LMO4 is required for central leptin control of fat metabolism and insulin sensitivity

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ABSTRACT

Metabolic homeostasis is orchestrated by the hypothalamus through the neuroendocrine and the autonomic nervous systems. The hypothalamic nuclei respond to the peptide leptin secreted from adipose tissue to suppress feeding and increase energy expenditure by promoting fat metabolism via sympathetic activity. Another important, but perhaps less appreciated function of central leptin signaling is to elevate peripheral insulin sensitivity. Environmental and genetic risk factors that affect hypothalamic leptin signaling can lead to obesity and type 2 diabetes mellitus (T2DM).

Here, we discovered that LIM domain only 4, LMO4, is a novel protein participating in central leptin signaling. In a process strikingly similar to T2DM in humans, CaMKIIα-Cre;LMO4flox/flox mice, which have LMO4 knocked out in the postnatal brain including the hypothalamus, develop visceral adiposity, reduced insulin sensitivity, obesity and diabetes when fed with regular chow. Central leptin signaling was significantly lost in key hypothalamic nuclei of mutant mice. Caloric restriction prevents obesity but not insulin resistance in these mice. Taken together, our results suggest that LMO4 function in the brain is required for central leptin signaling to control fat metabolism and peripheral insulin sensitivity.

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LIST OF ABBREVIATIONS

α-MSH: α melanocyte-stimulating hormone

ACC: acetyl-CoA carboxylase

Akt: protein kinase B

AgRP: agouti-related peptide

ARC: arcuate nucleus

Atgl: adipose triglyceride lipase

BAT: brown adipose tissue

bHLH: basic helix-loop-helix

BRCA1: breast-cancer susceptibility protein 1

CaMKIIα: calcium/calmodulin-dependent protein kinase II α

CLIM/NLI/LDB: LIM domain binding protein

CREB: cAMP response element-binding protein

CtBP: C-terminal binding protein

CtIP: CtBP-interacting protein 1

DIO: high-fat diet induced obesity

DMH: dorsomedial hypothalamic nucleus

ERα: estrogen receptor alpha

ERK: extracellular signal-regulated kinases

FAS: fatty acid synthase

FoxO1: forkhead box protein O1

gWAT: gonadal white adipose tissue

GFP: green fluorescent protein

GTT: glucose tolerance test

HSL: hormone sensitive lipase

i.c.v.: intracerebroventricular

i.p.: intraperitoneal

IL-6: interleukin-6

IRS: insulin receptor substrate

ITT: insulin tolerance test

JAK: Janus kinase

KO: knock out

LepR: leptin receptor

LHA: lateral hypothalamic area

LIM: Lin-11, Isl-1 and Mec-3

LMO: LIM domain only

LMO4: LIM domain only 4

MBH: mediobasal hypothalamus

MC4R: melanocortin receptor 4

MRI: Magnetic Resonance Imaging

NE: norepinephrine

NETO: norepinephrine turnover

NHLH2: nescient helix-loop-helix 2

NTS: nucleus tractus solitarius

PDK1: phosphoinositide dependent kinase 1

PI3K: phosphoinositide 3-kinases

POMC: pro-opiomelanocortin

PPARγ: peroxisome proliferator-activated receptor-γ

PTP1B: protein tyrosine phosphatase 1B

PVN: paraventricular nucleus

SF-1: steroidogenic factor 1

SHP2: src-homology-2 (SH2) domain-containing protein tyrosine phosphatase 2

Sim-1: single-minded homolog 1

Socs3: suppressor of cytokine signaling 3

SOD2: antioxidant gene superoxide dismutase 2

Stat3: signal transducer and activator of transcription 3

T2DM : type 2 diabetes mellitus

TAL1: T-cell acute leukemia 1

TGFβ: transforming growth factor beta

UCP1: uncoupling protein 1

Vglut2: vesicular glutamate transporter 2

VMH: ventromedial hypothalamic nucleus

VTA: ventral tegmental area

WT: wild type

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Chapter 1. Introduction

1.1 LMO4 and LIM domain only family.

LIM domain only 4 (LMO4) is a protein with 165 amino acids consisting exclusively of two tandem LIM domains (Fig.1) which were initially discovered in proteins **L**in-11, **I**sl-1 and **M**ec-3 (Freyd et al., 1990; Karlsson et al., 1990; Way and Chalfie, 1988). Approximately 55 residues in length, the LIM domain is a cysteine-rich structure containing two zinc-finger motifs which are separated by two amino acids (Sanchez-Garcia and Rabbitts, 1994). Although zinc finger motifs are typical DNA-binding structures, a DNA-binding function of the LIM domains has yet to be demonstrated. LMO4 is the most recently identified member in the LIM domain only (LMO) family (Kenny et al., 1998), of which the other members are LMO1, LMO2 and LMO3. Motifs other than the LIM domain have not been reported in this family (Sanchez-Garcia and Rabbitts, 1994). Proteins of this family are found in the nucleus and function as transcription cofactors. They play important roles in transcriptional regulation and are involved in development and oncogenesis. For example, LMO1 and LMO2 have been identified as oncogenes in T-cell acute lymphoblastic leukemia, which are activated by chromosomal translocation (Chervinsky et al., 1999; Ferrando et al., 2004; Rabbitts, 1998). LMO2 is also required for erythropoiesis in the embryonic stage and hematopoiesis in the adult (Warren et al., 1994). Both LMO1

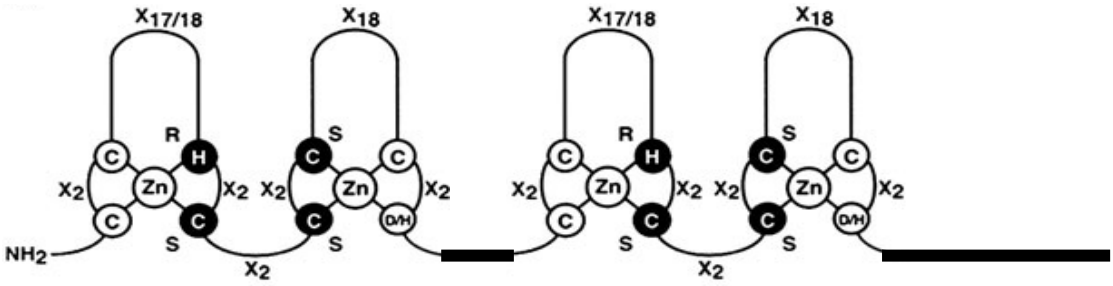


Figure 1. Topology of the LMO4 Protein Structure.

LMO4 has two tandem LIM domains which are generally 50-60 amino acids in size and share two characteristic zinc fingers that are separated by two amino acids.

and LMO3 are involved in the progression of neuroblastoma (Aoyama et al., 2005; Wang et al.). Lacking DNA-binding activity, LMO proteins exert their functions in the transcriptional regulation by forming complexes with other transcription factors. LMO1 and LMO2 have been demonstrated to interact with T-cell acute leukemia 1 (TAL1), a basic helix-loop-helix (bHLH) transcription factor in thymocytes (Herblot et al., 2000). LMO3 can bind to a neuronal specific transcription factor, nescient helix-loop-helix 2 (NHLH2), in the regulation of neurogenesis (Aoyama et al., 2005).

LMO4 shares only 50% homology with the LIM domains of other LMO proteins (Fig.2) and is widely expressed in embryonic and adult tissues (Kenny et al., 1998). Because of its various functions in tumorigenesis and neuronal differentiation, LMO4 has attracted increasing attention in the studies of LMO proteins. The first well-known function of LMO4 is to inhibit differentiation of mammary epithelial cells and it is reported that LMO4 is overexpressed in breast cancer (Visvader et al., 2001). Studies have identified the interaction of LMO4 with breast-cancer susceptibility protein 1 (BRCA1) and CtBP (C-terminal binding protein)-interacting protein 1 (CtIP). LMO4 acts as a negative regulator of BRCA1-mediated transcription activation in breast cancer (Sum et al., 2002). Further studies show that LMO4 also binds to estrogen receptor alpha (ERα) and represses ERα transactivation functions in the process of breast cancer progression (Singh et al., 2005). LMO4 function in the epithelial oncogenesis also involves the interaction with Smad proteins in transforming growth factor beta (TGFβ) signaling (Lu et al., 2006). Overexpression of

Kenny, Proc. Natl. Acad. Sci. 1998

Figure 2. Sequence Analysis of LMO4.

Sequence alignment of mouse LMO4 with LMO1, LMO2 and Drosophila LMO. Shading shows conservation of amino acids, and asterisks indicate the zinc-coordinating residues of each LIM domain. LMO4 shares only 50% homology with the LIM domains of other LMO proteins.

LMO4 is also found in some other human tumors (Mousses et al., 2002; Murphy et al., 2008; Taniwaki et al., 2006; Yu et al., 2008). The function of LMO4 involved in tumorigenesis is associated with its binding and modulation of different transcription factors, like other members of the LMO family.

Most recent research about LMO4 has focused on its role in the embryonic brain development, as the null mutation of LMO4 causes embryonic lethality and gives rise to exencephaly in mouse (Lee et al., 2005; Tse et al., 2004). Accumulating evidence indicates that LMO4 plays a important role in the regulation of proliferation and differentiation of developing neurons. LMO4 expression is associated with the morphogenesis of the developing mouse inner ear and required for the formation of vestibular structures (Alvarado et al., 2009; Deng et al., 2006). LMO4 is also required for the normal mammalian eye development in which LMO4 is important in regulating the inhibitory neuron differentiation (Duquette et al.). Similar function was also reported in the spinal cord. As a transcriptional factor, LMO4 participates in a regulatory network to establish the motor neurons identity in cooperation with Hb9 and controls the cell fate of V2-interneurons in developing spinal cord (Joshi et al., 2009; Lee et al., 2008). LMO4 is also involved in development of the somatosensory barrel field (Huang et al., 2009). Through a calcium-dependent transactivation trap assay, LMO4 was identified as a transcription activator in response to calcium influx and shown to form a complex with cAMP response element-binding protein (CREB) to pattern thalamocortical connections during cortex development (Kashani et al.,

2006). All of these functions are based on the interaction of LMO4 with LIM domain binding protein (CLIM/NLI/LDB) (Deane et al., 2002; Sugihara et al., 1998). Together, LMO4 participates in the gene expression regulation by interacting with multiple transcription factors and cofactors to form transcription complexes.

In addition, LMO4 is expressed in the adult brain (Hermanson et al., 1999) but the adult functions of LMO4 are poorly investigated. Previous studies in our laboratory have identified LMO4 as an essential hypoxia-inducible cofactor for peroxisome proliferator-activated receptor-γ (PPARγ) signaling in the neurons. This, for the first time, revealed a novel function of LMO4 in adult cortex. Elevated expression of LMO4 after stroke upregulates the antioxidant gene superoxide dismutase 2 (SOD2) and is an important determinant of neuron survival (Schock et al., 2008). However, LMO4 function in other regions of adult brain remains unknown.

1.2 Leptin signaling in the brain.

Leptin is a 16 kDa adipose derived hormone encoded by the obese gene (Zhang et al., 1994). It plays a key role in regulating food intake and energy expenditure by interacting with certain brain regions (Buchanan et al., 1998). Leptin exerts its function through binding to the leptin receptor (LepR) and activating several intracellular signaling cascades. There are at least six different types of leptin

receptors encoded by a single gene, among which the long form LepRb is the only isoform that can transduce the signal intracellularly via its long intracellular domain (Bjorbaek et al., 1997; Tartaglia et al., 1995). In the brain, LepRb is mainly expressed in the hypothalamus with the highest expression in the arcuate nucleus (ARC) (Bohlender et al., 2003). LepRb expression is also found in the extrahypothalamic sites in brain such as the vagal complex and the ventral tegmental area (VTA) (Williams et al., 2007; Hommel et al., 2006).

The LepRb is a member of the type I cytokine receptor family which also includes interleukin-6 (IL-6) receptor. The receptors of this family do not have inherent enzymatic activity but perform signal transduction by activating the tyrosine kinases of the Janus kinase (JAK) family. Once leptin binds LepRb, the intracellular domain of the receptor recruits JAK2 and initiates three different signal cascades, the JAK2/Stat3, SHP2/ERK and PI3K/Akt pathways, to activate the transcription of downstream target genes. The leptin signaling pathway in the ARC has been fully elucidated in the regulation of food intake. The ARC consists of two distinct neuronal populations which exert opposing effects on food intake and energy expenditure. The AgRP neurons synthesize agouti-related peptide (AgRP), which functions as a strong orexigenic neurotransmitter, while the POMC neurons produce pro-opiomelanocortin (POMC), which is cleaved to generate the α melanocyte-stimulating hormone (α-MSH), a powerful anorectic peptide (Fig.3) (Barsh and Schwartz, 2002). Leptin signaling induces POMC transcription and suppresses AgRP transcription by

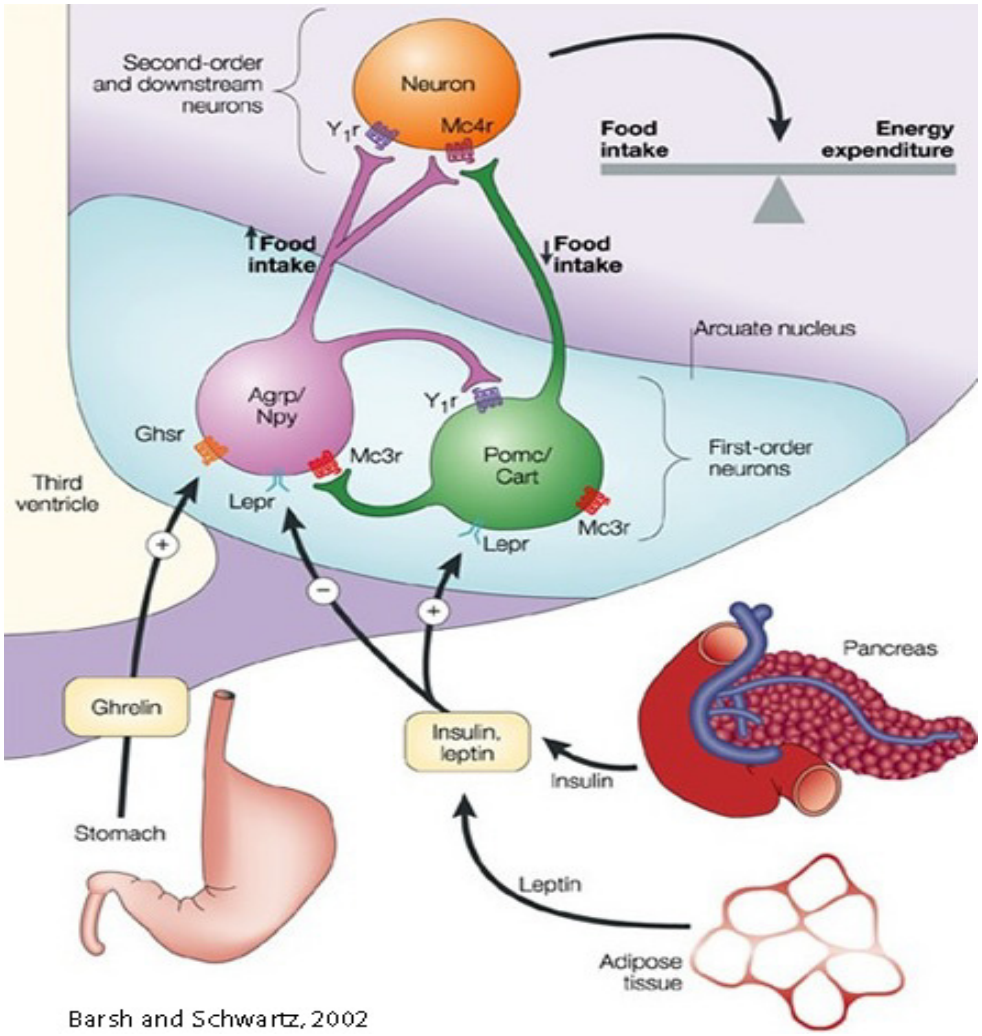


Figure 3. The Regulation of Food Intake and Energy Expenditure through the Arcuate Nucleus of Hypothalamus.

The Arcuate Nucleus (ARC) neurons receive different signals including ghrelin, insulin and leptin which are released from various peripheral tissues involved in metabolism. Leptin action on this region has been well investigated. The ARC consists of two distinct neuronal populations which exert opposing effects on food intake and energy expenditure in response to leptin. The AgRP neurons synthesize agouti-related peptide (AgRP), which functions as a strong orexigenic neurotransmitter, while the POMC neurons produce pro-opiomelanocortin (POMC),

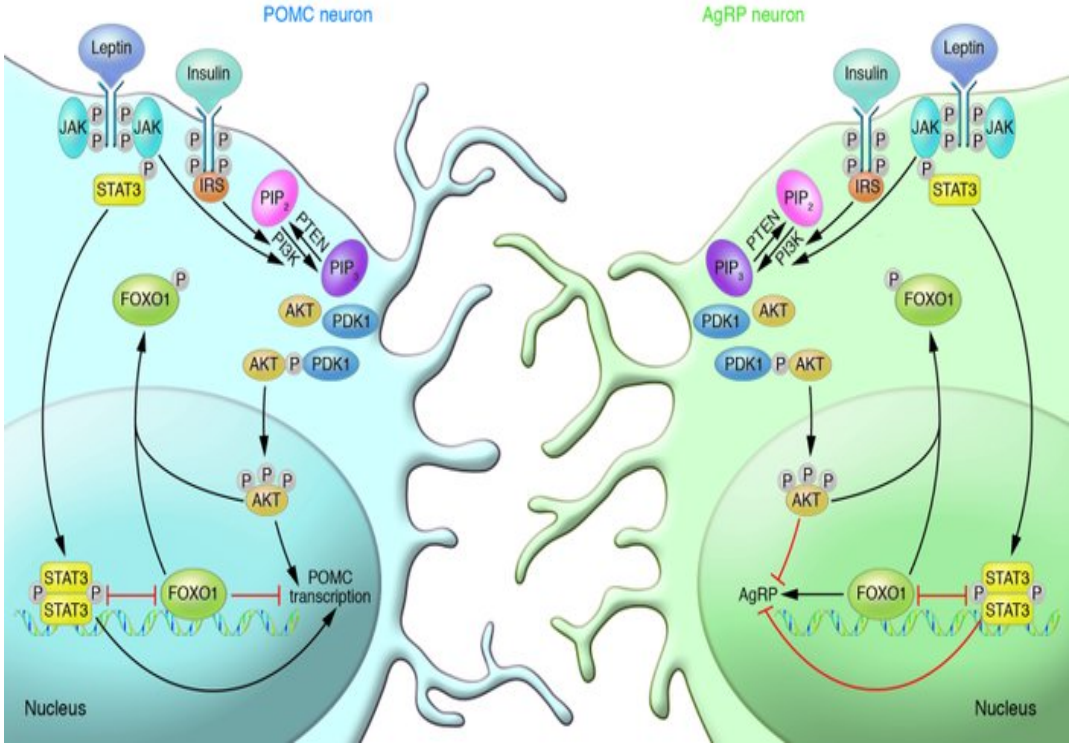
which is cleaved to generate the α melanocyte-stimulating hormone (α-MSH), a powerful anorectic peptide. The ARC neurons project to second-order neurons in the paraventricular nucleus (PVN) by releasing AgRP and α-MSH which bind the melanocortin receptors to modulate neuronal activity in the PVN. The PVN receives and integrates information from various hypothalamic regions and regulates food intake and energy expenditure through the sympathetic system.

phosphorylating both Stat3 and FoxO1 through the two parallel pathways, namely the JAK2/Stat3 and PI3K/Akt, respectively. In response to leptin signaling, the ARC neurons project synapses onto neurons of the paraventricular nucleus (PVN) by releasing AgRP or α-MSH (Fig.4) (Plum et al., 2006).

Leptin is primarily synthesized in the adipose tissue proportionately to the fat content (Niijima, 1998). As a protein, it has to pass from the blood to the cerebrospinal fluid by crossing the blood brain barrier with the help of specific transporters to reach various regions in the brain (Levin et al., 2004). All the short form leptin receptors have been regarded as transporters (Bouret, 2008; Pan et al., 2008). However, peripheral leptin administration to the lepR-null mice (db/db mice) did not completely block the cerebral leptin intake (Kastin et al., 1999), suggesting that some leptin transporters might be encoded by separate genes.

1.3 Leptin circuit in the hypothalamus.

The hypothalamus is one of the most complicated regions in the adult brain with a number of well defined nuclei. These nuclei play different roles in metabolic regulation. For example, destruction of the mediobasal hypothalamus (MBH), including the ARC and ventromedial hypothalamic nucleus (VMH), induces hyperphagia and Obesity (Elmquist et al., 1999) while lesion of the lateral



Plum, J. Clin. Invest. 2006

Figure 4. The Leptin and Insulin Signaling Pathways in the ARC of Hypothalamus.

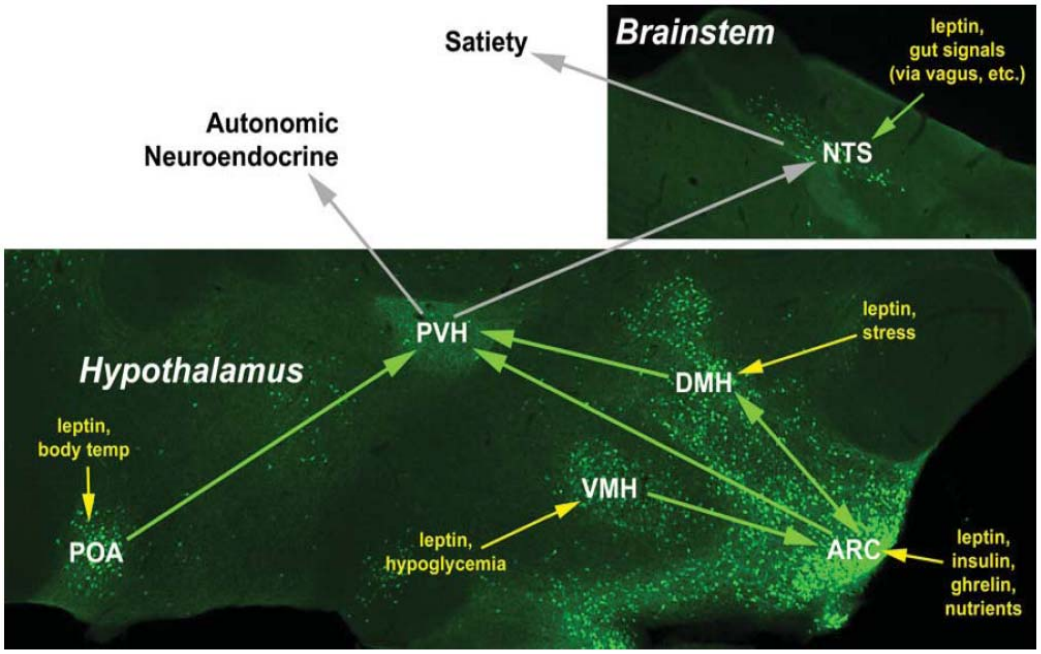
Leptin signaling induces POMC transcription and suppresses AgRP transcription by phosphorylating both Stat3 and FoxO1 through the two parallel pathways, the JAK2/Stat3 and PI3K/Akt pathways. Insulin plays a similar role as leptin in the ARC. FoxO1 and Stat3 are also regulated by insulin signaling. Stat3 is activated by leptin signaling and translocates into the nucleus to regulate gene transcription, Akt is phosphorylated by either leptin or insulin signaling and transduces the signal into the nucleus by phosphorylating FoxO1 which is then inactivated and excluded out to cytoplasm. The DNA binding sequences for both FoxO1 and Stat3 exist close to one another in both AgRP and POMC promoters, and the effect of FoxO1 on transcription of these genes is opposite to that of Stat3.

hypothalamic area (LHA) results in hypophagia (Kelley et al., 2005). Ablation of the lateral ARC and VMH or mediobasal ARC also dramatically increases food intake. Since the discovery of leptin and leptin receptor, the central role of leptin signaling in the regulation of food intake and energy expenditure has been revealed by tissue-specific deletion of leptin circuits. Most studies have focused on the ARC leptin circuit. Deletion of AgRP neurons reduces food intake while ablation of POMC neurons or the POMC gene induces hyperphagia (Morton et al., 2006; Smart et al., 2006). The same results have been observed through different transgenic mouse models in which leptin signaling is ablated in the ARC (van de Wall et al., 2008). It is also noted that not all ARC neurons respond to leptin, as some POMC neurons do not express functional LepRb (Munzberg et al., 2003). Instead, these neurons express the short form LepR with unknown functions. Other factors derived from peripheral metabolism-related tissues also act on the ARC neurons. For example, the gut peptide ghrelin activates AgRP neurons and plays an opposite function to leptin (Chen et al., 2004). In addition, insulin signaling mediated by PI3K pathway modulates the membrane potential of both AgRP and POMC neurons to regulate energy expenditure and glucose homeostasis (Niswender et al., 2004).

It is noted that the ARC neurons only account for a portion of leptin signaling in the hypothalamus. The db/db mice (that lack all leptin receptors) show dramatic obesity compared to the modest obesity observed in mice with leptin receptor ablated only in the ARC (Balthasar et al., 2004; van de Wall et al., 2008). Thus, hypothalamic leptin

signaling in nuclei other than the ARC also contributes to the regulation of energy homeostasis. It has been reported that ablation of LepRb in the steroidogenic factor 1 (SF-1) positive neurons in the VMH induces modest obesity in terms of increased fat stores without significant hyperphagia (Dhillon et al., 2006). The leptin circuit in the VMH also contributes to the regulation of glucose homeostasis (Tong et al., 2007). In other nuclei of the hypothalamus, however, functional studies of the leptin circuit are limited because of the lack of markers to guide neuron-specific LepR knockout in mouse models. For instance, the function of leptin signaling in the dorsomedial hypothalamic nucleus (DMH) has been largely ignored although altered feeding behavior has been observed from mice with DMH lesions (Elmquist et al., 1999).

LepRb expression levels in the PVN are relatively low. But neurons in this nucleus receive dense innervation from other nuclei such as the ARC and the DMH where leptin receptors are highly expressed. The VMH neurons also innervate the ARC POMC neurons with excitatory projections in response to leptin and may modulate PVN indirectly (Thompson and Swanson, 2003). The PVN regulates food intake as well as energy expenditure by integrating input from different regions and sending out a final signal to modulate sympathetic activity. Thus, the PVN functions with the ARC, VMH and DMH to form a complicated leptin circuit in the regulation of whole body metabolism (Fig.5) (Myers et al., 2009). How these different nuclei communicate in response to leptin remains to be fully investigated. Of note, melanocortin receptor 4 (MC4R) is highly expressed in the PVN to sense the neurotransmitters released from



Myers, cell metabolism 2009

Figure 5. Proposed Model for the Integrative Regulation of Leptin Circuit in the Hypothalamus.

A simplified model "wiring" diagram of the medial hypothalamic circuitry is shown superimposed upon an image of green fluorescent protein (GFP)-containing LepRb neurons (green) from sagittal sections of the media hypothalamus and brainstem of mice that express GFP in LepRb neurons. Each set of lepRb neurons responds not only to leptin but also to additional stimuli. Thus leptin acts at a number of points to enable the integration of signals of body energy status with other physiologic variables that dictate the strength of the final output signal.

the ARC neurons. The hyperphagia observed in MC4R-null mice is completely rescued by inducing MC4R expression in the PVN while reduced energy expenditure is unaffected, which hints that leptin circuit from the ARC to the PVN is responsible for food intake regulation while a separate leptin circuit might contribute to the modulation of energy expenditure (Balthasar et al., 2005). It is reasonable to hypothesize that each leptin circuit regulates distinct metabolic processes and the long-term energy homeostasis results from the integrated information of these different leptin circuits.

1.4 Leptin action in the extrahypothalamic sites.

LepRb expression has been reported in the vagal complex by expressing green fluorescent protein under the control of the promoter from leptin receptor gene combined with *in situ* hybridization. In line with the c-fos expression induced by leptin in the nucleus tractus solitarius (NTS) (Elias et al., 2000), leptin infusion to either the fourth ventricle or the NTS reduces food intake. Leptin also hyperpolarizes most neurons and suppresses the overall glutamate release in the vagal complex (Williams et al., 2007). However, the role of leptin responsive neurons in this region remains unclear.

Leptin action is also found in the VTA of the midbrain. Leptin treatment reduces the

frequency of dopamine neuron firing in both fresh VTA tissue sections and anesthetized rodents. Direct leptin injection into the VTA suppresses food intake which is consistent with the observation that knocking down the leptin receptor in this region increases food consumption (Hommel et al., 2006).

Taken together, these studies suggest that leptin action on the regulation of food intake and energy homeostasis is a complex network of different hypothalamic nuclei and extrahypothalamic regions.

1.5 Leptin resistance in obesity.

It has been reported that leptin replacement treatment effectively reverses the symptoms of patients with lipodystrophic syndromes, in which serum leptin is quite low due to the lack of adipose tissue (Halaas et al., 1995). The same result has been obtained in laboratory research when the leptin-null mice (ob/ob mice) are given exogenous leptin (Chehab et al., 1996). Moreover, leptin decreases food intake and body weight acutely in normal mice (Ahima et al., 1996). However, food intake returns to normal after long-term leptin administration in normal animals (Halaas et al., 1995). In most obese patients and animals that don't have defects in the gene encoding leptin or its receptor, leptin treatment can not reverse the increased food intake and body weight. For common obesity in which serum leptin levels are high

due to increased fat mass, the state in which the body does not respond effectively to leptin is termed “leptin resistance”. In laboratory animal models, leptin resistance can be induced in animals fed with a high-fat diet (Halaas et al., 1997). In high-fat diet induced obesity (DIO), leptin signaling is attenuated after prolonged exposure to elevated circulating leptin. Possible mechanisms underlying diet-induced leptin resistance at the molecular level are that increased suppressor of cytokine signaling 3 (Socs3) or protein tyrosine phosphatase 1B (PTP1B) attenuate leptin signaling by suppressing Stat3 phosphorylation from JAK2. Socs3 expression is induced by phosphorylated Stat3. Socs3 then binds to Tyr985 of the leptin receptor in competition with JAK2, thereby disrupting leptin signaling through negative feedback regulation (Banks et al., 2000). PTP1B suppresses letpin signaling by dephosphorylating JAK2 (Klaman et al., 2000). Inactivation of Socs3 or PTP1B in transgenic mice increases leptin sensitivity and decreases food intake. Thus, Socs3 and PTP1B expression levels are two common indicators of leptin resistance. Once animals are leptin resistant, it is ineffective to inhibit feeding through leptin action on the hypothalamus, especially on the ARC, and these animals are hyperphagia. Thus, caloric restriction is a common treatment for obese patients.

1.6 Leptin action and sympathetic outflow.

Leptin action on the hypothalamus regulates peripheral energy homeostasis through

sympathetic outflow. The connection from specific hypothalamic nuclei to certain peripheral tissues has not been fully explored. One study showed innervation from MC4R-positive neurons in multiple hypothalamic nuclei to BAT using retrograde trans-synaptic tracing with pseudorabies virus combined with immunohistochemistry (Voss-Andreae et al., 2007). This study elucidated how leptin signaling regulates thermogenesis in BAT through sympathetic outflow. The PVN is one nucleus in the hypothalamus with high levels of MC4R expression which receives different innervation from other nuclei such as the ARC. Leptin signaling in the ARC activates MC4R-positive PVN neurons by releasing neurotransmitters that bind to MC4R. Activated PVN neurons send sympathetic outflow to BAT by releasing norepinephrine (NE) at sympathetic nerve terminals. NE, by binding to its receptor, increases intracellular cAMP levels and thus activates lipolysis. The increased free fatty acids levels from lipolysis of triglyceride activate expression of the mitochondrial uncoupling protein 1 (UCP1), one of the key contributors to the BAT thermogenesis (Cannon and Nedergaard, 2004). Conversely, reduced sympathetic activity activates lipogenesis in adipose tissue. In addition, a recent study showed that leptin signaling in the mediobasal hypothalamus including the ARC and VMH contributes to fat metabolism (increasing lipolysis and reduces lipogenesis) in peripheral white adipose tissue, and this function of leptin is PI3K-dependent (Morton et al., 2005). By the same token, defect in leptin signaling in this hypothalamic region would probably increase lipogenesis.

1.7 Leptin signaling and insulin sensitivity.

Insulin, a pivotal hormone in metabolic regulation, is secreted by groups of cells within the pancreas called β cells. Its well-known function is to decrease serum glucose levels after a meal and maintain glucose homeostasis (Choudhury et al., 2005). Like leptin, insulin also functions by binding to its specific receptor and initiating a signaling pathway. However, the insulin receptor belongs to the family of receptor tyrosine kinases that possess intrinsic kinase activity allowing autophosphorylation of its own intracellular domain. After binding insulin, the activated insulin receptor recruits and phosphorylates insulin receptor substrate (IRS) proteins on tyrosine residues. Several proteins are then sequentially activated such as PI3K, phosphoinositide dependent kinase 1 (PDK1) and Akt which inhibits the transcription activity of FoxO1 (Obici et al., 2002). In addition to its effects in peripheral tissues, insulin plays a similar role in the regulation of food intake and energy homeostasis as leptin in the hypothalamus. It has been demonstrated that two transcriptional factors, FoxO1 and Stat3, are controlled by insulin signaling in the ARC while they are also regulated by leptin signaling. The DNA binding sequences for both FoxO1 and Stat3 exist close to one another in both AgRP and POMC promoters. The effect of FoxO1 on transcription of these genes, however, is opposite to that of Stat3 (Yang et al., 2009). Thus, in the hypothalamus, insulin signaling complements leptin signaling by inhibiting the FoxO1-dependent transcription (Fig.4).

As in the case for leptin resistance, insulin resistance is the state in which the body has low sensitivity to circulating insulin despite high serum insulin levels. This condition almost always coexists with leptin resistance in obese patients. Two potential mechanisms underlying insulin resistance have been proposed (Lionetti et al., 2009). One is the inflammation hypothesis, in which chronic inflammation attracts macrophages to adipocytes. Various inflammatory signals are released from macrophages and circulate to skeletal muscle. The insulin signaling pathway is shut down by the activated immuno-response signaling via the inhibition of IRS in muscle cells. Another one is the lipid overload hypothesis, in which increased free fatty acids released from adipocytes accumulate in muscle cells and block the activity of IRS to suppress insulin signaling.

Accumulating evidence shows hypothalamic leptin signaling also affects glucose homeostasis and insulin sensitivity. Severe insulin resistance and diabetes have been observed in genetic rodent models with leptin signaling deficiency in which either the leptin gene or LepR is deleted. Leptin replacement restores these metabolic disorders through a separate pathway other than the one that regulates food intake and body weight (Balthasar et al., 2004). One study established that leptin signaling in the mediobasal hypothalamus via PI3K dependent pathway regulates insulin sensitivity and glucose homeostasis in the Koletsky rats. This research also revealed that caloric restriction normalizes the increased food intake and body weight gain but had no

effect on hyperinsulinemia, which confirms again that the regulation of feeding and glucose metabolism by leptin action on the mediobasal hypothalamus likely occurs through two different pathways (Morton et al., 2005).

1.8 The potential role of LMO4 in the hypothalamus.

LMO4 expression in the hypothalamus has been reported (Hermanson et al., 1999) but the function was not investigated. Another group has reported the interaction of LMO4 with interlukin-6 (IL-6) receptor subunit glycoprotein 130 complex and its modulation in IL-6 signaling (Novotny-Diermayr et al., 2005). The same study showed that LMO4 enhanced the transcription activity of Stat3 by interacting with glycoprotein 130, JAK1, SHP2 and Socs3. The IL-6 receptor is a member of the type I cytokine receptor family that also includes the leptin receptor. Work from our laboratory found that LMO4 and Stat3 are co-expressed in primary cultured cortical neurons. Furthermore, our group found that knocking down LMO4 expression using RNA interference reduces Stat3 phosphorylation levels (Chen et al., 2007). Since the IL-6 and leptin receptors share a similar mechanism of signal transduction, we hypothesized that LMO4 might also be involved in leptin signaling and be required for Stat3 activation in the hypothalamus.

Chapter 2. Materials and methods

2.1 Animal care.

All procedures carried out in mice were approved by the animal care and use committee of the University of Ottawa. All animals were bred and maintained in the University of Ottawa animal facility under a 12 hour light (0600-1800) and 12 hour dark (1800-0600) cycle. Food (regular chow) and water were provided ad libitum unless specified.

2.2 Generation of CaMKIIα-Cre;LMO4flox/flox mice.

CaMKIIα-Cre;LMO4flox/flox mice were generated by crossing the homozygous LMO4flox/flox mice (gift from Dr. Jane Visvader, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia) with heterozygote CaMKIIα-Cre transgenic mice (gift from Dr. Günter Schultz, German Cancer Research Center, Heidelberg, Germany) to obtain CaMKIIα-Cre mice heterzygous for the LMO4loxP allele. These mice were then backcrossed to the homozygous LMO4flox/flox mice to obtain CaMKIIα-Cre;LMO4flox/flox mice. All the mice were backcrossed and maintained on a CD-1 background. CaMKIIα-Cre;LMO4flox/flox (heterozygous for Cre and homozygous for LMO4flox allele) mutant mice and LMO4flox/flox littermate control mice were genotyped by PCR. In addition, mice carrying the

CaMKIIα-Cre transgene were also used as controls; no differences were seen between these controls and LMO4flox/flox only control mice for body weight and metabolic measurements. To determine the tissues from which LMO4 gene is ablated, genomic DNA was extracted from various tissues and PCR genotyped using the primer set: 5'-CGAGCTGAAATTGTCAGCAGCAAG-3'
5'-GCATTCACCAGCCACAGATAAG-3' yielding a 2.1 kb and a 1.7 kb fragment for LoxP-flanked LMO4 allele and ablated allele, respectively.

2.3 Brain fixation for immunofluorescence and *in situ* hybridization.

Mice were anesthetized via a lethal dose of a ketamine/xylazine/acepromazine cocktail given intraperitoneally. Mice were immediately perfused intracardially with PBS solution followed by 4% paraformaldehyde in PBS (pH 7.4). Brains were fixed in 4% PFA overnight and this solution was replaced with 30% sucrose for 3 days. Following this, brains were quick frozen in -40°C isopentane. 14µm coronal sections were made.

2.4 *In situ* hybridization.

8 week old male CaMKIIα-Cre;LMO4flox/flox mutant and littermate control mice were anesthetized via i.p. injection of a ketamine/xylazine/acepromazine cocktail and perfused with 4% paraformaldehyde (PFA). Brains were fixed in PFA overnight

followed by overnight cryo-protection in 30% sucrose. Finally, brains were frozen in -40°C isopentane, and 14 μm coronal serial sections were made. Prior to hybridization, the brain slides were warmed to room temperature for 30 minutes, rinsed with DEPC-treated PBS, and washed in 2xSSC. Pre-hybridization, hybridization and washing were performed as previously described (Jensen and Wallace, 1997). 300 nucleotides of the mouse LMO4 exon 2 anti-sense RNA probes were synthesized by T7 RNA polymerase from the linearized pBS-mLMO4 plasmid using ribonucleotide mix containing DIG-UTP. Sense probes were made and used as a negative control.

2.5 Immunofluorescent staining.

Slides for pSTAT3 staining were first warmed at 37°C for 15 minutes then washed in PBS solution. Slides were subsequently pre-treated with a 1% NaOH solution for 20 minutes, followed by a 0.3% glycine/PBS solution for 10 minutes and finally a 0.06% SDS/PBS solution for 10 minutes. Slides were washed with PBS 3 times for 10 min each between each of these steps. They were then blocked at room temperature for one hour in 5% donkey serum blocking solution containing 0.1% TritonX100 and 0.2% NaN3. Slides were then incubated for 48 hours at 4°C with pStat3 primary antibody in 1% donkey serum (Cat.# 9131, Cell Signaling,) at a 1:2000 concentration. Subsequent to this, the slides were washed 6 times for 10 min each in PBS before incubation for 1 hour at room temperature with donkey anti-rabbit-conjugated biotin. Slides were then washed again 6 times for 10 min each with PBS before incubating

them with cy3-conjugated streptavidin for 30 minutes at room temperature.

2.6 Magnetic Resonance Imaging (MRI).

6-month-old littermate male mice anesthetized by i.p. injection of ketamine/xylazine/acepromazine (10mg/kg for ketamine, 2mg/kg for xylazine, and 1mg/kg for acepromazine) were scanned in pairs to compare differences in WAT volume between littermate control and knockout mice. Due to their small size, to maximize the signal acquisition the images were acquired using a multichannel wrist coil adapted to the 3T GE MRI scanner. To further assess the content of visceral fat present in each of the animals, “water + fat” and “water only” excitation turbo spin echo images were acquired. This allowed for the subtraction of adipose tissue from other surrounding tissues. The total acquisition time did not exceed 12 minutes. Axial slices were assessed, taken between the abdomen and the thorax. Ten 2mm thick slices were collected and "fat" pixels were quantified by Dixon imaging for each slice.

2.7 Quantitative real-time RT-PCR.

Total RNA from the different tissues (hypothalamus, white adipose tissue or liver) was extracted using TRIzol reagent (Invitrogen) followed by ethanol purification, reverse-transcribed to cDNA with random decamers and reverse-transcriptase (Ambion). An aliquot of cDNA was used for qPCR with specific primers or actin

primers together with Taq DNA polymerase/SYBR Green PCR mix (New England Biolabs) with the Rotor-Gene 3000 System (Corbett Life Science). All mRNA levels were normalized to actin. Gene specific qPCR primers used included:

LMO4: 5'-GGACCGCTTTCTGCTCTATG-3' 5'AAGCACCGCTATTCCCAAAT-3'

ACC: 5'-AGCAGATCCGCAGCTTGGT-3' 5'-ACTTCTGCTCGCTGGGTGG-3'

FAS: 5'-GCTGGCATTCGTGATGGAGTCGT-3'

5-AGGCCACCAGTGATGATGTAACTCT-3'

Hsl: 5'-GACTCACCGCTGACTTCCT-3' 5'-CTGTCTCGTTGCGTTTGTAG-3'

Atgl: 5'-TGGGTGACCATCTACCTTCC-3' 5'-CCCAGTGAGAGGTTGTTTCG-3'

UCP1: 5'-AAAGTCCGCCTTCAGATC-3' 5'-AGTTTCGGCAATCTTCT-3'

actin: 5'-GCTTCTTTGCAGCTCCTTCG-3' 5'-CCTTCTGACCCATTCCCACC-3'

2,8 Leptin and insulin ELISA.

All blood samples were collected between 14:00 and 16:00. Animals were fasted for 6 hours preceding blood collection intended for the insulin assay, whereas samples for leptin determination were obtained from normally fed mice. Plasma leptin and serum insulin levels were measured according to the manufacturer's protocols using the mouse Leptin (Cat. # 90030) and insulin (Cat. #90080) ELISA kits (Crystal Chem Inc., IL, USA).

2.9 Glucose tolerance test (GTT).

Mice were fasted overnight (~16 hours) in fresh cages with free access to water. Tests were performed at 10:00. Basal blood glucose was measured prior to mice receiving a glucose bolus (2 grams/kg body weight of 20% D-glucose) by intra-peritoneal (i.p.) injection. At 15, 30, 60, and 120 minutes blood glucose was sampled from the saphenous vein using a standard glucometer.

2.10. Insulin tolerance test (ITT).

Mice were fasted for 6 hours prior to ITT. The test was performed between 14:00 and 17:00. Human recombinant insulin (Sigma, MO, USA; Cat. #91077C) diluted in sterile saline was administered by i.p. injection at 0.75U/kg. Blood glucose levels were monitored in the same manner as described for the GTT protocol at 4 different time points: prior to injection (T0), and at 15, 30, and 60 minutes after injection. Data were expressed as % T0 blood glucose vs. time.

2.11 Thermogenesis study.

For the cold challenge, mice were fasted overnight preceding the test (~16 hours). They were then housed individually, without food and a minimum amount of cage bedding and placed at 4°C in a cold room for 4 hours. Every half hour rectal temperature was measured. To determine the contribution of non-shivering

thermogenesis, mice at room temperature were anesthetized using an i.p. injection of a ketamine/xylazine/acepromazine cocktail and rectal core temperature was measured.

2.12 NETO.

8 week old male KO and littermate control mice were used. Norepinephrine turnover rate (NETO) is an index of sympathetic activity to peripheral tissues and is extrapolated from the rate of decline in tissue NE content after inhibiting catecholamine synthesis using DL-a-methyl-p-tyrosine (a-MPT, Sigma). Mice received 2 intraperitoneal injections of a-MPT at time 0 (250 mg/kg; 25mg/ml) and 2 hours (125 mg/kg; 2.5 mg/ml). Two hours following the second a-MPT injection tissues were harvested after decapitation and quick frozen on dry ice. NETO rate was determined as described (Shi et al., 2004).

2.13 Immunoblot analysis.

Protein extracts of brain samples and western blot analysis were done as described previously (Chen et al., 2007). Anti-Stat3, phosphorylated Tyr705-Stat3, Akt, phosphorylated Ser473-Akt, Socs3 antibodies were obtained from Cell Signaling Technologies and anti-actin was from Sigma. Immunoblots were scanned and quantified using the Image J software (NIH).

2.14 Acute and chronic intracerebroventricular (i.c.v.) leptin infusion.

Two month old male mice were anesthetized with isofluorane gas and the heads were secured in a stereotaxic instrument. A single 0.5 mm burr hole was drilled using a dental drill to the following coordinates: 0.5 mm rostral to the bregma, 1.0 mm lateral to sagittal suture, and 2.5 mm below the dural surface. 1μg in 1μl sterile saline of recombinant mouse leptin (Invitrogen, Cat #PMP0013) was infused using a 27-gauge Hamilton syringe at a rate of 0.25 μl/min controlled by a syringe pump. Brains were either fixed for histology or fresh hypothalamic tissue was quickly removed and frozen after decapitation for Western analysis. For chronic infusion, the pedestal of a 30 gauge osmotic pump cannula (Alzet, CA, USA; Brain Infusion Kit 3) was attached to the mouse's skull using Vet-Bond. The depth was adjusted using height adjustment spacers. An Alzet osmotic pump (Alzet, CA, USA; 1007D) was surgically implanted subcutaneously between the mouse's scapulae. 10μg recombinant mouse leptin was infused at a rate of 0.5μl/hour over a period of 7 days.

2.15 Pair feeding.

Mice were housed individually from the age of 9 to 17 weeks. 24 hour food intake was measured in littermate control mice for 8 weeks. Each day LMO4 mutant and littermate control mice were provided the average amount of food consumed by age-matched control mice. Mice were fed twice daily, 1/3 of food was given at 9 am

and 2/3 at 5 pm to ensure that mice never underwent long periods of fasting. Body weight was measured every other day over the pair-feeding period, after which mice were subjected to GTT or ITT tests. Another group of mice was sacrificed to measure the individual organ weights at the end of pair-feeding studies.

2.16 Statistical analysis.

All results are expressed as mean ± SEM. Statistical analyses were performed using Statistical Package for the Social Science (SPSS). For between group comparisons, a two-tailed unpaired Student's t-test was used. Where appropriate, data were analyzed by ANOVA followed by LSD posthoc test to compare means between groups. P values of < 0.05 were considered significant.

Chapter 3. Results

3.1 Neuronal specific ablation of LMO4.

We have generated an LMO4 conditional knockout (KO) mouse line (CaMKIIα-Cre;LMO4flox/flox) using the conventional Cre-loxP recombination system. In this mouse line, the LMO4 gene is ablated by Cre recombinase. The cre expression is under the control of the CaMKIIα promoter. CaMKIIα is expressed postnatally in glutamatergic neurons and the expression intensity of CaMKIIα-Cre is not exactly the same among different mouse lines (Casanova et al., 2001). Therefore, we first analyzed its expression pattern by PCR amplification across the LoxP-flanked exon2 of the LMO4 gene using genomic DNA extracted from various dissected tissues and specific brain regions. We found Cre recombinase to selectively ablate the LMO4 gene in nervous tissue, for example the cortex, hypothalamus, cerebellum, brain stem and spinal cord, but not in any peripheral tissues associated with metabolism such as the liver, muscle, adipose tissue, stomach et cetera (Fig.6A). *In situ* hybridization was also performed to show loss of LMO4 expression in the hippocampus, cortex, amygdala and hypothalamus (Fig.6B). Quantitative RT-PCR of hypothalamic RNA extracts confirmed that LMO4 expression was reduced by 90% in KO mice at the mRNA level (Fig.6C), consistent with the *in situ* hybridization results which showed LMO4 was completely knocked out in discrete hypothalamic nuclei (PVN and VMH) but it was undetectable in the arcuate nucleus of hypothalamus

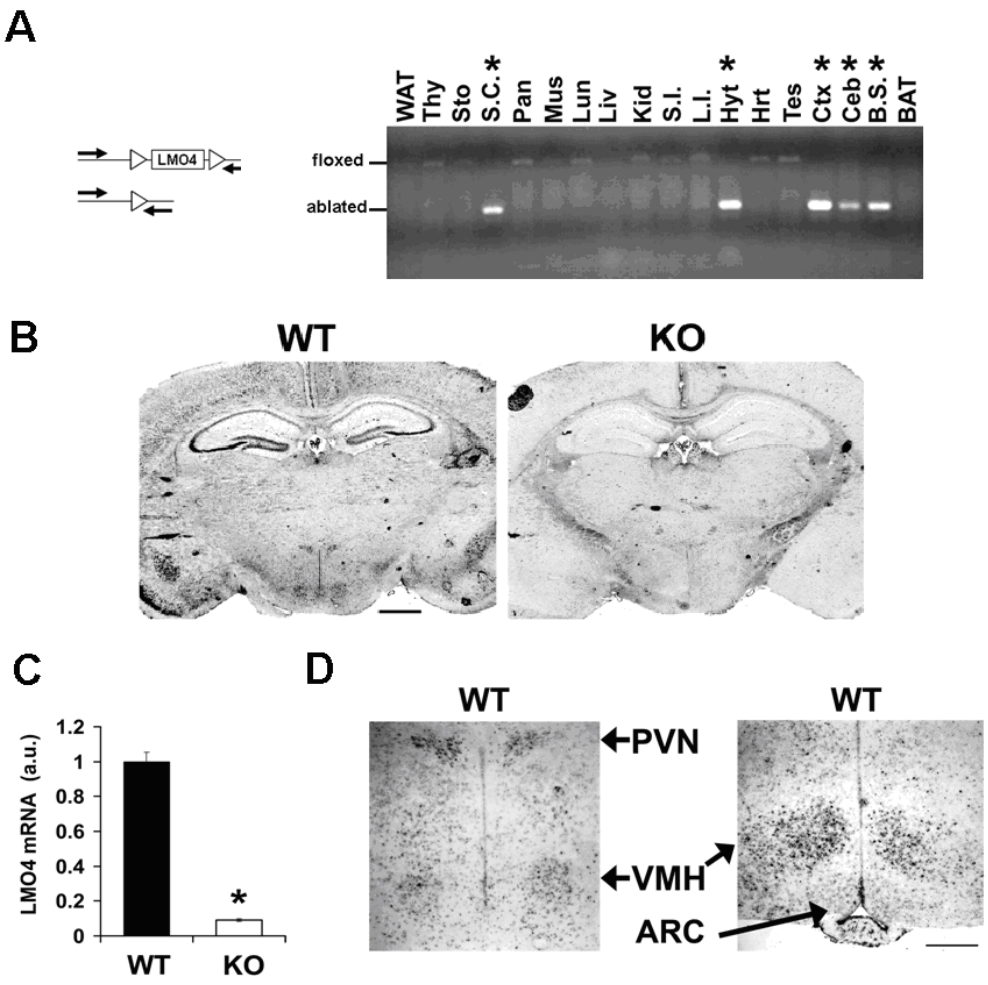


Figure 6. Neuronal Specific Ablation of LMO4.

(A), PCR amplification of genomic DNA from various tissues from CaMKIIα-Cre;LMO4flox/flox mice at weaning revealed selective ablation of LMO4 in neuronal tissues (asterisks). Diagram shows amplified floxed fragment is larger than the ablated allele. (WAT: white adipose tissue, Thy: thyroid, Sto:stomach, S.C.: spinal cord, Pan: pancreas, Mus: muscle, Lun: lung, Liv: liver, Kid: kidney, S.I.: small intestine, L.I.: large intestine, Hyt: hypothalamus, Hrt: heart, Tes: testicle, Ctx: cortex, Ceb: cerebellum, B.S.: brain stem, BAT: brown adipose tissue)

(B), Low power magnifications of *in situ* hybridization revealed LMO4 expression in cortex, hippocampus, amygdala and hypothalamus of the littermate control (WT) but

not in the CaMKIIα-Cre;LMO4flox/flox mouse (KO). Scale bar=400μm.

(C), Quantitative RT-PCR from RNA purified from the hypothalamus showed a 90% reduction in LMO4 levels (asterisk, $p<0.05$, n=6 per group) in CaMKIIα-Cre;LMO4flox/flox mice (a.u., arbitrary units, normalized to littermate control).

(D), *In situ* hybridization revealed LMO4 mRNA expression in the PVN and the VMH but not in the ARC in littermate control mice (WT). Scale bar=200μm.

(ARC) (Fig.6D). Taken together, we show that LMO4 is specifically ablated from the hypothalamus in CaMKIIα-Cre;LMO4flox/flox mice.

3.2 Adult onset obesity is due to dysregulated fat metabolism.

We found that the KO mice exhibit an obese phenotype compared to their wild type (WT) littermates. The male KO mice fed on regular chow developed obvious obesity by 4 months of age (Fig.7A). To further characterize the obese phenotype we observed in LMO4 KO mice, we measured the body weight increase of these mice after weaning at 3 weeks old. Male KO mice fed on regular chow developed significant obesity by 3 months of age, compared to their WT littermates (Fig.7B). To confirm that the increased body weight gain was from excessive fat deposition and not due to excess overall growth of the whole body, we performed MRI scanning on 6 months old mice. The result showed prevalence on abdominal adipose tissue in KO mice (Fig.7C). We also dissected and measured the weight of different tissues on these mice. At 4 months of age, weights of gonadal white adipose tissue (gWAT) and interscapular brown adipose tissue (BAT) in KO mice were twice those of littermate controls, while weights of other tissues including liver, pancreas, kidney, heart and thyroid gland were not different (Fig.7D). Indeed, increased gWAT weight in KO mice was detected as early as 2 months of age when the whole body weight was not significantly between KO and controls (Fig.7E). Increased fat deposition suggested

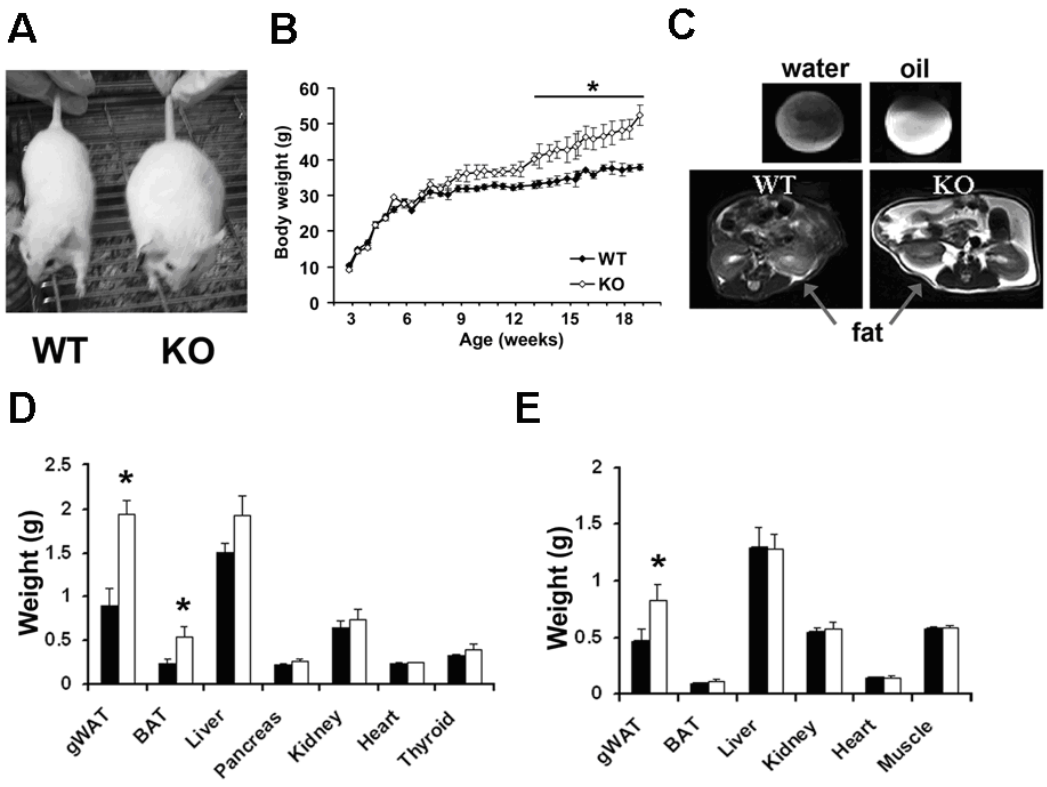


Figure 7. Increased Body Weight and Adiposity in CaMKIIα-Cre;LMO4flox/flox Mice.

(A), Male CaMKIIα-Cre;LMO4flox/flox mice (KO) at 4 months of age were obviously obese compared to littermate controls (WT).

(B), The onset of obesity was significant after 12 weeks (asterisk indicates $p<0.05$, $n\geq12$ per group), WT (black diamonds), KO (open diamonds).

(C), Magnetic resonance images revealed extensive adiposity (white) of KO mice compared to WT littermate at 6 months of age. Water and oil standards are shown for comparison (representative scan of 3 mice per group). This experiment was performed by Mariana Gomez-Smith in collaboration with Dr. Arturo Cardenas-Blanco.

(D), Gonadal white adipose tissue (gWAT) weights and brown adipose tissue (BAT)

weights were both increased at 4 months of age in KO mice (asterisk, $p<0.05$, n=6 per group), WT (filled bars), KO (open bars).

(E), Gonadal white adipose tissue (gWAT) weights were increased at 2 months of age in KO mice (asterisk, $p<0.05$, n=6 per group), WT (filled bars), KO (open bars).

dysregulated lipid metabolism. Quantitative RT-PCR of gWAT and liver RNA extracts was performed and showed that gene expression of the two key enzymes in the lipogenic pathway, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), was significantly elevated in gWAT of KO mice at 1 month of age and trended higher in liver though it was not significant (Fig.8A,8B). The expression levels of the lipolytic genes hormone sensitive lipase (HSL) and adipose triglyceride lipase (Atgl) were also elevated in gWAT but not in the liver at this age (Fig.8A, 8B).

The plasma leptin level of KO mice at 2 months of age was not different from that of WT littermate controls even though KO mice had increased gWAT at this time point (Fig.8C). However, KO mice developed hyperleptinemia by 4 months of age (Fig.8C), suggesting they become resistant to leptin signaling. In line with the appearance of leptin resistance, food intake in the KO mice was not different at 2 months of age (Fig.8D) but was elevated at 4 months (Fig.8E). Normally elevated leptin levels suppress feeding, but hyperphagia occurs when body is leptin resistant. Thus, these observations suggest that the obesity of KO mice can largely be explained by increased food intake. To summarize, we have described that LMO4 KO mice display elevated lipogenesis in gWAT as early as 1 month old. This is followed by increased abdominal adiposity which finally leads to the development of obesity.

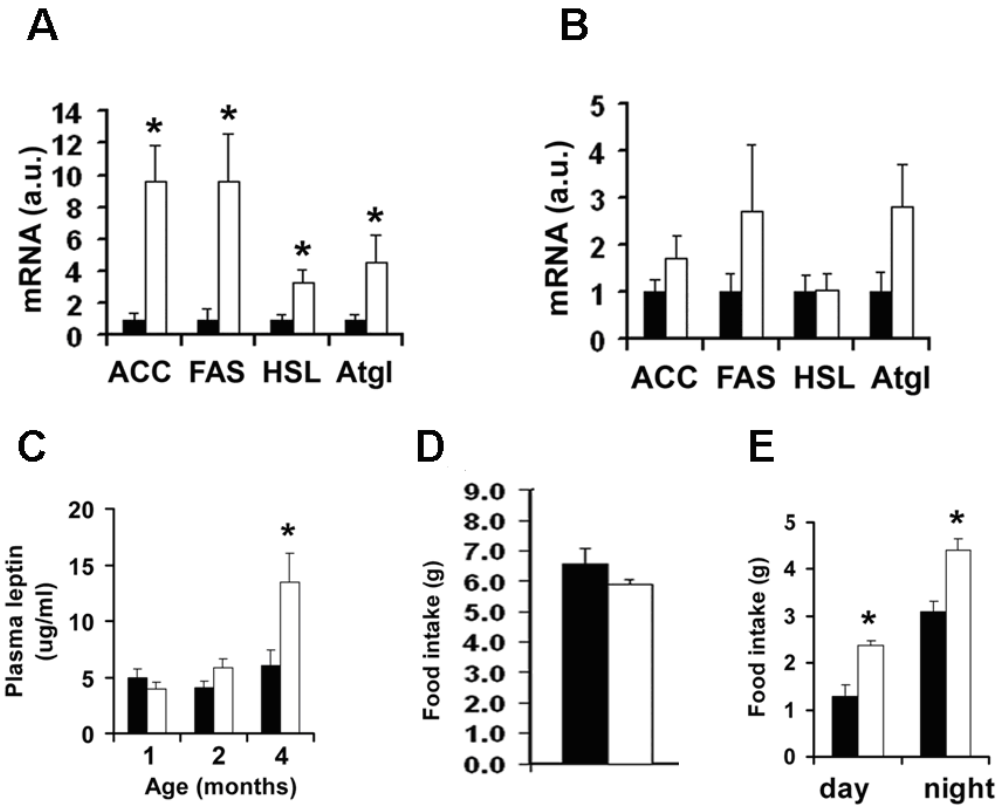


Figure 8. Dysregulated Fat Metabolism and Hyperleptinemia Followed by Hyperphagia in CaMKIIα-Cre;LMO4flox/flox Mice.

(A), Quantitative RT-PCR measured lipogenic (ACC and FAS) and lipolytic (HSL and Atgl) gene expression in gWAT at 1 month of age (asterisk, $p<0.05$, $n\geq4$ per group), WT (filled bars), KO (open bars), significantly elevated levels were detected in gWAT of LMO4 KO mice.

(B), Quantitative RT-PCR measured lipogenic (ACC and FAS) and lipolytic (HSL and Atgl) gene expression in liver at 1 month of age ($n\geq4$ per group), WT (filled bars), KO (open bars).

(C), Plasma leptin levels were significantly elevated in KO mice at 4 months of age (asterisk, $p<0.05$, n= 8-11 mice per group), WT (filled bars), KO (open bars). This

experiment was performed by Mariana Gomez-Smith.

(D), Food intake averaged over 3 days was normal in 2 month old KO mice ($n \geq 9$ mice per group), WT (filled bars), KO (open bars).

(E), Daytime and nighttime food intake averaged over 3 days was elevated in 4 month old KO mice (asterisk, $p < 0.05$, $n \geq 9$ mice per group), WT (filled bars), KO (open bars). This experiment was performed in collaboration with Mariana Gomez-Smith.

3.3 LMO4 KO mice have reduced insulin sensitivity.

Obesity is one of the most important risk factors for the development of diabetes. To test whether these obese mice also have a defect in glucose metabolism, the glucose tolerance test (GTT) was administered to male KO mice and their littermate controls at different ages. There was no difference of glucose tolerance at 2 months old (Fig.9A). But the KO mice showed impaired glucose tolerance at 4 months of age (Fig.9B) and developed obvious hyperglycemia by 6 months of age (Fig.9C). An insulin tolerance test (ITT) was also performed to compare the response to a peripheral insulin injection between KO and WT mice. Although the KO mice responded normally to the GTT glucose injection at 2 months of age (Fig.9A), they showed reduced sensitivity to insulin at this same age (Fig.9D). Consistent with the reduced response to insulin, serum insulin levels were elevated more than 2 folds in KO mice at 2 months and 4 months of age. There was no significant difference between WT and KO mice at 1 month of age (Fig.9E). All together, neuronal ablation of LMO4 is associated with reduced insulin sensitivity and hyperinsulinemia.

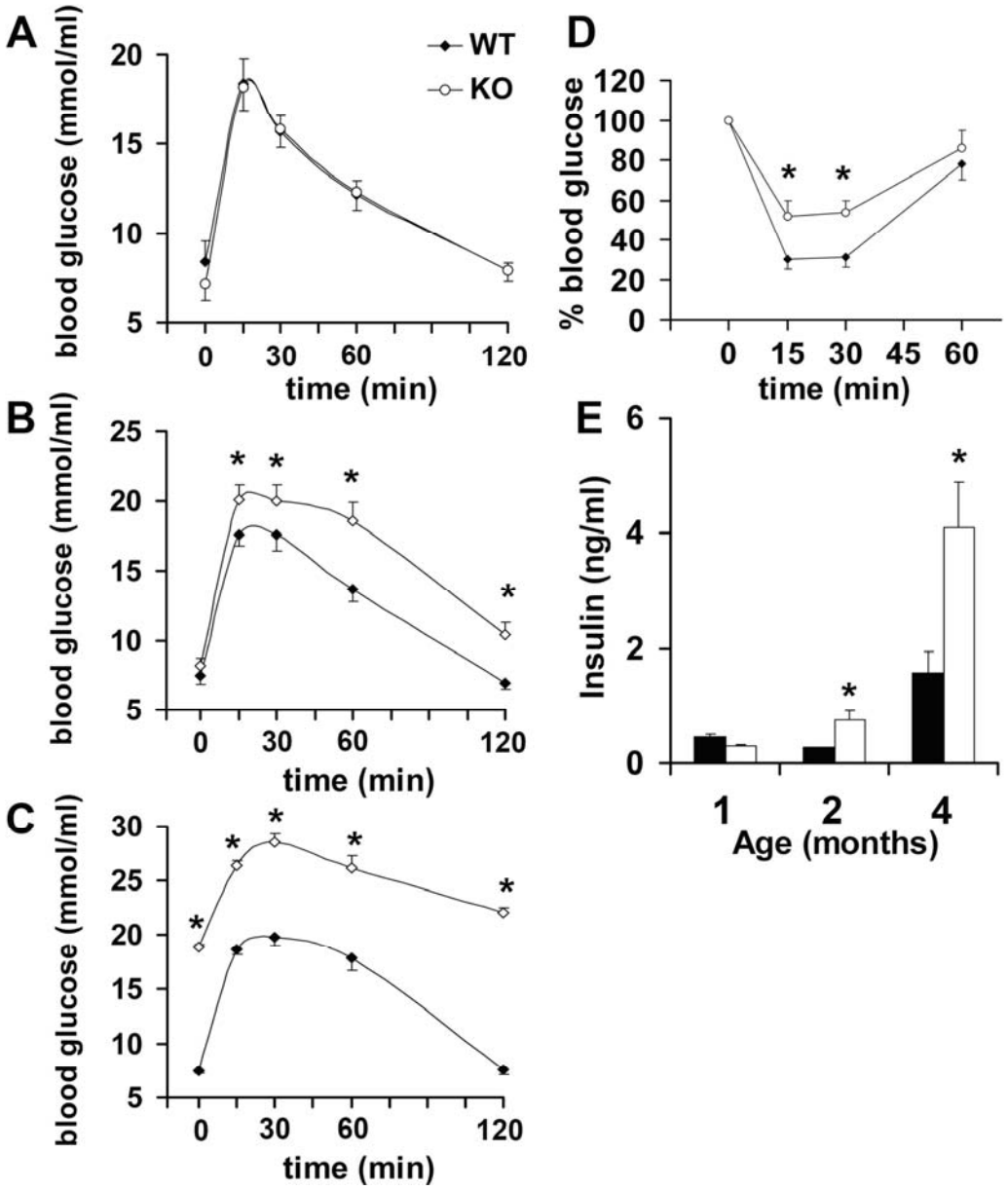


Figure 9. CaMKIIα-Cre;LMO4flox/flox Mice Exhibit Impaired Glucose Handling and Insulin Resistance.

Glucose tolerance test by bolus intraperitoneal (i.p.) injection of glucose after an overnight fast was measured at 2, 4 and 6 months of age.

(A), CaMKIIα-Cre;LMO4flox/flox mice showed normal response to glucose injection at 2 months of age (n=9–12 per group), WT (black diamonds), KO (open diamonds).

(B), Impaired glucose handling was detected at 4 months of age in LMO4 KO mice (asterisk, $p<0.05$, n=9–12 per group), WT (black diamonds), KO (open diamonds).

(C), LMO4 KO mice were hyperglycemic at 6 months of age (asterisk, $p<0.05$, n=9–12 per group), WT (black diamonds), KO (open diamonds).

(D), Insulin tolerance test showed insulin resistance at 2 months of age (asterisk, $p<0.05$, n=9–12 per group), WT (black diamonds), KO (open diamonds).

(E), Circulating insulin levels were increased in KO mice by 2 months of age (asterisk, $p<0.05$, $n\geq8$ per group), WT (filled bars), KO (open bars).

These experiments were performed in collaboration with Mariana Gomez-Smith.

3.4 Reduced sympathetic tone to adipose tissue in LMO4 KO mice.

In the CaMKIIα-Cre;LMO4flox/flox mouse model, LMO4 was only ablated from the nervous system. We did not manipulate any genes in peripheral tissues. Nonetheless, we observed dysregulated fat metabolism in WAT and reduced peripheral insulin response. The brain regulates whole body metabolism and energy homeostasis through sympathetic innervation to different tissues. Reduced sympathetic activity has been reported in obese patients (Lustig, 2008). To test sympathetic activity in LMO4 KO mice, cold challenge was performed. During cold exposure, mammals maintain their core body temperature by increasing heat production which is achieved by upregulating sympathetic activity to BAT. When exposed to 4℃, the KO mice showed significantly lower body temperature which suggested impaired thermogenesis (Fig.10A). To determine whether the impairment was affecting shivering or non-shivering thermogenesis, the former being supplied by muscle shivering and the later generated by BAT thermogenesis, we anesthetized mice at room temperature to suppress muscle shivering. Under this condition, we observed a quick drop in body temperature of KO mice (Fig.10B). In line with this observation, basal levels of mitochondrial uncoupling protein 1 (UCP1) mRNA were reduced in BAT of LMO4 KO mice (Fig.10C). Together, these results suggest reduced sympathetic outflow to BAT. We also performed norepinephrine turnover (NETO) assay on these mice. NETO is an index of sympathetic activity to peripheral tissues which is determined by

the rate of decline in tissue norepinephrine content after tyrosine hydroxylase inhibition. Here we found significant decrease of NETO in both BAT and gWAT of KO mice, which further confirmed lower sympathetic outflow to BAT and gWAT (Fig.10D).

3.5 Ablation of LMO4 impairs leptin signaling in the hypothalamus.

To elucidate molecular mechanism underlying the observed obese phenotype, we asked whether loss of LMO4 function in postnatal brains contributes to loss of leptin signaling. Leptin signaling has been well investigated in the hypothalamus. Stat3 is one of the downstream transcription factors mediating leptin signaling in the brain. LMO4 has been reported to modulate IL-6 signaling by affecting the phosphorylation of its down stream molecule Stat3 in the liver (Novotny-Diermayr et al., 2005) and previous studies in our laboratory also revealed that LMO4 knockdown in primary cultured neurons reduces Stat3 phosphorylation (Chen et al., 2007). We hypothesized that leptin signaling in the hypothalamus is impaired in the absence of LMO4. To test this hypothesis, recombinant leptin was injected into the hypothalamus. As expected, acute i.c.v. injection of leptin increased phosphorylation of Stat3 in the ARC, VMH and DMH of hypothalamus of the WT control mice (Fig.11A-C). However, Stat3 phosphorylation was only detected in the ARC not in the VMH or DMH of LMO4 KO mice (Fig.11D-F). This suggests that leptin signaling is only affected in the VMH

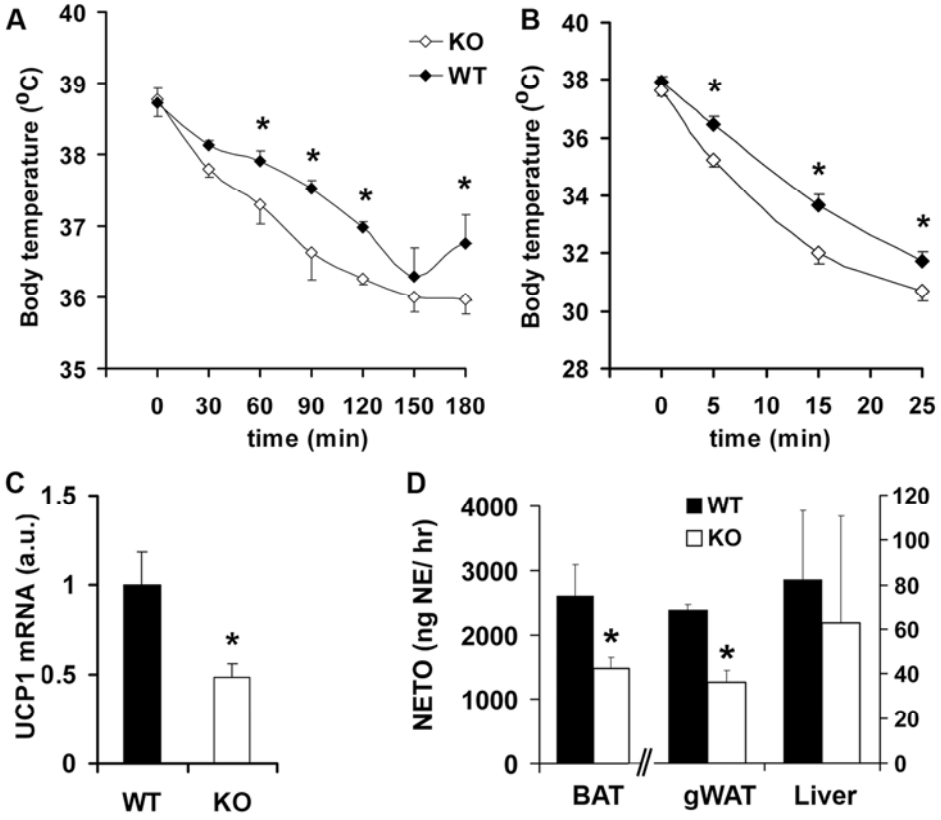


Figure 10. Reduced Sympathetic Tone to Adipose Tissues in CaMKIIα-Cre;LMO4flox/flox Mice.

(A), Reduced thermogenic ability was observed in CaMKIIα-Cre;LMO4flox/flox male mice upon cold challenge of conscious exposure at 4°C (asterisk, $p<0.05$, n=6 per group), WT (black diamonds), KO (open diamonds). This experiment was performed in collaboration with Mariana Gomez-Smith.

(B), Reduced thermogenic ability was observed in anesthetized KO mice at room temperature (asterisk, $p<0.05$, n=6 per group), WT (black diamonds), KO (open diamonds).

(C), Quantitative RT-PCR revealed lower levels of uncoupling protein-1 (UCP1)

mRNA in BAT LMO4 KO mice (asterisk, $p<0.05$, $n\geq4$ per group), WT (filled bars), KO (open bars).

(D) Norepinephrine turnover (NETO) assay revealed reduced sympathetic outflow to BAT and gWAT but not to liver in KO mice at 2 months of age (asterisk, $p<0.05$, $n=8$ per group), WT (filled bars), KO (open bars). This experiment was performed by Mariana Gomez-Smith in collaboration with Dr. Hyme Anisman.

and DMH nuclei of the KO hypothalamus. This result is consistent with what we have observed by *in situ* staining of LMO4. Ablation of LMO4 is only detected in the VMH, DMH and PVN of the hypothalamus (Fig.6B, 6D). We did not see LMO4 expression in the ARC. This result indicates that there are some neurons where leptin signaling is LMO4-dependent (VMH, DMH) and some neurons where leptin signaling is LMO4-independent. Immunoblot analysis of hypothalamic protein extracts from KO and WT mice was also performed to further confirm the impaired leptin signaling. Phosphorylation of both Stat3 and Akt, the two major downstream molecules activated by leptin signaling, were reduced in KO mice 45 minutes after i.c.v. injection of leptin (Fig.11G). Importantly, we did not observe any change in the protein level of Socs3, which has been reported to suppress Stat3 phosphorylation and is commonly shown as an indicator of leptin resistance (Fig.11G). The unchanged Socs3 indicated that the reduced Stat3 phosphorylation is likely directly related to the loss of LMO4, not due to elevated Socs3 expression.

To confirm the leptin signaling deficiency in KO mice, leptin was also administered to the brains of 2 month old mice by 7-day continuous infusion using Alzet osmotic pumps (Fig.12A). As LMO4 is not expressed in the hypothalamic nuclei responsible for the regulation of food intake by leptin (i.e. the ARC), we did not expect that ablation of LMO4 in the hypothalamus influences food intake behavior after chronic leptin infusion. Food intake was suppressed to the same extent in both KO and WT mice as we expected (Fig.12B). However, despite a similar suppression of food intake

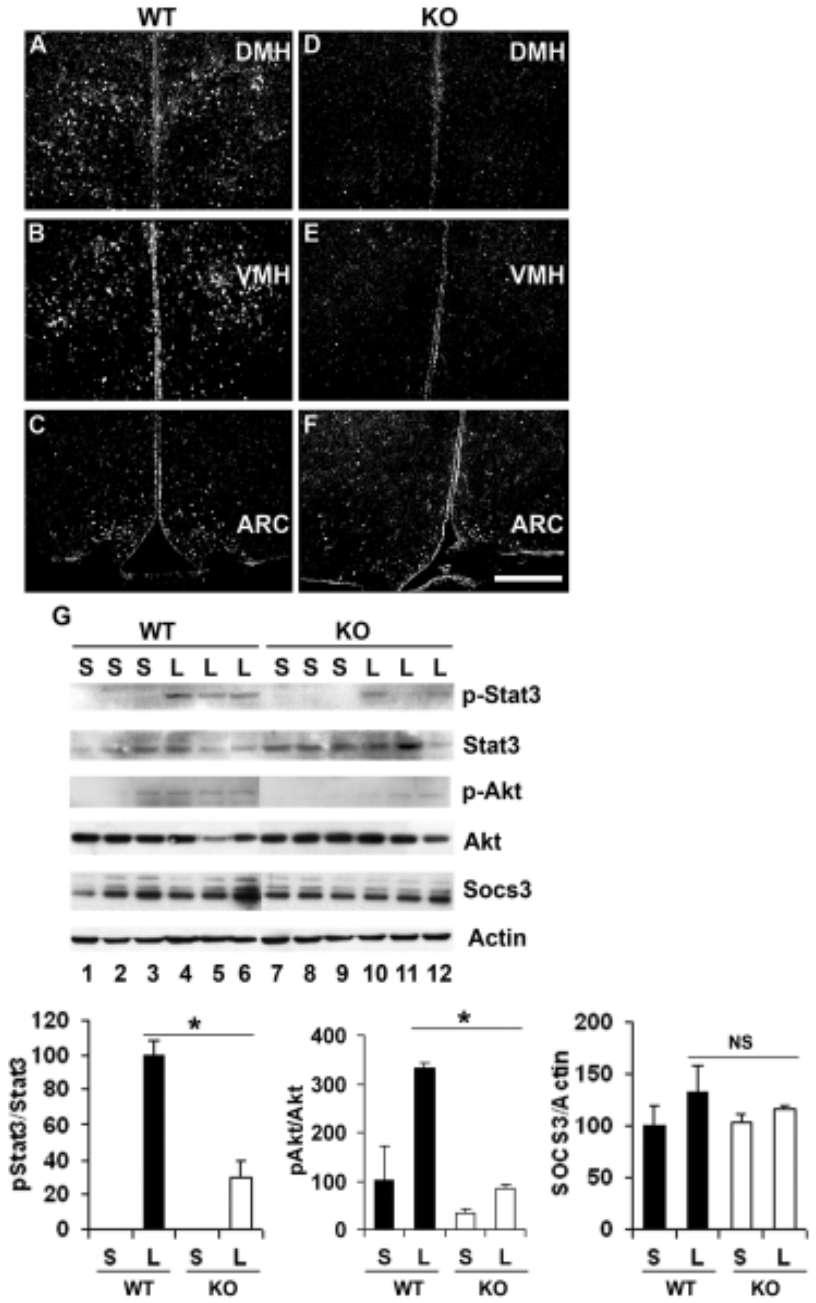


Figure 11. Altered Central Leptin Signaling in CaMKIIα-Cre;LMO4flox/flox Mice.

(A-F), Immunofluorescent staining of phospho-Stat3 revealed markedly reduced response to acute intracerebroventricular (i.c.v.) leptin injection in 2-month-old male KO mice in the VMH and DMH, but a similar response in the ARC. WT (A-C), KO (D-F). Scale bar=200μm.

(G), Representative immunoblot analysis of whole hypothalamic extracts (n=3 per group) revealed higher levels of phospho-Stat3 and phosphor-Akt in littermate WT than in KO after leptin injection (2-month-old male). Scanned blots were quantified with Image J software and normalized to leptin treated WT for pStat3 or saline treated WT for pAkt and Socs3. S, saline; L, leptin. WT (filled bars), KO (open bars).

by chronic leptin infusion, body weight and fat loss was slower in KO mice (Fig.12C). After 7 days of leptin infusion, gWAT loss approached 50% in WT controls while only 20% of gWAT was lost in KO mice (Fig.12D). Quantitative RT-PCR revealed that chronic leptin infusion also significantly downregulated lipogenic gene expression (i.e. ACC and FAS) in gWAT of littermate controls but not in gWAT of KO mice (Fig.12E). Furthermore, hypothalamic leptin administration normally elevates UCP1 mRNA expression in BAT through increased sympathetic outflow. In our experiment, chronic central leptin infusion failed to upregulate UCP1 expression in KO mice (Fig.12E). Together, these results suggest that LMO4 is required for central leptin signaling in the hypothalamus. In the absence of LMO4, some brunch of leptin signaling is impaired and fails to regulate sympathetic outflow to adipose tissue to control fat metabolism.

3.6 Pair feeding prevents adiposity and weight gain, but does not restore insulin sensitivity in LMO4 KO mice.

To test whether hyperphagia accounts for obesity in LMO4 KO mice, we sought to rescue the obese phenotype by restricting the caloric intake of these mice. The KO mice were pair-fed with littermate controls for a period of 2 months starting when they were 2 months of age. Pair feeding prevented excess body weight gain in KO mice (Fig.13A) and also normalized fat content (Fig.13B). In line with these results,

quantitative RT-PCR showed similar expression levels of lipogenic genes in gWAT of both KO and WT mice (Fig.13E). Although pair feeding improved glucose handling in KO mice at 4 months of age (Fig.13C), it did not rescue the impaired insulin response (Fig.13D). Furthermore, serum insulin levels of KO mice were still higher than that of WT controls (Fig.13F). Thus, caloric restriction only partially rescued the metabolic defects observed in LMO4 KO mice. Of note, these findings indicate that LMO4 function in the hypothalamus is also important for insulin sensitivity.

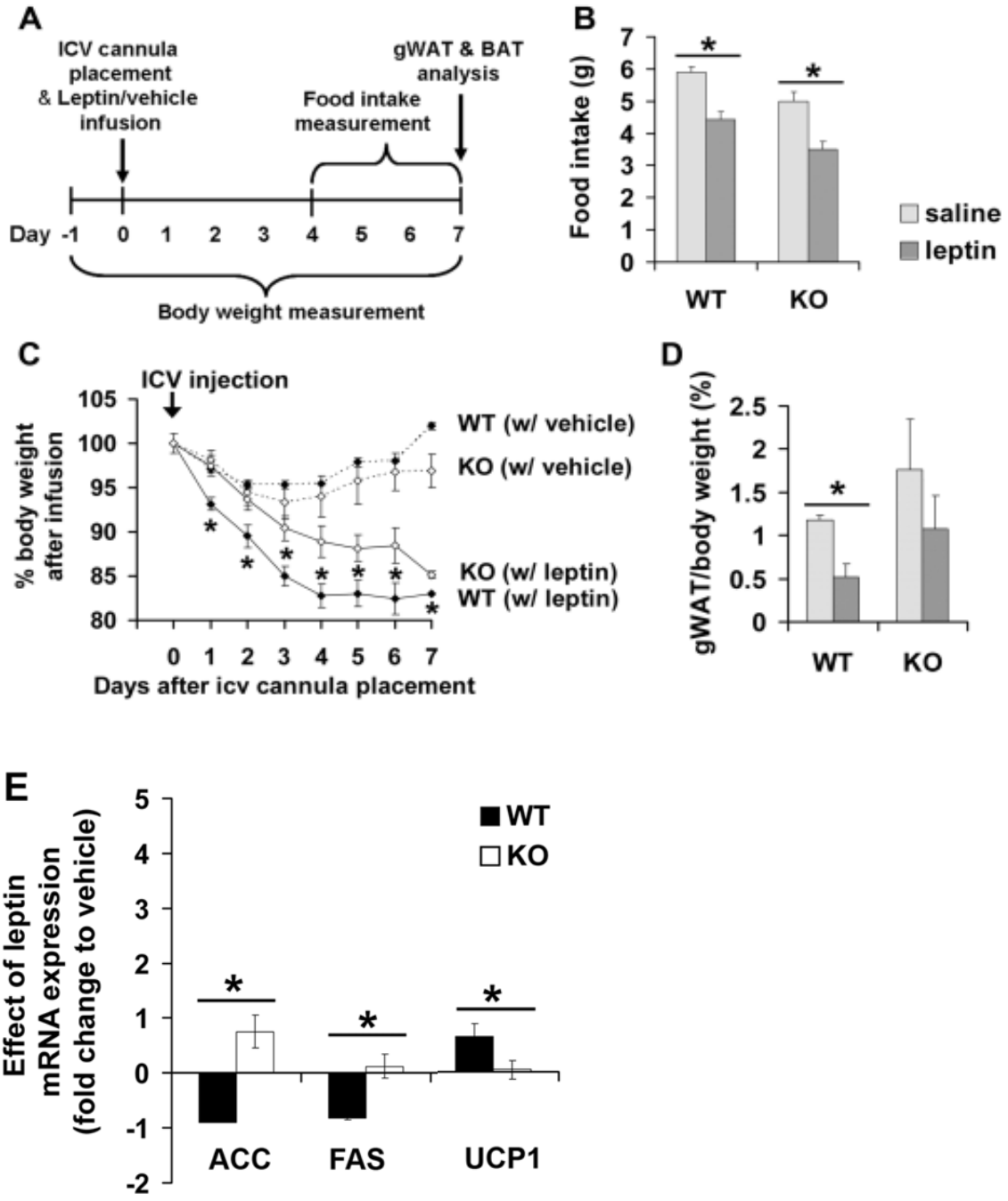


Figure 12. Effects of Chronic Leptin Infusion.

(A), Diagram of the chronic icv leptin infusion protocol using an osmotic minipump. Body weight changes were measured daily and food intake was measured from day 4 to day 7. (n=6-8 per group) (B), Leptin infusion suppressed food intake in both littermate controls and KO mice (asterisks, $p<0.05$, n=6-8 per group).

(C), Leptin-induced weight loss was not as great in KO mice as in WT mice (asterisks, $p<0.05$). WT (black diamonds), KO (open diamonds).

(D), Leptin induced a 50% gonadal WAT (gWAT) weight loss in WT but not in KO mice (asterisks, $p<0.05$, n=6-8 per group).

(E), Quantitative RT-PCR showed that leptin reduced lipogenic gene expression in WAT of WT but not in KO mice. Similarly, leptin increased UCP1 mRNA expression in BAT in WT but not in KO mice (asterisk, $p<0.05$, $n\geq4$ per group), WT (filled bars), KO (open bars).

These experiments were performed in collaboration with Mariana Gomez-Smith.

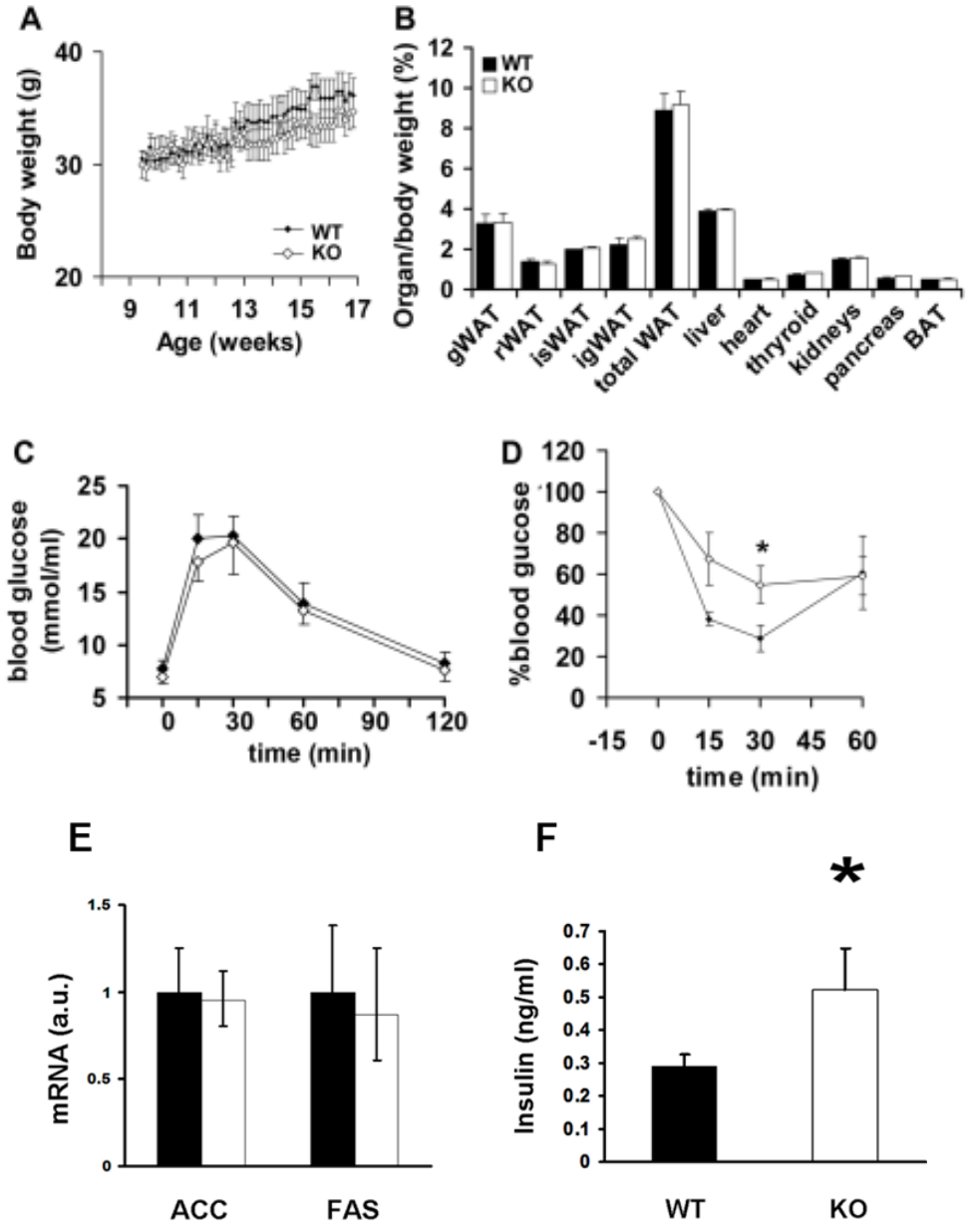


Figure 13. Pair Feeding Rescues Adiposity and Obesity but Not Insulin Resistance in CaMKIIα-Cre;LMO4flox/flox Mice.

(A), Pair feeding between the ages of 2 and 4 months prevented overt weight gain in KO mice (n=9 per group).

(B), Lean and fat mass was unchanged in KO mice (n=9 per group, rWAT retroperitoneal WAT, isWAT intrascapular WAT, igWAT inguinal WAT) after pair

feeding.

(C), Glucose tolerance test appeared normal after 2 month pair feeding, WT (black diamonds), KO (open diamonds).

(D), Insulin tolerance test revealed persistence of insulin resistance in KO mice after pair feeding (asterisk, $p<0.05$, n=9 per group), WT (black diamonds), KO (open diamonds).

(E), Quantitative RT-PCR showed similar levels of lipogenic gene expression in gWAT of both KO and WT mice (n=5 per group), WT (filled bars), KO (open bars).

(F), Serum insulin levels were still high in KO mice after 2 month pair feeding, (asterisk, $p<0.05$, n=4 per group), WT (filled bars), KO (open bars).

Chapter 4. Discussion

4.1 The LMO4 expression pattern in the hypothalamus.

LMO4 has been reported to be expressed in the brain during the embryonic stage and to play a very important role in brain development. In our laboratory, we generated a conditionally knockout mouse model of LMO4 using the Cre-LoxP system in which cre expression was under the control of CaMKIIα promoter. As CaMKIIα is expressed postnatally (Zou et al., 2002), the possibility that LMO4 is ablated during the brain development stage and affects the proliferation and differentiation of neurons in the hypothalamus can be largely excluded. Indeed, LMO4 KO mice we generated were born in expected Mendelian ratios and showed normal development. Previous research from our laboratory and other groups has already shown that LMO4 is only expressed in neurons not in glial cells (Chen et al., 2007). However, LMO4 expression in the hypothalamus has not been well studied. Our present study reveals that LMO4 mRNA is expressed in the PVN, VMH and DMH of hypothalamus. LMO4 expression is undetectable in the ARC by *in situ* hybridization, which suggests that neurons in the ARC might not express LMO4 or LMO4 expression in the ARC is too weak to be detected by *in situ* hybridization. Quantitative real-time RT-PCR showed that LMO4 mRNA levels were reduced by 90% in the hypothalamus and almost 100% in the hippocampus of KO mice (data not shown). Based upon the high

expression of CaMKIIα in glutamatergic neurons and enrichment of glutamate immunoactivity in the hippocampus (Blackshaw et al.), it is likely that in our KO mice LMO4 is ablated in excitatory neurons. And the real-time PCR results also hint that LMO4 might be expressed in other subpopulations of neurons in which CaMKIIα is not expressed. In the hypothalamus, the VMH has a high density of glutamatergic neurons. In the DMH there are fewer glutamatergic neurons than GABAergic neurons. The PVN contains multiple subpopulations of neurons including some glutamatergic neurons. Most neurons in the ARC release either AGRP/NPY or POMC as neurotransmitters (Chou et al., 2003). All of these might explain the *in situ* hybridization results that LMO4 reduction was detected in the VMH, DMH and PVN, and not in the ARC. To further confirm the LMO4 expression pattern in the hypothalamus, other methods such as immunochemical or immunofluorescent staining are needed to colocalize LMO4 with different neuronal markers.

4.2 The partially impaired leptin signaling in the hypothalamus.

The present study demonstrates that LMO4 contributes to central leptin signaling. Neuronal specific ablation of LMO4 attenuated leptin signaling in the hypothalamus, which leads to leptin resistant, obesity and eventually to diabetes in mice. To analyze leptin signaling in the absence of LMO4, both acute and chronic leptin infusion were administered to the hypothalamus of these KO mice. Stat3 is the most important

downstream molecule of leptin signaling and its phosphorylation level is commonly used as an indicator of acute leptin response (Vaisse et al., 1996). In our study of acute leptin infusion, we observed Stat3 phosphorylation was diminished in the VMH, DMH and PVN of KO hypothalamus, but not in the ARC. This suggests that the ARC does not need LMO4 to respond to leptin. Thus, the leptin signaling is partially impaired in LMO4 KO mice. The western blot of whole hypothalamic protein extracts also showed reduced Stat3 phosphorylation in KO mice after acute leptin infusion. The remaining detectable phosphorylated Stat3 is likely from the ARC.

Of note, when we performed the leptin infusion, we injected leptin into the lateral ventricle. Then leptin diffused to different nuclei in the hypothalamus. The ARC has been claimed to be outside of the blood brain barrier, though it is still controversial. Our results indicates that at least some if not all of the leptin responsive neurons are located inside of the blood brain barrier which might also partially explain why we could not observe very strong signal of Stat3 phosphorylation as the ARC has the most density of leptin responsive neurons. Furthermore, the acute leptin response of the hypothalamic neurons has been demonstrated to affect membrane potentials by modulating potassium channel activity. This effect is mediated by PI3K-Akt signaling pathways (Bjorbaek et al., 1997). This is consistent with our observation that reduced Akt phosphorylation in the hypothalamus of KO mice. As LMO4 has been identified as a calcium responsive early immediate gene in the matured neurons (Kashani et al., 2006), it is possible that the firing activity of hypothalamic neurons is abnormal in the

absence of LMO4. To elucidate whether LMO4 ablation affects hypothalamic neuronal activity via disrupting the membrane potentials, electrophysiological approaches are required for further investigation.

In agreement with the results from acute leptin injection, 7-day continuous leptin infusion also reveals that the central leptin signaling is partially impaired in KO mice. Chronic central leptin administration has been reported to regulate whole body metabolism and energy homeostasis through suppressing food intake and inhibiting lipogenesis in adipose tissues. The molecular mechanism of the food intake regulation by leptin signaling in the hypothalamus has been well investigated since the discovery of leptin. The ARC is the main nucleus in the hypothalamus that mediates leptin action on the regulation of food intake. Recent study indicated that the control of food intake and energy expenditure is divergent through different nuclei in the hypothalamus. Our findings supported this conclusion. After chronic leptin infusion to the hypothalamus, we observed similar suppression of food intake between LMO4 KO mice and WT controls. This implied intact leptin signaling in the ARC of these mice. However, central leptin action on the regulation of peripheral lipogenesis, especially in white adipose tissue, was attenuated in the absence of LMO4. Therefore, leptin signaling responsible for the control of fat metabolism is impaired. As a result, KO mice are not obese until the onset of hyperphagia due to leptin resistance, which also explains why pair-feeding rescued obesity in our KO mice.

The early manifestation of increased lipogenesis and abdominal adiposity in KO mice also suggested that impaired central leptin action on energy homeostasis is mediated via sympathetic outflow to peripheral tissues. First, our NETO assay revealed reduced basal sympathetic tone to gWAT and BAT in the absence of LMO4. As a consequence, the UCP1 expression level, a common indicator of BAT thermogenesis, was downregulated in LMO4 KO mice and the non-shivering thermogenesis generated from BAT was defective in these mice. In addition, chronic central leptin infusion failed to stimulate UCP1 expression in the BAT of KO mice. Taken together, leptin signaling in the hypothalamus regulates food intake and energy expenditure through at least two different pathways, one of which regulates fat metabolism and is impaired by neuronal ablation of LMO4. It is likely that central leptin regulates the function of WAT and BAT through the same signaling circuit in the hypothalamus.

4.3 The potential role of LMO4 in the hypothalamic leptin signaling.

We have previous reported that knocking down of LMO4 reduced Stat3 phosphorylation in primary cultured neurons (Chen et al., 2007). Here, we provide evidence in vivo that LMO4 is involved in leptin signaling by affecting Stat3 phosphorylation. The molecular mechanism underlying this effect remains unknown. LMO4 can interact with the IL-6 receptor and its associated kinases Jak1/2 to promote Stat3 signaling in response to IL-6 in the liver (Novotny-Diermayr et al., 2005).

Although a few studies show LMO4 is localized in the cytoplasm (Schaffar et al., 2008), LMO4 is mostly known as a transcription cofactor that recruits different transcription factors and other components to form a complex in the nucleus. Stat3 is a well established transcription factor. Once activated by an upstream kinase, the phosphorylated Stat3 translocates into the nucleus to stimulate target gene expression. Phosphorylated Stat3 is then dephosphorylated by phosphatase and transported out of the nucleus. It is possible that LMO4 behaves in a similar fashion to mediate leptin-induced Stat3 interaction with JAK. It should be noted that LMO4 does not interact with Stat3 directly as Co-IP and two-hybrid assay failed to reveal any interaction (our unpublished results).

Leptin signaling has been reported in different nuclei of the hypothalamus. In the ARC, this pathway is fully elucidated and the target genes of phosphorylated Stat3 have been identified as POMC and AgRP in different neuronal subpopulations (Kitamura et al., 2006). However, the downstream events of Stat3 in the nuclei other than the ARC, such as the VMH and the DMH, remain to be investigated. Our LMO4 KO mice provide opportunities for the identification of downstream targets in these nuclei and are good model for the functional study of the different nuclei in the hypothalamus. Of note, another group has generated mice lacking leptin receptors on steroidogenic factor 1 (SF-1)-positive neurons in the VMH. Those mutant mice lose leptin signaling in the VMH and display the very similar phenotype as our LMO4 KO mice. The mice have increased fat stores without an early onset hyperphagia (Dhillon

et al., 2006). Together with our current studies, these findings suggest that leptin signaling in the VMH specifically regulates fat metabolism in adipose tissue. Most neurons in the VMH release glutamate as a neurotransmitter and the excitatory projection from the VMH to the ARC has been found recently (Sternson et al., 2005). This raised the possibility that the leptin signaling in the VMH exerts its function through the ARC by releasing glutamate to the ARC neurons. However, the mutant mice lacking vesicular glutamate transporter (Vglut2) in SF1-positive neurons generated by the same group did not reveal the similar phenotype (Tong et al., 2007). The PVN is of importance in regulating autonomic and neuroendocrine function to modulate energy homeostasis by receiving projections from a number of other regions in the hypothalamus. Previous research has established that the neuronal circuit in the PVN regulates food intake while that in other nuclei of the hypothalamus is responsible for energy expenditure such as fat metabolism in adipose tissues (Balthasar et al., 2005). Our findings indicate that the VMH might be one of those hypothalamic nuclei that regulate peripheral fat metabolism through sympathetic outflow. Meanwhile, the leptin signaling in the DMH can not be excluded since we do detect LMO4 ablation in this region of our KO mice and the DMH innervates to the PVN. To further elucidate the function of LMO4 in leptin signaling in these different nuclei, we need to generate conditional knock out mouse models under the control of more specific promoters such as the SF1 which is a VMH specific factor or Sim1 of which the expression is restricted in the PVN of the hypothalamus.

4.4 Caloric restriction and leptin signaling in the hypothalamus.

It is of interest to see that 2 month pair feeding not only prevents the obesity but also normalizes the increased lipogenesis in adipose tissue. The obesity in our KO mice is due to an early elevation in lipogenesis in adipose tissue that leads to elevated leptin production from fat and eventually to resistance to leptin signaling and hyperphagia. Central leptin signaling responsible for the control of food intake is not impaired in KO mice at 2 months but is impaired by 4 months since the mice are hyperleptinemic and eat more. This is the most likely reason for weight gain and obesity in our mutant mice. Thus, caloric restriction by paired feeding was sufficient to rescue the obesity phenotype in our mice.

4.5 Leptin signaling and insulin sensitivity.

In our LMO4 KO mice, we observed reduced insulin sensitivity as early as 2 months of age even though the serum glucose levels are normal, as well as the body weight. Reduced insulin sensitivity has been reported in obese patients as a consequence of increased body fat content. Leptin signaling in the hypothalamus has also been demonstrated to regulate insulin sensitivity and glucose metabolism through a distinct pathway than the one controls food intake and body weight. After 2 months of paired

feeding, not only was body weight completely normalized, so was the fat mass. However, reduced peripheral insulin sensitivity still persisted despite normal serum glucose levels. These results suggest that the reduced insulin sensitivity in KO mice is caused by impaired leptin signaling in the hypothalamus but not due to the increased fat content. Consistent with this concept, another group had already demonstrated that leptin signaling in the mediobasal hypothalamus regulates insulin sensitivity and glucose metabolism in a PI3K-dependent pathway (Morton et al., 2005). In agreement with their findings, we also observed reduced Akt phosphorylation which is the downstream target in the PI3K pathway in response to leptin. Moreover, the previous studies identified the mediobasal hypothalamus that includes the ARC and the VMH to be the region that controls insulin sensitivity and glucose metabolism in response to leptin. Here, in our KO mice, leptin signaling was impaired in the VMH but not in the ARC, which suggests that leptin signaling in the VMH is sufficient for the regulation of insulin sensitivity. Future studies that restore LMO4 expression and leptin signaling in the VMH of CaMKIIα-Cre;LMO4flox/flox mice will be needed to confirm this possibility.

4.6 Conclusions:

In our present study, we generated a novel mouse model in which LMO4 was ablated in nervous system postnatally. The LMO4 KO mice show impaired leptin signaling in

the VMH, DMH and PVN of the hypothalamus which results in increased lipogenesis in white adipose tissue and reduced insulin sensitivity, followed by hyperleptinemia, hyperinsulinemia, hyperphagia and hyperglycemia, leading to obesity and eventually diabetes. This is the first study to document the LMO4 expression pattern in the adult hypothalamus. We observed that LMO4 is expressed in the hypothalamic nuclei such as the VMH, DMH and PVN but not the ARC. We also found that LMO4 is essential for leptin signaling in the hypothalamus. Loss of LMO4 attenuates leptin signaling by reducing Stat3 phosphorylation. The molecular mechanism underlying the requirement for LMO4 on Stat3 phosphorylation in discrete regions of the hypothalamus remains to be investigated. Another important finding from our study is that leptin signaling in the VMH, DMH and PVN regulates lipid metabolism in white adipose tissue and insulin sensitivity through sympathetic systems. Impaired leptin signaling in these regions reduced sympathetic outflow to white adipose tissue and brown adipose tissue, results in increased lipogenesis and decreased thermogenesis, respectively. We also found the VMH, DMH and PVN, other than the ARC, are the nuclei in the hypothalamus which regulate insulin sensitivity in response to leptin. The impaired leptin signaling in the VMH, DMH and PVN reduced insulin sensitivity thus results in diabetes. A similar mechanism might explain why some patients develop diabetes before overt obesity. Finally, we have generated a new transgenic mouse model for obesity and diabetes research, in which we only manipulate a small gene in the brain that induces all the symptoms mimicking the progressive process of human disease. This is a good model for the research of all the obesity and diabetes

related diseases, such as the development of heart disease, stroke and the late vascular disease in diabetes.

Chapter 5. References

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