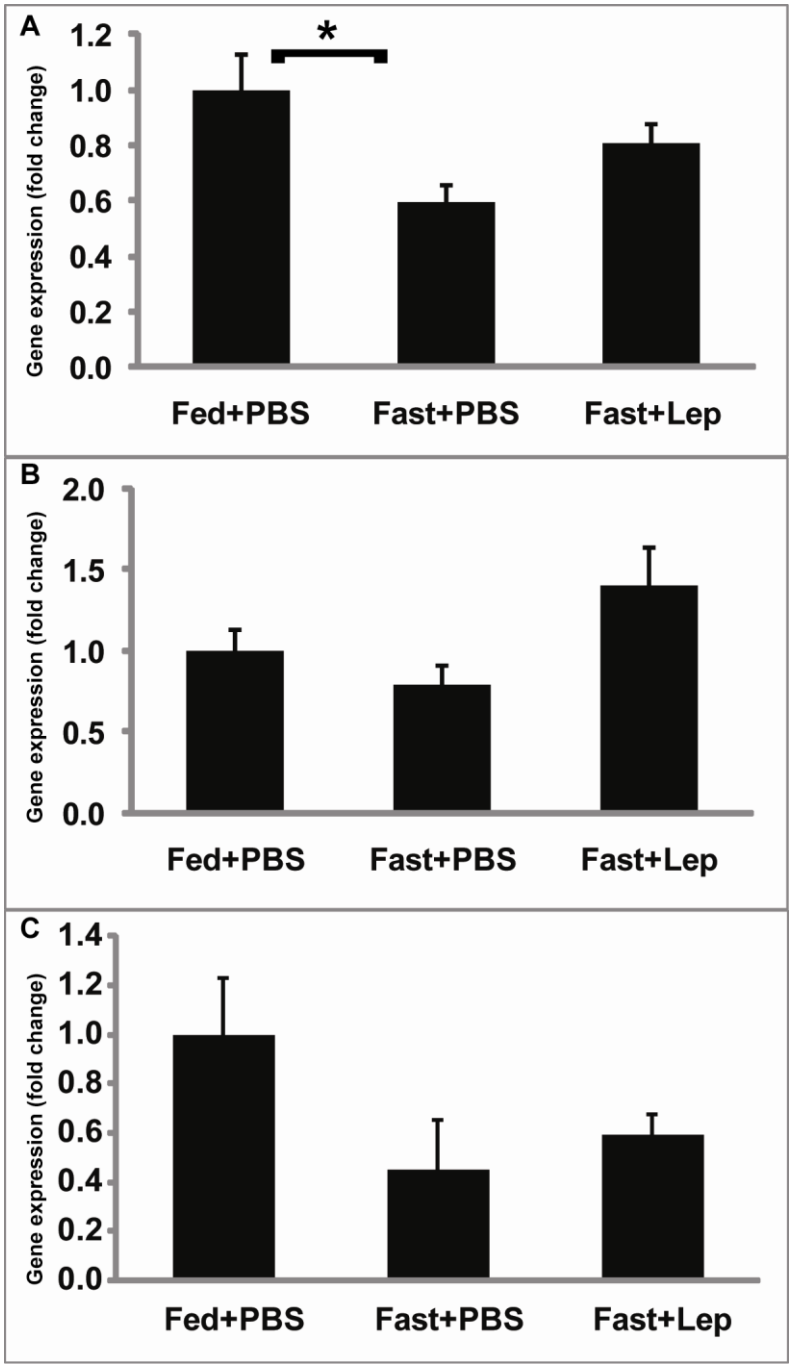

Figure 3.6 Leptin increases fasting-induced changes in *Kiss1* and *Tac2* mRNA expression in the ARC and AVPV. OVX wild-type mice were fed or fasted and treated with PBS or leptin (i.p.) for 48hr. Microdissected ARC tissue was analyzed for *Kiss1* (A) and *Tac2* (B) gene expression by qRT-PCR. Changes in *Kiss1* expression in the AVPV was also analyzed (C). Expression data are plotted relative to *Gapdh* expression (calculated by $2^{-\Delta\Delta Ct}$ method) +/- SEM. N=7-9 animals per cohort. *=$p<0.05$ (by one way ANOVA with Bonferroni's post-testing).

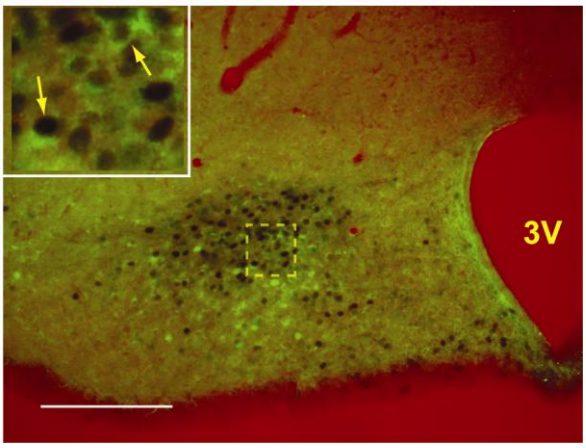

Figure 3.7 LepRb neurons in the PMv project directly to GnRH neurons. Immunohistochemical analysis of BIG mice reveal that LepRb neurons in the PMv (as indicated by pSTAT3; black) colabeled with WGA (green), suggesting that PMv LepRb neurons project directly to GnRH neurons. Results are representative from 5 animals. Arrows indicate colocalized neurons. Inset=digital zoom of boxed region. Scale bar = 10uM. 3V=third ventricle.

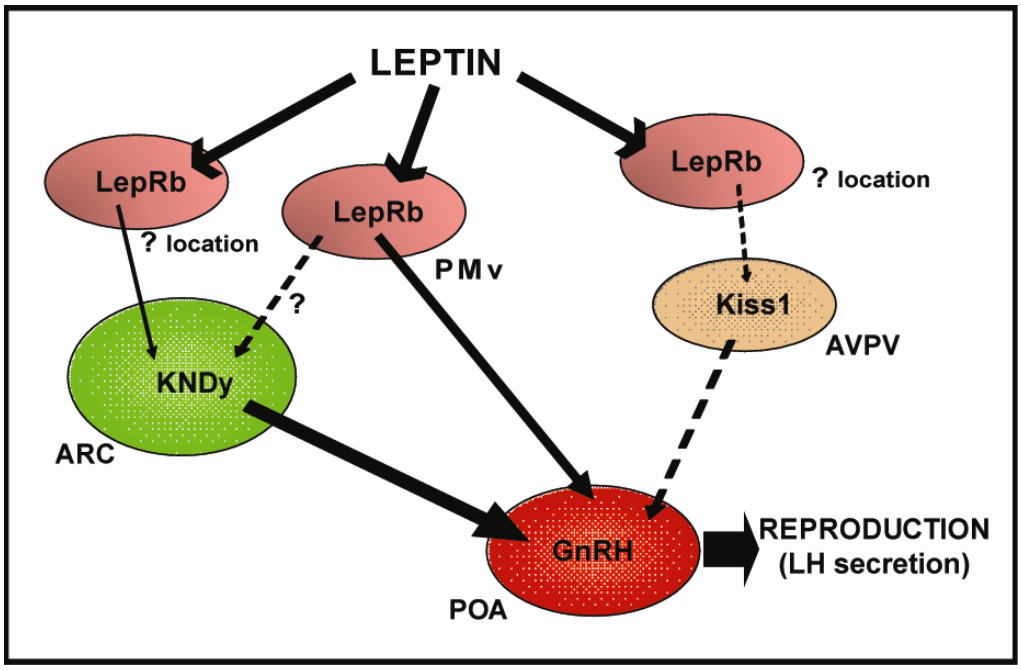

Figure 3.8 Leptin action on LepRb neurons in the PMv or other locations modulate reproductive function indirectly via Kiss1 neurons or directly via GnRH neurons. While GnRH and Kiss1 populations do not express LepRb, they do receive synaptic input from LepRb neurons. LepRb neurons in the PMv directly synapse with GnRH neurons, whereas leptin may be modulating ARC KNDy neurons indirectly via LepRb populations. Overall, this complex circuitry pattern links leptin signaling of changes in nutritional status to reproductive function. Dotted arrows indicate weaker or more questionable connections.

CHAPTER FOUR

SUMMARY AND CONCLUSIONS

GLOBAL PERSPECTIVE OF LEPTIN'S REGULATION OF PHYSIOLOGY

Leptin in neuroendocrine function

The discovery of leptin has advanced the understanding of the mechanisms that coordinate energy balance and physiological function. Leptin is secreted from adipocytes in proportion to body adiposity and thus in energy-replete states. Leptin binds to LepRb-expressing neurons in the CNS to modulate energy homeostasis by suppressing food intake and increasing/permitting energy use on numerous physiological functions, such as locomotor activity, growth and reproduction. While LepRb populations are distributed throughout the hypothalamus and other brain regions, ARC LepRb neurons are the most well-defined. Leptin activates POMC neurons to suppress feeding, while inhibiting orexigenic AgRP/NPY neurons (Figure 4.1). The ARC also contains a population of *Kiss1* expressing neurons, which project to GnRH neurons and play a role in fertility and are controlled by LepRb neurons, however the mechanism(s) by which leptin regulates Kiss1 neurons are poorly defined.

Only a small fraction of leptin action is mediated via the ARC POMC and AgRP cells, and thus, other LepRb neuronal populations in other hypothalamic nuclei must contribute crucial aspects of total leptin action. For example, the VMH contains a population of LepRb neurons that may be functionally defined by their co-expression of BDNF or steroidogenic factor-1 (SF-1). BDNF is an important factor for neurite outgrowth (Cronin et al., 2004) and is controlled by nutritional states (Duan et al., 2003; Nakagawa et al., 2003). SF-1 neurons are required for body weight homeostasis and glycemic control (Dhillon et al., 2006). Another population of LepRb neurons in the PMv have been reported to project onto, and presumably regulate, GnRH neurons (Leshan et al., 2009), contributing to the regulation of sexual and mating behaviors (Simerly, 1990; Kollack-Walker and Newman, 1995; Coolen et al., 1996; Yokosuka et al., 1999). Lastly, LepRb neurons are also found in areas associated with the mesolimbic dopamine reward system, such as the VTA and LHA, which are important for controlling motivation and rewarding behaviors (DiLeone et al., 2003; Leinninger et al., 2009). Thus, leptin acts via many unique LepRb-containing loci in the brain to mediate separate and specific physiological functions such as feeding, growth, reproduction, and immune response.

This dissertation has expanded our knowledge of the distinct central mechanisms of leptin action, in particular, by identifying and analyzing the circuits by which several LepRb-containing neuronal populations have the potential to mediate different physiological processes. We first explored

LepRb neurons in the LHA and identified two circuits by which they regulate participants in the mesolimbic DA system; these circuits represent novel pathways to potentially control hedonic feeding and other motivated behaviors (Figure 4.1). We next studied the connectivity between LepRb neurons and hypothalamic Kiss1 and GnRH neurons; these novel circuits are likely important in regulating reproduction (Figure 4.1). Collectively, these studies have utilized sophisticated tract tracing tools and mouse models to identify novel LepRb-neuronal circuits with unique contributions to leptin action, thus verifying our overall hypothesis: that distinct sets of LepRb-containing loci potentially have discrete contributions to physiology.

Leptin circuits regulating food reward pathways

In order to define the neural mechanisms by which leptin contributes to hedonic feeding, we first generated novel tract tracing tools to specifically study LepRb circuitry. Detection of WGA tracing from LepRb neurons throughout the CNS determined that LHA OX and MCH neurons (which do not express LepRb) receive synaptic input from LepRb expressing neurons. Furthermore, we showed that LHA LepRb neurons project to local OX neurons (but not to MCH neurons) and indeed, leptin in the LHA induces OX expression. This LHA-LepRb regulated circuit presumably modulates the mesolimbic reward system. In the future it will be important to identify the LHA LepRb neurons that project to LHA OX neurons thereby illuminating how leptin collectively modulates the LHA to alter hedonic feeding. The populations of

LepRb neurons that lie upstream of MCH neurons remain unclear, although previous work suggest a potential role for ARC melanocortin neurons in this regulation (Elias et al., 1998; Hanada et al., 2000; Kim et al., 2005). However, these studies exceed the scope of this dissertation.

We also showed that LHA LepRb neurons project to DAergic neurons in the VTA, and intra-LHA leptin activates neurons in the VTA to presumably modulate the mesolimbic system. Given that VTA neurons are activated by LHA-leptin, we need to determine if this activity is mediated by LHA LepRb neurons projecting directly to the VTA or via indirect projections onto LHA OX neurons that innervate the VTA. One means of parsing this is to determine whether antagonists of OX receptors block the leptin-induced activation of VTA neurons, to determine whether leptin acting on the LHA regulates the mesolimbic DA system indirectly via OX neurons. Additionally, it will be useful to determine whether the VTA neurons that are activated by intra-LHA leptin treatment are DAergic; this data would suggest a mechanism by which LHA LepRb neurons regulate DA levels, and thus mesolimbic DA signaling. It is important to understand the contributions of these direct and indirect pathways from LHA LepRb neurons to the mesolimbic DA system and energy balance.

The mesolimbic DA system controls hedonic feeding, which contributes to over-consumption of calories beyond energy needs (overeating). Thus, hedonic feeding promotes obesity and possibly-linked reproductive deficits. Understanding these LepRb → mesolimbic circuits and how hedonic intake is regulated may help develop therapies to curb hedonic drive and stem

overeating of highly palatable, energy dense foods that promote obesity. Identification of this circuit (both the direct and indirect targets) is an important step forward in understanding leptin regulation of hedonic pathways in food intake and their contribution to physiology.

Leptin circuits controlling reproductive physiology

The studies herein shed light on the neural pathways by which leptin controls the central reproductive axis. While LepRb populations synapse with both ARC and AVPV Kiss1 neurons (that do not express LepRb), only the ARC KNDy neurons appear to be in substantial direct synaptic contact with GnRH neurons to potentially mediate leptin action on GnRH neurons. Furthermore, we showed that fasting blunts and leptin increases *Kiss1* expression in the ARC, while these effects are not seen in the AVPV. In order to confirm this LepRb → ARC KNDy neuronal circuit, we will analyze Tac2-GFP/LepRb$^{cre/WGA}$ mice (Tac2-GFP mice interbred with LepRb$^{cre/WGA}$ mice) to confirm with better techniques that ARC KNDy neurons receive synaptic input from LepRb neurons. Since leptin mediates hypothalamic *Kiss1* expression (Castellano et al., 2006), we expect immunofluorescent detection will reveal colocalization of GFP and WGA in the majority of ARC Tac2-GFP neurons, confirming that LepRb neurons synapse with ARC KNDy neurons. However, the role for AVPV Kiss1 neurons in leptin action remains unclear.

While outside the scope of this report, it will be imperative to determine which LepRb neurons project to and regulate ARC and AVPV Kiss1 neurons.

In order to do this, we have begun to generate Tac2^{cre} and Kiss^{cre} mice, with which to perform neuron-specific retrograde tracing studies. While awaiting the generation of these novel mouse models, anterograde Ad-iN/WED tracing from candidate LepRb populations (e.g. PMv, ARC) in LepRb^{cre} mice may reveal some connections. With regards to the neurochemical makeup of LepRb-expressing neurons that innervate ARC KNDy neurons, we speculate that LepRb GABAergic (e.g. ARC) neurons project to and suppress Kiss1 ARC KNDy neurons and leptin action could blunt this inhibitory input, thus potentiating the activation of Kiss1 neurons by other factors and activation of GnRH neurons.

In addition, we showed herein that leptin may directly influence GnRH neurons via PMv LepRb neurons. Indeed, we have recently showed that LepRb neurons in the PMv are activated by sexual odorants (Leshan et al., 2009), however the neurochemical makeup of these PMv LepRb neurons remains in question. Future studies to identify these PMv LepRb neurons are underway in our laboratory.

These studies just described trace LepRb neuronal circuitry to reproductive neurons in a simplistic model, as we initially analyzed intact female mice in diestrus for WGA or BL tracing. While this standardizes for cyclic changes, the low estrogen levels may hinder the number of Kiss1 neurons detected in the AVPV as others have previously shown. Thus, we will verify the tracing of AVPV Kiss1 neuronal efferents and afferents (or the lack there of) in animals in the estrus stage. But, we presume that acute cyclic

changes do not substantially change the wiring circuit since WGA accumulation occurs over several days and is not likely to be affected by day to day changes in cycle stage. When determining the regulation of *Kiss1* gene expression by leptin, we analyzed OVX female mice to standardize for estrogen-induced gene expression changes. However, low estrogen levels suppress *Kiss1* expression in the AVPV and increase expression in the ARC, thus it would be useful to repeat this experiment using both OVX and intact animals.

When evaluating the neural systems that regulate feeding behavior or reproduction, one must consider the physiological, environmental and ecological variations between rodents and humans. For one thing, humans have a much greater body mass and therefore a greater capacity for energy storage compared to a small mouse. As a result, larger mammals with greater fat storage capacities are better adept to counter acute environmental energetic challenges. Rodents are acutely influenced by environmental changes such as in temperature, rainfall, photoperiod or food availability, while humans are less affected by these situations except in extreme cases such as drought or prolonged freezing temperatures. Another factor to consider is that humans, at least modern day humans, obtain food with relative ease compared to foraging rodents, therefore the energy requirements vary among species. Amongst rodent species, there also exist energy utilization differences experienced by animals in the wild (with natural fluctuations in environmental factors) versus domesticated animals in stable and controlled

conditions. Nevertheless, food restriction or prolonged exercise can suppress pubertal development and ovulation in all mammals, ranging from rodents to livestock to primates and humans, while depleting fat stores and suppressing growth (Armstrong et al., 1987; Foster et al., 1989; Manning and Bronson, 1989; Kurz et al., 1990). The underlying neural pathways that link energetics and reproduction, while more acutely regulated in rodents, are likely to be similar to that in humans. Overall these systems remain poorly defined and have only begun to be deciphered herein in mouse models.

Additionally, one must also contemplate sex differences when studying feeding behaviors and reproduction. Male and female rodents differ essentially in body size, but also the nature and in the degree of the energetic costs of reproduction. A male's energetic costs are primarily behavioral, in order to physically dominate over other males and to attract female mates. Meanwhile, the physical demands of pregnancy, partuition and lactation in females is highly energetically demanding. Thus, there exists a fundamental difference between the sexes with different strategies for balancing the energetic demands for successful reproduction. We speculate that the LepRb → Kiss/GnRH circuitry pattern is more profound and robust in females than males. Overall, the bioenergetic relationship with reproductive success which varies between species, between populations and between sexes is important to consider.

Undernourishment and over-exercising can contribute to unbalanced energy homeostasis and are linked to reproductive deficits in all species.

Understanding how the hypothalamic reproductive system is regulated by energy levels may help develop therapies to initiate and promote reproductive competence. Identification of this circuitry (both the direct and indirect targets) is an important step forward in understanding leptin regulation of reproduction and their contribution to physiology.

Importance and clinical applications

Herein, we have determined that LHA LepRb neurons are connected to neural circuits that regulate motivational aspects of feeding via LHA OX neurons projecting to the mesolimbic DA system, which play into the whole of energy balance regulation. Additionally, we determined that leptin acts on Kiss1-ARC neurons indirectly, which are known to modulate GnRH neurons and subsequently reproductive physiology. Identification of these LepRb circuits and their direct and indirect pathways to modulate different functions is consistent with our overarching hypothesis that separate populations of LepRb neurons regulate different aspects of leptin action. Characterization of these individual neuro-circuits is crucial for understanding the totality of leptin action in physiology.

Defining these neural pathways will enable us to understand how changes in energy balance can lead to pathologies that affect various physiological functions. For example, understanding how low body adiposity can be linked to amenorrhea and infertility may lead to pharmaceutical treatments which can restore normal physiological function. Alternately,

understanding the pathways underlying overeating are crucial to understand the pathogenesis of obesity and identify potential pharmacological therapies.

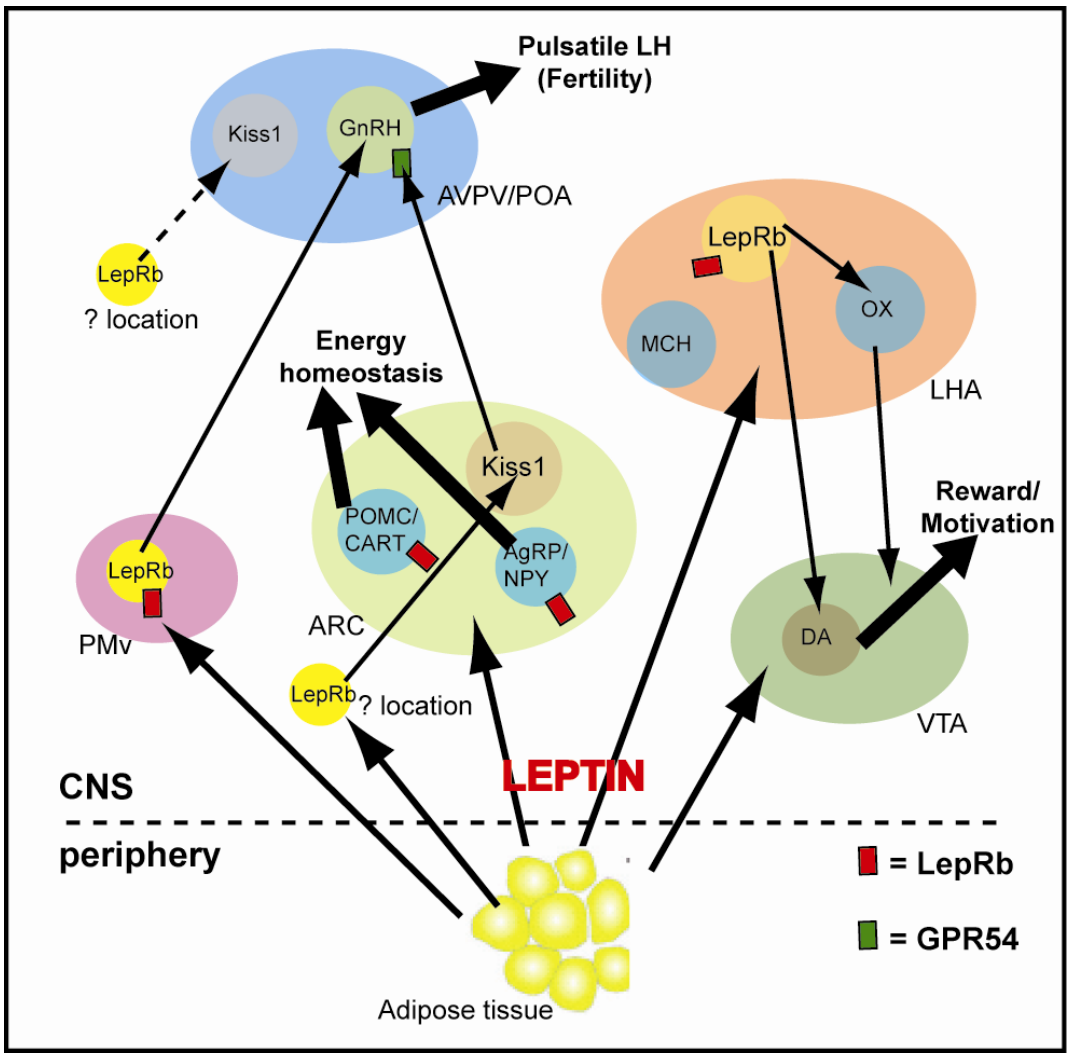

Figure 4.1 Distinct LepRb populations regulate different neural systems and physiological functions. Leptin secreted from adipose tissue acts on LepRb-expressing neurons in the PMv, ARC, LHA, and VTA (amongst other regions). LepRb populations in the ARC that express POMC/CART or AgRP/NPY are well-known for their role in the regulation of energy homeostasis, while LepRb neurons in the LHA modestly project directly to DAergic neurons in the VTA to modulate the mesolimbic DA system. LHA LepRb neurons also innervate local OX neurons that control DAergic neurons in the VTA. LepRb neurons synaptically project to ARC KNDy neurons, which project to GnRH neurons. Also, LepRb neurons in the PMv directly project to GnRH neurons to collectively presumably control reproduction. Dotted arrows indicate weaker or more questionable connections.

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