INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

EFFECTS OF DIETARY FAT TYPE AND ENERGY RESTRICTION ON HYPOTHALAMIC MEMBRANE STRUCTURE AND LEPTIN RECEPTOR FUNCTION

Jodi T. Heshka

School of Dietetics and Human Nutrition
McGill University
Montrèal, Canada
September, 2001

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the degree of Master of Science

Short Title: Dietary fat, energy restriction, and the leptin receptor.

In memory of my Grandmother, Mary Sokoloski

For my family

"The brain, the locus of thought, is made up of the most elaborate network we know. It is this mass of connections and junctions which allows us to solve problems, to feel a wide variety of emotions or simply, to be moved."

- Anonymous

ABSTRACT

The objectives of the present study were to examine the effects of dietary fat type and level of energy intake on hypothalamic leptin binding affinity and membrane fatty acid composition, circulating leptin levels, and body weight homeostasis in rats. Animals were fed diets containing tallow, safflower oil, or menhaden oil (20% wt/wt) for 10 wks, *ad libitum* or at 60% of *ad libitum* intakes. Specific leptin binding could not be detected in hypothalamic membrane homogenates; hypothalamic leptin levels were unaffected by diet or energy intake. Levels of tracer exceeding assay reference values were found in homogenates, suggesting intra-membrane binding. Excess tracer levels were weakly associated ($p<0.07$) with the sum of hypothalamic phospholipid monounsaturates. Restriction lowered weight gain and food intakes ($p<0.0001$ for both). In hypothalamic phospholipids, energy restriction lowered levels of 18:3(n-3) and increased levels of 20:1(n-9), 20:4(n-6), and 22:4(n-6) ($p<0.05$, $p<0.02$, $p<0.05$, and $p<0.04$, respectively). Fat type and energy level interactively affected hypothalamic levels of 20:4(n-6), 22:5(n-3) and 22:6(n-3) ($p<0.05$, $p<0.006$, and $p<0.05$, respectively). Restriction lowered circulating leptin levels ($p<0.0001$); overall plasma leptin levels were marginally associated ($p<0.07$) with hypothalamic 16:0 concentrations. The results of the study support previous findings suggesting that leptin binding at the level of the hypothalamic membrane may not be detectable. The results also support the lack of a dietary fat effect on plasma leptin levels and levels of certain hypothalamic fatty acids, such as 20:4(n-6), 22:4(n-6), and 22:5(n-3), with energy restriction. The findings of the study suggest a link between increased membrane fluidity, increased binding affinity, and lower circulating leptin levels, promoting the possibility that the biological actions of leptin can be controlled through dietary effects on OB-Rb or its signaling.

RÉSUMÉ

Le but de cette étude était d'examiner les effets des types de gras alimentaires et du niveau de consommation en énergie sur l'affinité de la liaison de la leptine hypothalamique, la composition en acides gras des membranes hypothalamiques, le taux de leptine circulant, et l'homéostasie du poids corporel chez le rat. Les animaux ont été nourris avec des diètes composées de suif, d'huile de tournesol, ou d'huile de menhaden (20% p/p) pendant dix semaines *ad libitum*, ou à 60% de la prise alimentaire *ad libitum*. La liaison spécifique de la leptine n'a pas été détectée dans les homogénats de membranes hypothalamiques; les taux de leptine hypothalamique n'ont pas été affectés par la diète ou par la prise énergétique. Des taux de traceur de la leptine dépassant les valeurs de référence ont été détectés dans les homogénats, suggérant ainsi une liaison intra-membranaire. Les taux excessifs de traceur ont été faiblement associés ($p<0.07$) à la somme des phospholipides monoinsaturés. La restriction énergétique a diminué la prise de poids corporel ($p<0.0001$). Dans les phospholipides hypothalamiques, la restriction énergétique a diminué les concentrations de 18:3(n-3) et a augmenté les concentrations de 20:1(n-9), 20:4(n-6), et 22:4(n-6) ($p<0.05$, $p<0.02$, $p<0.05$, et $p<0.04$, respectivement). Le type de gras et le niveau de restriction énergétique ont affecté de façon interactive les niveaux de 20:4(n-6), 22:5(n-3) et 22:6(n-3) ($p<0.05$, $p<0.006$, et $p<0.05$, respectivement) dans l'hypothalamus. La restriction a également entraîné une diminution des concentrations de leptine circulante ($p<0.0001$); la leptine plasmatique totale a été associée marginalement ($p<0.07$) aux concentrations hypothalamiques de 16:0. Les résultats de cette étude sont en accord avec d'autres ayant suggéré que la liaison de la leptine ne puisse pas être détectable au niveau de la membrane hypothalamique. Ces résultats supportent également une absence d'effet du type de gras sur la leptine circulante et sur les concentrations de certains acides gras hypothalamiques, tels 20:4(n-6), 22:4(n-6), et 22:5(n-3), lorsque la consommation énergétique est restreinte. Les résultats de cette étude suggèrent un lien entre l'augmentation de la fluidité membranaire, l'augmentation de l'affinité de la liaison entre la leptine et son récepteur, et les faibles taux de leptine circulante, étant ainsi en accord avec la possibilité que l'action

biologique de la leptine circulante pourrait être contrôlée par des effets alimentaires sur OB-Rb ou sur sa voie de signalisation cellulaire.

ACKNOWLEDGMENTS

I acknowledge my supervisor, Dr. Peter Jones, for his continuous support of this project and for providing feedback during the writing of the resulting manuscripts.

I also acknowledge and thank my committee members, Dr. David Bissonnette, Dr. Errol Marliss, and Dr. Louise Thibault, for their guidance, their insights, and their time. In particular, I would like to thank Dr. Thibault for dissecting the rat brains.

I give a word of gratitude to Geoff Hynes, my partner in this project. I admire him for his strength and dedication. It has been a pleasure to work with him.

A special mention must be made of the wonderful staff at the School. I will always remember the considerable help and many kindnesses of Francine Tardif, Leslie Anne LaDuke, Anne Houston, and Lise Grant. Additionally, I would like to thank Mr. Gordon Bingham for his generous help with the animals during the course of the feeding trial. I thank Drs. Kris Chadee and Adam Belley, of the McGill Parasitology Institute, for their time, expertise, and instruction with the execution of the binding assays. I give a special word of thanks to Dr. Katherine Gray-Donald. She has been a source of guidance and friendship, and remains a mentor.

I must also thank the other Faculty members of the School and the many friends who made my graduate experience so memorable. In particular, I thank my fellow students in the lab who supported me in my work. My gratitude is extended to Mahmoud Raeini-Sarjaz and Nirupa Matthan for their assistance with the operation of the gas chromatograph. I also thank Catherine Vanstone and Julia Krasevec for helping me perfect my laboratory techniques. My thanks are extended to everyone in the Jones lab group for their care and comraderie. I especially thank Christine Bourque for her friendship, encouragement, sense of humour, and kindness.

Finally, my love and thanks to the people who care about me the most. I give my deepest thanks to my parents for always believing in me. Their strength, encouragement, wisdom, and faith help make me complete, and their sacrifices made all of this possible. This work could also not have been completed without the constant enthusiasm and inspiration of my sister, Jade. Last, thank you to Sanjay. You challenged and encouraged me, and always gave me something to look towards.

TABLE OF CONTENTS

LIST OF TABLES

Chapter III. Manuscript II.

LIST OF FIGURES

Chapter II. Manuscript I.

Chapter III. Manuscript II.

CONTRIBUTIONS OF AUTHORS

This thesis consists of two manuscripts. The first is a review paper that was coauthored with my supervisor, Dr. Peter Jones. The second is an original research manuscript, coauthored with a fellow graduate student, Geoff Hynes, and Drs. Louise Thibault and Jones. Both Dr. Jones and I developed the ideas for the project. Dr. Jones supervised the project, provided financial resources for the laboratory work as well as personal financial support during my studies. He also provided suggestions pertaining to the experimental design. Additionally, Dr. Jones corrected the manuscripts. Dr. Thibault dissected the rat brains. Otherwise, I was responsible for the overall experimental design, protocol development (in regards to the binding assays), execution (the feeding trial responsibilities and plasma leptin analyses were shared with Geoff Hynes), data analysis, and texts of the manuscripts.

CHAPTER I. INTRODUCTION

Obesity remains to be one of today's most prevalent health concerns. Since the initial identification of leptin and its receptor in 1994 and 1995 (Zhang et al., 1994; Tartaglia et al., 1995), respectively, the leptin/receptor system has been largely implicated in the development of obesity. Leptin, a hormonal product of the *ob* gene, is produced by adipocytes and reduces food intake, increases energy expenditure, and results in weight loss in mammals (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995; Zhang et al., 1994). Leptin exerts its physiological actions via receptors located in various areas of the hypothalamus, a region of the brain well known for its involvement in body weight homeostasis. A number of splice variants of the receptor have been cloned; to date, six isoforms have been identified, termed OB-Ra-f (Lee et al., 1996). Of these isoforms, however, it is the long transmembrane form of the receptor, OB-Rb, that is generally considered to be fully functional, thus able to mediate the biological actions of leptin (Baskin et al., 1999, Cheung et al., 1997). OB-Rb signaling occurs through a ligand-induced conformational change of the receptor components, initiating a cascade of downstream signals, including activation of the JAK/STAT system of signal transduction.

Since the initial identification of leptin and its receptor, research has suggested that obesity in both humans and animals may be caused by a state of leptin resistance, where individuals have high circulating leptin levels, pronounced hyperphagia, and obesity. Leptin resistance is due to decreased peripheral or central leptin sensitivity, occuring either alone or in combination. While much is known about the structure and localization of OB-Rb, the precise mechanism by which the receptor regulates body composition remains unknown. As well, whether leptin resistance occurs at the level of uptake in the hypothalamus or the level of leptin production in the adipocytes remains to be elucidated.

Previous work has identified that both dietary fat type and energy restriction have individual and interactive effects on weight gain and circulating leptin levels, consequently influencing leptin resistance. Energy restriction has been shown to decrease both circulating leptin levels and hypothalamic OB-Rb mRNA (Baskin et al.,

1998; Cha and Jones, 1998; Lin and Huang, 1997; Mizuno et al., 1998; Saladin et al., 1995). As well, both the type and amount of dietary fat have been shown to affect circulating leptin levels (Raclot et al., 1997; Cha and Jones, 1998; Hun et al., 1999).

While the fatty acid composition of the brain was once thought to be static, it is now well known that dietary fatty acid composition alters membrane fatty acid composition through de novo membrane phospholipid synthesis and acyl group turnover in membrane phospholipids (Clandinin et al., 1994). Altering the fatty acid make-up of membranes could lead to an impairment of the structure and/or function of any membrane-associated receptor complexes, such as OB-Rb. The possibility that the biological actions of leptin and the development of leptin resistance can be controlled through diet-induced modifications at the level of leptin uptake and OB-Rb function in the hypothalamus has important implications for the control of obesity and obesity-related disorders.

Based on the above considerations, the objectives of the present project were to examine, in rats, the interactive effects of dietary fatty acid composition and level of energy intake on: (i) changes in hypothalamic fatty acid composition, (ii) hypothalamic leptin receptor binding, (iii) circulating leptin levels, and (iv) body weight homeostasis. Results from the study have the potential to more clearly define the mechanism by which leptin acts in the hypothalamus to control energy balance.

CHAPTER II. MANUSCRIPT I.

A Role for Dietary Fat in Leptin Receptor, OB-Rb, Function*

Jode T. Heshka and Peter J.H. Jones

School of Dietetics and Human Nutrition
McGill University
21,111 Lakeshore Road, Ste Anne de Bellevue, QC
Canada H9X 3V9

Running Title: Dietary Fat and Ob-Rb Function

Key Words: dietary fat, effects on leptin receptor function, OB-Rb, leptin, membrane, neuropeptides, Janus kinase (JAK), signal transducers and activators of transcription (STAT)

* *Life Sciences* 2001;69:987 (reprinted with permission)

2.1. ABSTRACT

Leptin is a hormone believed to control appetite and regulate body weight via its own receptors and other leptin receptor containing neurons in the hypothalamus. Much is known about the structure of the functional, or long, form of the leptin receptor, OB-Rb, which is a class I cytokine receptor. However, the mechanism by which the receptor regulates the biological action of leptin remains to be elucidated. Both the type and amount of dietary fat have been shown to affect factors involved in OB-Rb binding and signaling, as well as the morphology of hypothalamic cell membranes. Thus, the purpose of the following review article was to examine possible mechanisms by which dietary fat may affect the functioning of the long form of the leptin receptor at the hypothalamic level. Dietary fatty acid composition can alter membrane fatty acid composition, such that membrane fat reflects that found in the diet. Altering the fatty acid make-up of membranes, such as the polyunsaturated:saturated fat ratio, changes membrane fluidity and could lead to an enhancement or impairment of the structure and/or function of any membrane-associated receptor complexes. Dietary fat has also been shown to interfere in the biochemical pathways involving leptin, OB-Rb, and other neuropeptides under OB-Rb's control, such as neuropeptide Y (NPY), proopiomelanocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART). Considering the effects of dietary fat on neurons containing these neuropeptides, research is beginning to show an emerging link between dietary fat, obesity, and decreased sympathetic nervous system (SNS) activity in hypothalamic areas, the main areas believed to play critical roles in body weight regulation. At a biochemical level, increased monounsaturated fat intake has been shown to increase cyclic adenosine monophosphate (cAMP) levels, possibly reducing mitogen-activated protein kinase (MAPK) activation. As a result, there is the potential for interruption of signals and gene transcription mediated by Janus kinase/signal tranducers and activators of transcription (JAK/STAT) pathways, such as c-fos and, in particular, STAT3, as well as decreased protein kinase activity. Dietary induced alterations in hypothalamic cell membranes or other factors involved in OB-Rb function, such as neuropeptides or signaling intermediates, or even SNS activity, form a possible basis for the control of leptin's effects on body composition and appetite. Improving the

biological activity of leptin by diet modification may exist as a practical strategy for the treatment of obesity and obesity-related disorders.

2.2. INTRODUCTION

Leptin, the product of the *ob* gene (Zhang et al., 1994), is produced in the adipose tissue and reduces food intake, increases energy expenditure, and results in weight loss in mammals (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995). The 16-kDa protein is believed to exert its effects over appetite and energy expenditure via leptin receptors and leptin-receptor-immunoreactive neurons in the hypothalamus (Baskin et al., 1999a; Cheung et al., 1997; Kristensen et al., 1998; Tartaglia et al., 1995). Since the initial identification of the leptin receptor in 1995 (Tartaglia et al., 1995), much research has characterized the structure and localization of the leptin receptor. A number of splice variants of the receptor have been cloned. To date, six different alternatively spliced isoforms have been identified, referred to as OB-Ra-f (Lee et al., 1996). Of these isoforms, only the long form of the receptor, OB-Rb, and possibly the shorter form, OB-Ra, appear capable of signaling and are thus able to mediate the biological effects of leptin, although OB-Ra is currently believed to act as a transport form (Chen et al., 1996; Lee et al., 1996; Murakami et al., 1997). One of the other isoforms, OB-Re, is thought to be a soluble form of the receptor since it lacks the typical transmembrane and intracellular portions of the receptor (Chen et al., 1996). However, exactly how OB-Rb functions to mediate the effects of leptin remains unknown. Since its discovery, the study of leptin and its receptor in relation to other factors known to affect body weight and appetite, such as neuropeptide Y, insulin, and glucocorticoids, has been a primary research focus (Flier and Maratos-Flier, 1998; Wolf, 1997). Yet an important area of research into the function of the leptin receptor generally remains ignored; that of the effect of changes in diet and in the receptor's environment on the binding and signaling of OB-Rb itself. Of the factors that may influence the surroundings of the receptor, especially the cell membrane, dietary fat may have the most important implications.

Modulation of dietary fatty acids alters the composition of cellular membrane phospholipids through their incorporation into membrane lipids by de novo synthesis and acyl group turnover. Increasing the proportion of polyunsaturated fatty acids in membranes will alter their fluidity and may consequently affect leptin binding or OB-Rb signaling. Dietary fat has also been shown to affect a number of other factors involved in

the OB-Rb-leptin binding and signaling pathway, including downstream signaling targets and gene expression of other neuropeptides regulated by leptin and known to affect appetite and body weight. Thus, the purpose of the present review was to examine evidence that dietary fat may influence leptin receptor activity through a number of different mechanisms.

2.3. CHARACTERIZATION OF THE LONG FORM OF THE LEPTIN RECEPTOR (OB-Rb) – A CLASS I CYTOKINE RECEPTOR

The human leptin receptor is a member of the class I cytokine receptor superfamily (Tartaglia et al., 1995), which includes not only OB-Rb but receptors for various interleukins (Ils-2, -3, -4, -6, and –7) (Bazan, 1989; Goodwin et al., 1990; Idzerda et al., 1990; Itoh et al., 1990), granulocyte colony stimulating factor (G-CSF) (Gearing et al., 1989), growth hormone (GRH) (Bazan, 1989), prolactin (PRL) (Bazan, 1989), erythropoeitin (EPO) (Bazan, 1989), and leukemia inhibitory factor (LIF) (Tartaglia et al., 1995). Ultrastructural localization studies have shown OB-Rb to be predominantly located in the hypothalamus, a region of the central nervous system long known to be highly involved in body weight regulation. In particular, OB-Rb has been detected in the arcuate, ventromedial, paraventricular, dorsomedial, and ventral premammillary nuclei, as well as the lateral hypothalamic area (Hiroike et al., 2000; Mercer et al., 1996).

2.3.1. OB-Rb Structure and Ligand Binding

Nucleotides for the entire cDNA of the leptin receptor have been sequenced (Tartaglia et al., 1995). The human leptin receptor gene is located on chromosome 1q31 and consists of 20 exons. Many of the OB-R intronic sequences are still unknown. OB-Rb has a 4 helix bundle structure similar to that of the G-CSF receptor, and possesses both a major and minor interface, when examined as an OB-Rb/leptin complex (Hiroike et al., 2000). As a transmembrane glycoprotein, all isoforms of the receptor have extracellular and membrane spanning domains, with, of course, the exception of OB-Re (Figure 2.1). The extracellular domains of the receptors consist of 820 amino acids, and are characteristic of the family to which the leptin receptor belongs. Recent research has

demonstrated that OB-Rbs expressed on COS-1 cells exist as both homodimer and homooligomer complexes without any evidence for heterodimer formation (Devos et al., 1997), unlike many of the other class I receptors, which undergo ligand-induced heterodimerization (Banks et al., 2000). The fact that the expressed extracellular domain of OB-Rb is a dimer suggests that OB-Rb exists as a preformed homodimer, even in the absence of ligand (Devos et al., 1997). OB-Rb appears to be activated via ligand-induced conformational changes and not heterodimerization or oligomerization with other receptors of the class I family (Banks et al., 2000; Devos et al., 1997).

Further analysis of the extracellular domain typical of class I cytokines identifies the existence of modules containing approximately 200 amino acids (Bazan, 1990). These modules are characterized by a conservation of four cysteine terminals in the N-terminal half and a Trp-Ser-x-Trp-Ser box, with the x residue non-conserved, near the C-terminal end (Bazan, 1990). The Trp-Ser-x-Trp-Ser region consists of two fibronectin type III molecules (Patthy, 1990) each of which consists of about 100 amino acids and seven β-strands in antiparallel folds to form a barrel-like structure (Devos et al., 1996). The contact surface of the class I cytokine receptor, including OB-Rb, to the ligand lies in the hinge region between the two fibronectin domains (Devos et al., 1996). As opposed to other members of the class I family, the two fibronectin domains (F3) in OB-Rb are each associated with a cytokine receptor domain (CK), thus forming two separate, homologous CK-F3 domains (Fong et al., 1998). The first CK-F3 domain is not required for leptin binding or receptor activation, but rather the second domain is the likely receptor binding site (Fong et al., 1998). OB-Rb binds leptin in a 2:2 ratio (Devos et al., 1997). Any modifications in the second CK-F3 domain can result in decreased or abolished leptin binding (Fong et al., 1998). Additionally, any mutation in the extracellular receptor domain, particularly in the Trp-Ser-x-Trp-Ser region, eliminates or greatly reduces the capacity of the receptor to bind to leptin (Patthy, 1990).

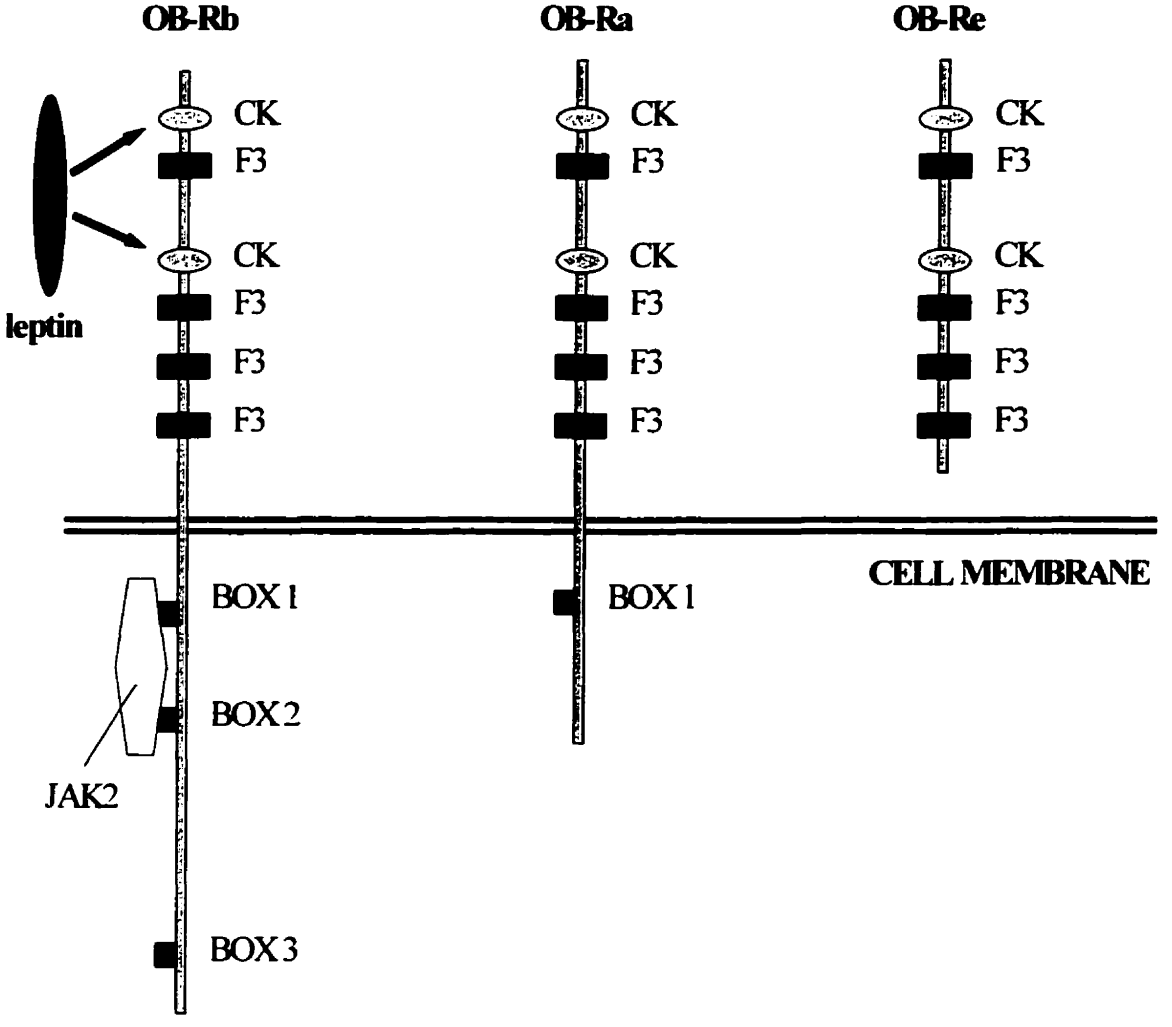

Figure 2.1. Comparison of different leptin receptor isoforms.

The repeating CK-F3 domains in OB-Rb are located in the extracellular portion of OB-Rb, and thus are near the cytosolic carboxy-terminal tail (Misra and Garg, 1996). Also located near the repeating sequences is a potential consensus sequence (YXXQ) for Signal Transducers and Activators of Transcription (STAT) binding (Chen et al., 1996).

Following the extracellular domain in OB-Rb is a short transmembrane domain consisting of 23 amino acids; itself followed by a cytoplasmic domain whose length varies among the isoforms. OB-Ra and OB-Rb are the two major forms of the receptor expressed in human cells. OB-Rb exon 20 encodes a cytoplasmic domain consisting of 303 amino acids (Kapitonov and Jurka, 1999). It is this long intracellular domain of OB-Rb that contains two sequences for the binding of Janus kinase (JAK) and thus possesses the potential for activation of the JAK-STAT system.

2.3.2. OB-Rb Signaling

Given the number of different OB-R isoforms that exist, signals specific to OB-Rb's intracellular tail are crucial for the biological action of leptin. Leptin binding to its hypothalamic receptor induces a conformational change in the receptor and triggers two unique mechanisms, in addition to activating a number of other downstream proteins with possible roles in OB-Rb signaling (Figure 2.2). First, the JAK family of tyrosine kinases is activated by transphosphorylation. Research has demonstrated that the JAK2, and possibly JAK1, tyrosine kinases are activated during OB-Rb signaling (Ghilardi and Skoda, 1997). JAK activation is followed by the phosphorylation of tyrosine residues located on the intracellular tail of OB-Rb (Bjorbaek et al., 1997). Of all the OB-R isoforms, OB-Rb is the only form to contain tyrosine residues on its intracellular tail, partially explaining OB-Rb's unique role as the major signaling isoform (Tartaglia, 1997; Tartaglia et al., 1995). The tyrosine phosphorylation of OB-Rb results in the activation of signals located within the hypothalamic cell. Essentially, different signaling proteins with different SH2 domains (domains containing specific phosphotyrosine binding sites) are engaged and bind phosphotyrosine based on its surrounding amino acid motif (Koch et al., 1991; Songyang et al., 1993). It is known that OB-Rb activation of the SH2 domain on tyrosine phosphatase 2 influences OB-Rb signal transduction (Li and Friedman, 1999). The intracellular portion of murine OB-Rb contains three different

tyrosine residues that have been implicated in OB-Rb signaling (Banks et al., 2000). Tyr^{985} and Tyr^{1138} have been shown to be surrounded by hydrophilic sequences, whereas Tyr^{1077} is located within the folded protein, surrounded by hydrophobic sequences (Banks et al., 2000). Recent research has demonstrated not only that Tyr^{985} and Tyr^{1138} are accessible to JAK2 tyrosine kinase, but that mutation of these residues with intact Tyr^{1077} prevented tyrosine phosphorylation of OB-Rb (Banks et al., 2000). Using a model of an EPO receptor with mutant intracellular OB-Rb domains, researchers have been able to further demonstrate that Tyr^{985} controls the tyrosine phosphorylation of the tyrosine phosphatase SHP-2, and Tyr^{1138} controls STAT3 activation (Banks et al., 2000). Incidently, while it was originally thought that OB-Rb signaling may occur through activation of different members of the STAT family, researchers have now concluded that OB-Rb acts primarily through the activation of STAT3 (Banks et al., 2000; Ghilardi et al., 1996; Vaisse et al., 1996).

Activation of STAT3 represents the second major mechanism triggered by conformational change of OB-Rb. The STAT group of proteins are DNA binding transcription factors that contain serine-rich homology sequences that also interact with the receptor molecules through phosphorylated tyrosine residues (Ghilardi et al., 1996). The STAT proteins are activated by tyrosine phosphorylation, form hetero- or homo-dimers, translocate to the nucleus, and modulate transcription of target genes (Ghilardi et al., 1996). Specifically, STAT3 activation is highly dependent on the YXXQ motif of OB-Rb. Coincidently, STAT3 has been shown to be present in high levels in a number of hypothalamic nuclei and other areas within the central nervous system, suggesting that leptin action may be mediated specifically by STAT3 (Hakansson and Meister, 1998; Stromberg et al., 2000).

OB-Rb activation also mobilizes a number of other downstream signals and events, not only the direct recruitment of JAK2, SHP-2, and STAT3. As a tyrosine kinase-based signaling system, OB-Rb has been shown to activate the extracellular factor-regulated kinases (ERKs), a family of serine-threonine kinases that include the MAPKs (mitogen-activated protein kinases) (Takahashi et al., 1997). The activation of ERKs represents a critical event in receptor tyrosine kinase signal transduction. Following JAK-induced tyrosine phosphorylation, ERKs (or MAPKs) translocate to the

nucleus where they mediate gene expression and control various aspects of cellular physiology, including signal transduction (Banks et al., 2000; Blenis, 1993; Hill and Treisman, 1995). In CHO cells stably expressing OB-Rb, leptin stimulation has been shown to increase MAPK phosphorylation (Hill and Treisman, 1995; Yamashita et al., 1998). In OB-Rb, the activation of ERK is mediated by the adapter protein, GRB-2, which binds to JAK2 via the SH2 domain it contains (Banks et al., 2000; Blenis, 1993). ERK also appears to act as a mediator in the leptin-stimulated gene expression of the immediate early response gene, c-fos (Banks et al., 2000; Yamashita et al., 1998).

Recently, research has identified another factor influenced by, and influencing, OB-Rb signaling. The newly identified family of cytokine-inducible inhibitors of signaling include small proteins referred to as SOCS (suppressor of cytokine signaling) that contain central SH2 domains that are thought to be able to bind to phosphorylated tyrosine residues on JAK2s (Bjorbaek et al., 1999). Researchers have demonstrated that leptin can specifically induce SOCS-3 mRNA expression in the same regions of the hypothalamus that contain OB-Rb (Bjorbaek et al., 1999). However, these same researchers have also shown that forced expression of SOCS-3 inhibits leptin-induced tyrosine phosphorylation of JAK2 by binding to phosphorylated Tyr^{985}, and that JAK2 and SOCS-3 co-immunoprecipitate in lysates from leptin-treated COS cells (Bjorbaek et al., 1999). As well, the researchers showed that phosphorylated ^{Tyr985} bound to SHP-2 and mediated both the activation of ERK and inhibition of OB-Rb-induced STAT3 activation (Bjorbaek et al., 1999). Based on their findings, it was concluded that SOCS-3 expression is leptin-induced, that SOCS-3 is a negative regulator of proximal leptin signaling, and that SOCS-3 binds to JAK2 in a manner that is leptin-dependent, thereby inhibiting OB-Rb signaling by inhibiting JAK-induced tyrosine phosphorylation of OB-Rb (Bjorbaek et al., 1999).

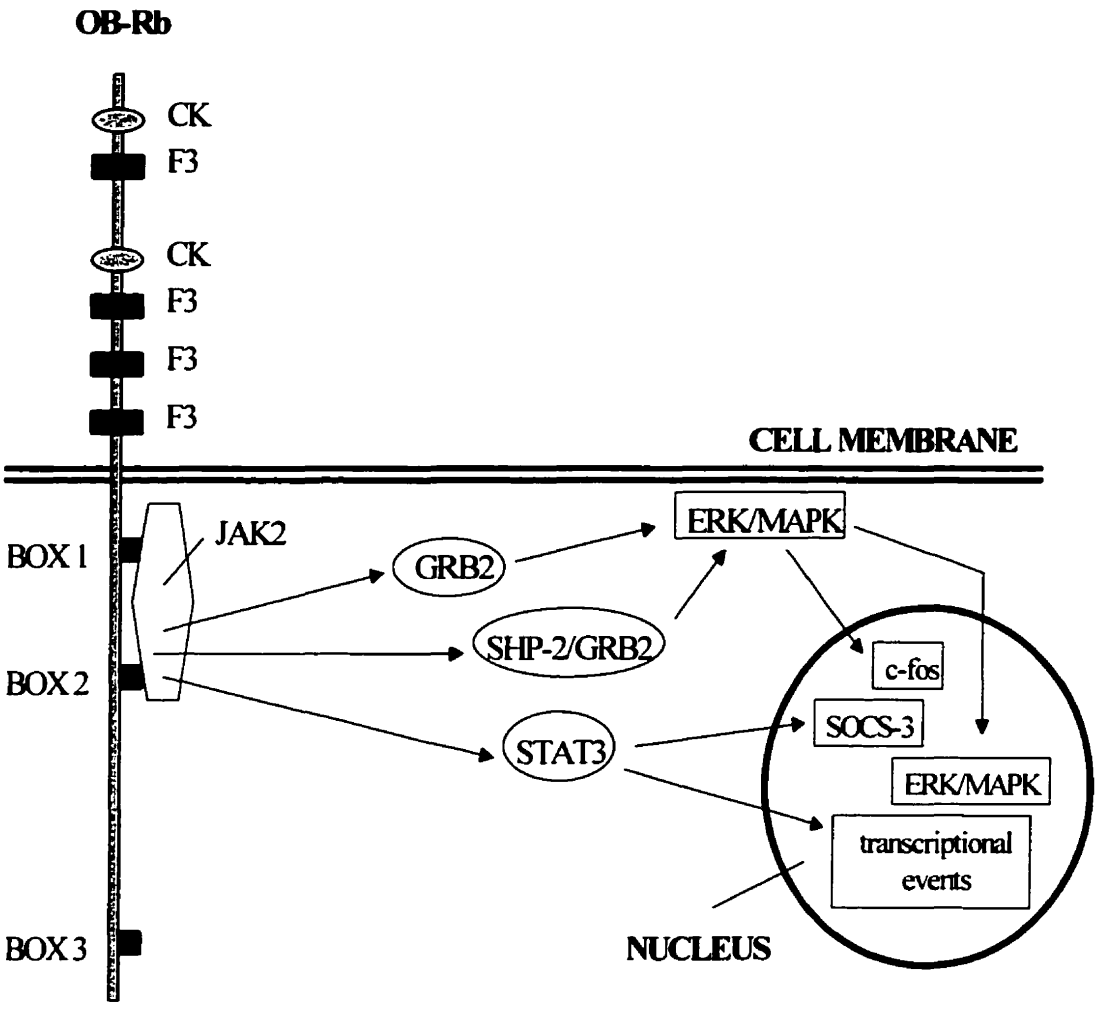

Figure 2.2. Signaling by OB-Rb via the JAK/STAT pathway in the hypothalamus.

2.3.3. OB-Rb Function, Neuropeptides, and SNS Activity

The effects of leptin on food intake and energy expenditure may not only occur via direct interaction with its hypothalamic receptor. Recent studies have shown that a number of neurons also present in the hypothalamus are targets of brain leptin, including NPY (neuropeptide Y), POMC (proopiomelanocortin), and CART (cocaine- and amphetamine-regulated transcript) (Kristensen et al., 1998; Schwartz et al., 1998; Schwartz et al., 1996a; Thornton et al., 1997). Additionally, leptin treatment has recently been associated with decreased levels of MCH (melanin-concentrating hormone) and AGRP (agouti-related protein) transcripts in the hypothalamus (Ebihara et al., 1999; Huang et al., 1999). Interestingly, all of these neurons are present in the arcuate nucleus where OB-Rb is most abundant. Moreover, many researchers believe that these neuropeptides are downstream factors involved in carrying the message induced by leptin signaling via OB-Rb. NPY is known to be one of the most potent stimulators of energy intake (Fei et al., 1997; Stanley et al., 1992). POMC encodes the precursor for α-MSH, or α-melanin stimulating hormone (Fan et al., 1997). Intracerebral injections of α-MSH inhibit food intake in both a time- and dose-dependent manner (Fan et al., 1997). Activation of CART neurons has been shown to reduce feeding and results in loss of body weight in rats (Kristensen et al., 1998; Koylu et al., 1997; Lambert et al., 1998). Leptin has been shown not only to co-localize with these neurons in the hypothalamus but also to actually regulate the expression of NPY, POMC, and CART (Kristensen et al., 1998; Mobbs and Mizuno, 2000; Schwartz et al., 1996b; Widdowson and Wilding, 2000).

The roles of leptin and OB-Rb in the sympathetic nervous system (SNS) are important aspects of the control of food intake and body composition that cannot be ignored. The SNS has long been implicated as forming part of a feedback mechanism regulating satiety and body composition based on the fact that neuropeptides and hormones, including leptin, NPY, POMC, and CART, known to regulate body weight are all located in the SNS (Bray, 1991; Collins et al., 1996; Vrang et al., 2000). Leptin, CART, and α-MSH have been shown to increase SNS activity, whereas NPY and MC4 have the opposing effect (Bray, 1991; Collins et al., 1996; Haynes et al., 1999; Smith et al., 1996; Vrang et al., 2000). While the effects of chronic administration of NPY and

MC4 have been studied in detail, the exact effect of chronic central or peripheral leptin administration on SNS activity is a new, ongoing area of study. Researchers have demonstrated that any interference in leptin signaling results in reduced sympathetic outflow and increased parasympathetic activity (Haynes et al., 1997). When examined as a whole, OB-Rb's function in the hypothalamus and interaction with other nearby neuropeptides represents an important aspect in the overall SNS control of food intake, body fat accumulation, and energy expenditure.

2.4. FACTORS INVOLVED IN THE FUNCTION OF THE OB-Rb/LEPTIN SYSTEM

Any defect in factors involved in OB-Rb signaling or any interference in the signaling pathway could ultimately result in obesity and, eventually, leptin resistance. Since identification of both leptin and its receptor, genetic mutations resulting in decreased leptin production or reduced OB-Rb activity have been identified in both animals and humans, ultimately leading to obesity. Other than defects at the leptin gene or leptin receptor level, there are a number of other possible causes of decreased leptin action or leptin resistance, such as reduced transport across the blood brain barrier or OB-Rb-down-regulation of leptin gene expression in the adipose tissue (Burgeura et al., 2000; Zhang et al., 2001; Zhang et al., 1997). The present discussion will focus on events occurring at the receptor level and the role that dietary fat may play in the course and outcome of these events. To our knowledge, there have been no studies directly examining the effect of dietary fat on OB-Rb function to date. Recent research provides us with information that can allow us to speculate on mechanisms by which dietary fat could affect OB-Rb function at the cellular level in the hypothalamus. Such mechanisms include up- or down-regulation of OB-Rb gene expression, changes in hypothalamic membrane morphology, or any interference in the pathways underlying OB-Rb signaling.

2.4.1. Dietary Fat, OB-Rb, Neuropeptides, and SNS Activity

In both animals and humans, a chronic high fat diet without a compensatory increase in energy expenditure leads to the progressive development of obesity,

hyperleptinemia, and leptin resistance. It is now well known that diet-induced obese rodents exhibit up-regulation of leptin mRNA expression (Huang et al., 1997). Many researchers have speculated that the insensitivity of obese rodents to exogenous leptin administration may be due to down-regulation of transporter or of OB-Rb gene expression (Baskin et al., 1999b; Lin et al., 2000b; Widdowson et al., 1997). Recently, researchers proved that obese mice have significantly increased levels of leptin receptor mRNA in both the arcuate nucleus (66%) and choroid plexus (98%) after 8 weeks of high-fat feeding (59% of total energy) as compared to low-fat (10% of total energy) fed mice (Lin et al., 2000a). This finding was consistent with those of other investigators who had already shown up-regulation of OB-Ra in the blood-brain-barrier in rats fed high fat diets (Boado et al., 1998). In the same study by Lin et al. (2000a), OB-Rb mRNA levels were 26 and 33% lower in the arcuate nucleus and choroid plexus, respectively, after 19 weeks of high-fat feeding. These results confirmed earlier findings by the same group (Chang et al., 1999; Huang et al., 1997; Lin et al., 2000b). Thus, the researchers concluded that with shorter-term high-fat feeding, increased OB-Rb expression might play a role in preventing obesity. However, with long-term high-fat feeding, this system is overwhelmed and desensitized, and reduced OB-Rb expression leads to a breakdown of body weight homeostasis (Lin et al., 2000a).

Dietary fat has also been shown to affect the expression of NPY, POMC, and CART mRNA (Figure 2.3). Leptin has been shown to down-regulate NPY mRNA levels in rats (Wang et al., 1997). After 8 weeks of high-fat feeding, Lin et al. showed that NPY mRNA levels were 45% lower in the arcuate nucleus as compared to low-fat fed mice (Lin et al., 2000a), consistent with their earlier findings (Lin et al., 2000b). After 19 weeks of high-fat feeding, NPY mRNA levels were 32% lower than those in mice fed the low fat diet (Lin et al., 2000a), again consistent with the group's earlier findings (Lin et al., 2000b). In the same study, Lin et al. demonstrated that at 8 weeks of high-fat feeding, POMC mRNA levels were no different between the high- and low-fat fed groups. However, after 19 weeks of feeding, mRNA expression in the arcuate nucleus was 55% lower in the high-fat fed group (Lin et al., 2000a). Possibly, the effects of high-fat feeding on NPY and POMC mRNA levels are largely the results of the effects of altered OB-Rb expression, as influenced by dietary fat, on these neuropeptides. Given

that POMC and NPY are both downstream of OB-Rb signaling and are important in energy balance regulation, it has been speculated that with chronic high-fat feeding, first peripheral, then central leptin resistance develops, accompanied by obesity, hyperphagia, and decreased energy expenditure. Associated with this leptin resistance are declines in NPY mRNA levels and failure of leptin to stimulate the POMC system via OB-Rb (Lin et al., 2000a).

The precise effects of dietary fat on CART, MCH, and AGRP remain inconclusive, although research is active in these areas. One recent study found that high-fat feeding had no effect on levels of CART in the rat vagus nerve and nodose ganglion (Broberger et al., 1999). Others have found that CART mRNA levels are low at times when leptin levels are low, such as during starvation (Schwartz et al., 2000a). Research is currently scarce on the effects of diet on MCH and AGRP, so the role of dietary fat in the function of these peptides is not well understood. However, taken as a whole, leptin's interactions with OB-Rb and other neuropeptides in the hypothalamus may explain the apparent reciprocal relationship between SNS activity and food intake.

2.4.2. Dietary Fat and Cell Membranes

As a transmembrane receptor, the hypothalamic cell membrane can potentially play an important role in OB-Rb's function. All cell membranes contain a lipid bilayer composed of mostly polar lipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), cardiolipin, and sphingomyelin, which may be in alkyl-acyl or glycosylated forms (Clandinin et al., 1994). In addition, there are varying levels of fatty acids among different membrane types. Specific to the discussion of OB-Rb, brain membrane phospholipids contain large amounts of long chain, polyunsaturated fatty acids (Shimizu et al., 1997). It is now a well-known fact that dietary fat can influence the composition of cellular membrane phospholipids. New dietary fatty acids can be incorporated into membrane lipids through de novo membrane phospholipid synthesis and acyl group turnover in membrane phospholipids, and changes in membrane fatty acid composition will generally reflect the dietary fatty acid profile (Clandinin et al., 1994).

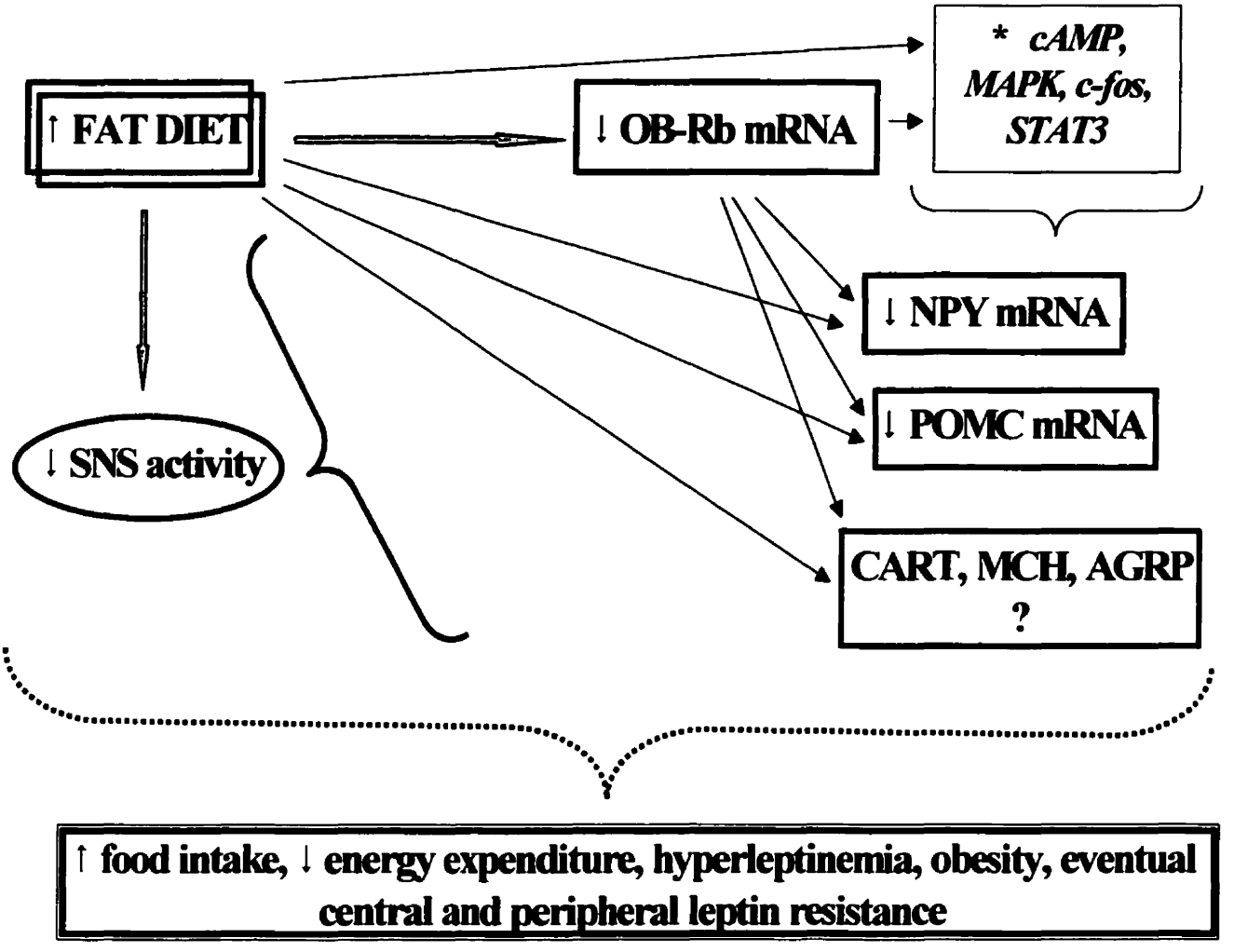

Figure 2.3. Effects of chronic high dietary fat intake on energy homeostasis

Innis and Clandinin were the first to demonstrate in the whole animal that subcellular membrane lipids rapidly change according to changes in dietary fat composition when animals are fed diets adequate in all nutrients (Innis and Clandinin, 1981). To investigate the effects of diet lipid on the incorporation of fatty-acyl chains into PC, PE, and cardiolipin in cardiac mitochondrial inner-membranes, rats were fed polyunsaturated-rich diets (soybean oil) for 12 days, then crossed over to monounsaturated-rich diets (rapeseed, or canola, oil) for 11 days, then returned to soybean oil for 11 more days. Additional rats were fed either soybean or rapeseed oil only throughout the study. In rats participating in the crossover, changing from soybean to rapeseed oil caused the fatty acid composition of PC, PE, and cardiolipin to resemble that of rats fed rapeseed only. These changes were reversed when rats were crossed back to soybean oil. Thus, membrane phospholipid fatty acyl chains respond rapidly to changes in dietary fatty acid balance.

The effects of diet fat on brain membrane fatty acid composition have also been demonstrated by feeding rats diets in which both the lipid content and polyunsaturated to saturated fatty acid ratio varied for 20 weeks (Gibson et al., 1984). The fat content of the diets was either 4% or 16%. The proportion of saturated fatty acids in membrane phospholipids was found to be only marginally affected by changes in dietary fat composition. However, it was found that n-6 to n-3 ratios of unsaturated fatty acids were higher in rats fed the unsaturated fat diets, and lower in rats fed saturated fat, even though total amounts of unsaturated fatty acids in the membranes did not change. Based on these results, it was concluded that, although a certain amount of homeostatic control over membrane fatty acid composition occurred, dietary fats could alter brain membrane phospholipid composition. Furthermore, as was suggested by Innis and Clandinin (1981), the extent or lack of homeostatic control over membrane fatty acid composition may have important consequences in terms of modulation of various membrane-receptor-enzyme activities.

As a transmembrane receptor, OB-Rb's immediate surrounding environment is the cell membrane, specifically the hypothalamic cell membrane. The effects of dietary fatty acid composition on the fatty acid profile of the membrane may be the most fundamental mechanism by which diet fat affects the function of leptin via its active

receptor. Transitions in dietary fat intake can alter any hormone-receptor-mediated mechanism in vivo by changing the surrounding lipid environment. As a receptor anchored within the hypothalamic cell membrane, it is highly conceivable that, like any other receptor complex, the function of OB-Rb is affected by changes in hypothalamic membrane composition.

Changing the fatty acid profile of the membrane can have major consequences on both the function of the membrane as well as that of proteins embedded in the membrane, such as receptors. The primary mechanism by which dietary fatty acids can have these effects is by altering membrane fluidity. The fluidity of the membrane bilayer at any given temperature depends on the extent of fatty acylation, the presence of cholesterol, and by the nature of the phospholipids, specifically the polar head group (Kinsella, 1990). The greater the degree of fatty acylation and unsaturation, the greater the membrane fluidity. As well, while cholesterol normally promotes rigidity in membranes, it can also increase fluidity by introducing spaces in the bilayer, due to its short length in comparison to the acyl chains of the phospholipids. The large amount of polyunsaturated fatty acids in brain membranes likely allows for both lateral mobility and rotational diffusion of inner or trans-membrane proteins and lipids, such as the leptin receptor (Kinsella, 1990).

Increased incorporation of certain fatty acids into the membrane has been shown to alter membrane morphology. For example, PE is capable of forming the hexagonal (II) phase, a particular arrangement of membrane lipids (Yeagle, 1989). Diacylglycerol has the ability to dramatically decrease the temperature of the hexagonal (II) phase transition and is therefore capable of disrupting the lipid bilayer of membranes in which it exists (Yeagle, 1989).

Whether diet induced changes in the brain membranes affect leptin receptor activity has not been studied in any great detail. However, the effect that membrane fluidity may have on previously mentioned factors strongly suggests that fluidity does influence OB-Rb activity. For example, the activities of a variety of protein kinases are very sensitive to membrane fluidity. Based on their studies on the effects of diet fat on protein kinase activities of rat liver membranes, Gavrilova and colleagues (Gavrilova et al., 1993) suggested that a more fluid lipid environment favoured higher plasma

membrane-bound protein kinase activities. Specifically, they speculated that phosphatidylglycerol and dieoylglycerophosphocholine significantly increased tyrosine kinase activity by increasing membrane fluidity to a greater extent than did PS and PE. Dipalmitoylglycerophosphocholine and sphingomyelin reduced tyrosine kinase activity.

The ability of dietary fat, primarily polyunsaturated fat, to change membrane morphology and fluidity and influence the function of membrane-related enzyme-receptor complexes leads to the suggestion that action of the OB-Rb-leptin complex may be directly controlled by diet. However, there is the possibility that the mechanism by which dietary fat influences leptin action is indirect. Dietary fat has been shown to affect a number of factors involved in OB-Rb binding and signaling, such as cAMP, MAPK, c-fos, and STAT3 (Cha and Jones, 1998; El-Haschimi et al., 2000; Tappia et al., 1997; Wang et al., 1999a). By changing the level of activity of any one of these factors, dietary fat has the potential to influence leptin action without changing hypothalamic membrane morphology itself.

2.4.3. Dietary Fat and Factors Involved in OB-Rb Binding and Signaling

To date, there has been very little study of the effects of dietary fat specifically on OB-Rb binding and function. Cha and Jones (1998) examined the effects of dietary fat on plasma leptin levels. In rats fed diets based on either beef tallow, safflower oil, or fish oil (20% wt/wt) for 10 weeks. Upon conclusion of the diet treatment, plasma leptin levels were measured. Rats fed the safflower oil diet had the highest leptin levels, while those fed beef tallow had the lowest. Based on their results, it was concluded that polyunsaturated fats had a strong hyperleptinemic effect. Although the researchers did not evaluate leptin binding or OB-Rb function, their results suggest that dietary fat, polyunsaturates in particular, does play a role in the leptin/OB-Rb system, either by increasing leptin production or decreasing leptin uptake in the hypothalamus. It is conceivable that fats in the rat diets altered hypothalamic membrane morphology, thus affecting the ability of OB-Rb to bind leptin or initiate signal transduction, and, certainly, our group plans to continue examining this hypothesis.

While the direct effects of dietary fat on OB-Rb function remains to be elucidated, there are a number of other ways in which dietary fatty acid composition may affect the

action of leptin via its receptor. One possibility is related to the characterization of the leptin receptor itself. Dietary fat has been demonstrated to affect a number of other cytokine/receptor systems that are related to leptin/OB-Rb, such as IL-6 and its receptor. Tappia et al. (1997) studied the relationship between IL-6 production in macrophages of rats fed diets varying in unsaturated fatty acid compositions. After feeding rats diets with corn, fish, or olive oil for 8 weeks, IL-6 production was found to relate in a curvilinear fashion to the total diacyl species with 20:4(n-6) and 18:2(n-6) at the sn-2 position. Moreover, the total diacyl species with 20:4(n-6) and 18:2(n-6) at this position related in a curvilinear fashion to total 18:2(n-6) intake. Thus, IL-6 production was dependent on the amount of unsaturated fatty acid intake. Researchers (Tappia et al., 1997) then speculated that diet fat-induced alterations in the cytokine biology occurred, affecting its ability to bind to its receptor. Similarly, Meydani et al. (1996) found that, in both animals and humans, long-chain (n-3) polyunsaturated fatty acids decreased cytokine production and their biological function. Given the degree of similarity between the IL-6/IL-6 receptor and leptin/OB-Rb systems (Sevetson et al., 1993), it might be expected that dietary fat would influence leptin binding in a similar fashion.

Dietary fatty acid composition has also been shown to influence cAMP production, and this may have effects on leptin receptor mRNA expression. cAMP interrupts cytokine-induced JAK/STAT signals. Specifically, cAMP has been demonstrated to inhibit STAT3 tyrosine phosphorylation (Sengupta et al., 1996), thus preventing the rapid transcriptional activation of genes by STAT. cAMP has also been shown to decrease activation of MAPK, likely in a counter-regulatory mechanism mediated by β-adrenergic antagonists (Shimizu et al., 1997; Meydani, 1996). cAMP production in peritoneal macrophage plasma membranes was highest in rats fed diets rich in corn or olive oils as opposed to fish oil for 4 weeks, but these differences changed considerably with continued dietary treatment (Tappia et al., 1997). After 8 weeks, cAMP production was greatest in rats fed corn and coconut oil diets, moderate in those fed fish oil diets, and lowest in rats receiving diets with olive oil or just rat chow (Cha and Jones, 1998). Corn and fish oils contain large amounts of different n-3 fatty acids. Thus, it was speculated that after long-term feeding, diets containing fats rich in n-3 fatty acids increased cAMP production.

Even stronger evidence that dietary fat affects leptin signaling has been demonstrated through studies examining the effects of high-fat feeding on c-fos-like immunoreactivity and leptin-induced STAT3 activation in rodent hypothalami. To our knowledge, no study has ever examined the effects of dietary fat on ERK expression. However, after feeding mice diets either high in fat (58% of total calories, supplied as either beef tallow, safflower oil, or fish oil) or low in fat (10% of total calories), Wang et al. (1999a) quantified c-fos like immunoreactive neurons in different parts of the mouse hypothalamus. The researchers found that neurons in the dorsal part of the hypothalamus were dramatically increased (367% after 1 week) but that ventromedial hypothalamic activity was decreased by saturated fat feeding as compared to low fat feeding (Wang et al., 1999a). Consistent with the findings of our lab group (Cha and Jones, 1998), weight gain and different fat pad weights were highest in the saturated fat-fed group, and lowest in the animals fed high n-3 diets (Wang et al., 1999a). Paraventricular hypothalamic activity only increased after substantial high fat feeding (7 to 11 weeks) (Wang et al., 1999a). Substitution of the saturated fat diets with n-3 diets partly reversed the increase in neurons in the paraventricular nucleus, but significantly increased c-fos-like immunoreactive neurons in the arcuate nucleus (400%) (Wang et al., 1999a). This finding is particularly important, as it may speak to the possible direct effects of fatty acids on arcuate nucleus OB-Rb activation and downstream signaling (Wang et al., 1999a). These results led to the conclusion that dietary saturated fat increased lateral hypothalamic neuronal activity and decreased ventromedial activity, and that this pattern was consistent with the obesogenic effects of saturated fat (Wang et al., 1999a). Furthermore, the researchers demonstrated that diets equally high in polyunsaturated fats (particularly n-3 fatty acids), were neither obesogenic nor altered lateral/medial hypothalamic neuronal activity balance (Wang et al., 1999a). These findings all add more weight to idea that leptin and OB-Rb, in their control over neuropeptides key to energy homeostasis, are central to the "dual-center hypothesis" – that the lateral hypothalamus acts as a feeding and parasympathetic center opposing the ventromedial hypothalamus, which acts as a satiety and SNS center (Bray, 1984; Bray, 1981).

In order to study the molecular basis for leptin resistance, El-Haschimi and colleagues (2000) examined the ability of leptin to activate hypothalamic STAT3

signaling in obesity-prone mice fed either low- or high-fat diets. The researchers found that prolonged high-fat feeding for 15 weeks induced profound resistance to peripherally administered leptin, as evidenced by the complete inability of leptin to activate hypothalamic STAT3 signaling (El-Haschimi et al., 2000). The same dose of leptin remained capable of inducing STAT3 signaling in the low-fat-fed mice. Based on these findings, the researchers tested the hypothesis that the absence of leptin signaling was due to a defect in access of peripheral leptin to hypothalamic sites of action. After administering leptin intracerebroventricularly in both groups of mice, El-Haschimi et al. (2000) found that, even at a much lower dose, the leptin was able to induce STAT3 activation in the high-fat-fed mice, although the extent of activation was 75% lower than in the low-fat-fed mice. As such, the researchers concluded that leptin resistance caused by chronic high fat feeding was caused by an apparent defect in access to hypothalamic sites of leptin action (i.e. OB-Rb or neurons with OB-Rb-like immunoreactivity) that reduced STAT3 activation, or an intracellular defect in neurons with OB-Rb-like immunoreactivity lying upstream of STAT3 activation (El-Haschimi et al., 2000).

2.5. CONCLUSION

The possibility that the biological actions of leptin can be controlled via its hypothalamic receptor has important implications for the control of obesity and obesity-related disorders. Presently, the mechanism by which the active form of the leptin receptor, OB-Rb, regulates the biological action of leptin remains, for the most part, unknown. What is known is that chronic intake of high fat diets without a compensatory increase in energy expenditure results in the obesogenic syndrome and, most often, leptin resistance. There are a number of potential causes of leptin resistance, such as decreases in leptin production, decreased transport via the blood brain barrier to hypothalamic sites of leptin action, or defects in leptin binding or signaling. Leptin action can occur not only through its own receptor, OB-Rb, but also through other neurons containing OB-Rb-like immunoreactivity, such as NPY, POMC, or CART neurons. Research has demonstrated that dietary fat can alter different factors involved in leptin binding and signaling. Dietary fat can up- or down-regulate OB-Rb, as well as the neuropeptides that are regulated by leptin and are possibly involved in OB-Rb signaling. Furthermore, dietary fat has been shown to have a reciprocal effect on sympathetic nervous system activity. Diet-induced alterations in the fatty acid content of brain membrane phospholipids, and thus membrane fluidity, can affect the action of membrane-receptor complexes. Dietary fat can also play a major role in the expression or action of other factors critical to OB-Rb function, such as cAMP, MAPK, c-fos, or STAT3. Indeed, certain researchers agree that a pattern is emerging linking the development of obesity with dietary fat, decreased sympathetic nervous system activity, altered membrane fluidity, and dysregulation of hypothalamic neuropeptide balance (Wang et al., 1999a).

Brain lipids are characterized by a high degree of unsaturation. Furthermore, research demonstrates a strong, direct association between highly unsaturated diets and the n-6 to n-3 polyunsaturated fatty acids ratio in brain membranes. The evidence presented in this review strongly advocates the benefits of both polyunsaturated fatty acids, particularly those of the n-3 family, and low-fat diets in promoting the anti-obesogenic actions of leptin and preventing leptin resistance. Dietary strategies aimed at

alterations in both the amount and type of fat consumed may present an important treatment for obesity and obesity-related disorders.

BRIDGE

The previous review paper provided strong evidence that, although the exact mechanism of leptin action remains to be elucidated, certain biochemical mechanisms, such as activation of the JAK/STAT pathway, STAT3 and c-fos activation, and NPY, POMC, and CART gene expression are clearly influenced by leptin. Furthermore, these mechanisms are not only involved in transmission of the leptin signal, but are influenced by dietary fat type and amount. Given leptin's interactions with its own hypothalamic receptor (OB-Rb) as well as with other neuropeptides located in the hypothalamus, it is generally believed that leptin and its receptor are central to the control of body weight maintenance via the hypothalamus.

Study Rationale

The rat is an animal model commonly used to study both human obesity and leptin metabolism. Perhaps the greatest advantage of using the rat in studies of leptin and obesity is the finding that rat OB-R mRNA is 83% identical to human OB-R mRNA (Masuzaki et al., 1995). In addition to this strong resemblance, rats are known to have similar relative rates of metabolism, development, brain/body ratios, and fatty acid composition of their neural structures as compared to humans. The larger brain size in the rat allows for easier examination of small brain structures, such as the hypothalamus. Additionally, previous work with similar diets and study design used rats as subjects; thus, choosing the same species of rat as used before allows for comparison of results from the proposed study with recently published research.

It is now well known that a number of membrane receptors are influenced by membrane fatty acid composition. While it was originally thought that the fatty acid composition of adult brain tissue remained constant, we now know that this is not the case. Brain fatty acid composition is subject to changes dependent on the fatty acid profile of the diet. Thus, as a transmembrane receptor, the functioning capabilities OB-Rb, like those of many other receptors, may be affected by changes in its membrane environment. Any interference in OB-Rb's ability to bind leptin, or in any secondary mechanisms involved in leptin receptor signal transduction, results in increased body

weight and decreased energy expenditure. Conversely, augmenting leptin binding or OB-Rb activity should lead to a reversal of the obese state.

Dietary fat type and level of energy restriction have already been demonstrated to influence circulating leptin levels. Particularly, saturates have been shown to be hyperleptinemic, while energy restriction to 70% has resulted in lower plasma leptin levels. Beef tallow has long been known to decrease SNS activity in the hypothalamus, while the high level of n-3 fatty acids present in fish oil have been associated with increased membrane fluidity.

Study Objectives

Based on the above considerations, the overall hypothesis of the following study was that dietary-induced changes in membrane morphology would affect leptin binding and OB-Rb signal transduction. As such, the objectives of the present project were to examine, in rats, the interactive effects of dietary fatty acid composition and level of energy intake on: (i) changes in hypothalamic fatty acid composition, (ii) hypothalamic leptin receptor binding, (iii) circulating leptin levels, and (iv) body weight homeostasis. Results from the study have the potential to more clearly define the mechanism by which dietary fat and energy intake level affect leptin's actions to control energy balance at the hypothalamic level.

CHAPTER III. MANUSCRIPT II.

Interactive Effects of Diet Fat Type and Energy Balance on Hypothalamic Fatty Acid Profile and the Hypothalamic Leptin Receptor*

Jode T. Heshka, Geoffrey R. Hynes, Louise Thibault, and Peter J.H. Jones*

School of Dietetics and Human Nutrition
McGill University, Ste Anne de Bellevue, Quebec, Canada

Running Title: Diet Fat, Energy, and Brain Leptin Receptor Function

Key Words: leptin, leptin receptor, hypothalamus, membrane, energy restriction, fatty acid, tallow, safflower oil, menhaden oil, rat

*Corresponding Author:

Dr. P. J. H. Jones
School of Dietetics and Human Nutrition
Macdonald Campus of McGill University
21,111 Lakeshore Road
Ste Anne de Bellevue, QC, Canada H9X 3V9

tel: 514 398-7547
fax: 514 398-7739
email: jonesp@macdonald.mcgill.ca

* Revised version of the manuscript submitted to *Lipids*, July, 2001.

* *To whom correspondence should be addressed*:

Dr. P. J. H. Jones
School of Dietetics and Human Nutrition
Macdonald Campus of McGill University
21,111 Lakeshore Road
Ste Anne de Bellevue, QC, Canada H9X 3V9

tel: 514 398-7547
fax: 514 398-7739
email: jonesp@macdonald.mcgill.ca

Abbreviations:

Ad Libitum, A
Beef Tallow, BT
Blood-Brain Barrier, BBB
Counts Per Minute, CPM
Janus Kinase, JAK
Menhaden (Fish) Oil, FO
Neuropeptide Y, NPY
Pro-opiomelanocortic, POMC
Restricted, R
Safflower Oil, SO
Signal Transducers and Activators of Transcription, STAT
Suppressor of Cytokine Signaling-3, SOCS-3

3.1. ABSTRACT

To examine effects of dietary fat type and energy balance on hypothalamic membrane fat accretion, circulating leptin levels, and leptin receptor function, adult rats were fed diets containing beef tallow, safflower oil, or menhaden oil (20% wt/wt) for 10 wks. *ad libitum* or at 60% of *ad libitum* intakes. Restriction was accomplished by reducing dietary carbohydrate. After 10 wks, restriction lowered weight gain and food intakes ($p<0.0001$ for both). Energy restriction lowered hypothalamic phospholipid fatty acid levels of 18:3(n-3) and increased levels of 20:1(n-9), 20:4(n-6), and 22:4(n-6) ($p<0.05$, $p<0.02$, $p<0.05$, and $p<0.04$, respectively). Interactions between fat type and energy level were observed for hypothalamic levels of both of 20:4(n-6), 22:5(n-3), and 22:6(n-3) ($p<0.05$, $p<0.006$, and $p<0.05$, respectively). Restriction lowered circulating leptin levels ($p<0.0001$); overall plasma leptin levels were marginally associated ($p<0.07$) with hypothalamic 16:0 concentrations. In hypothalamic membrane homogenates, leptin levels were unaffected by diet or energy intake, but were associated with both 18:3(n-3) and 22:5(n-6) levels ($p<0.0003$ and $p<0.001$, respectively). Hypothalamic homogenates were devoid of any measureable specific leptin binding, however, levels of tracer greatly exceeding the assay reference value (B_0) were found in homogenates, suggesting intra-membrane binding. Excess tracer levels were marginally associated ($p<0.03$) with the sum of monounsaturates. These results suggest that membrane fluidity may be a determinant of leptin resistance, and demonstrate changes in hypothalamic membrane fatty acid composition due to diet fat type and energy intake levels. The results also support the lack of a dietary fat effect on leptin levels with energy restriction.

3.2. INTRODUCTION

Leptin is the 16 kDa protein product of the obese (*ob*) gene (Zhang et al., 1994). Leptin is produced and secreted primarily by the white adipocytes, released into circulation, and transported across the blood-brain-barrier to the hypothalamus. Leptin has been shown to reduce food intake, increase energy expenditure, and promote weight loss in mammals (Campfield et al., 1995; Pelleymounter et al., 1995). Leptin exerts these peripheral effects through the sympathetic nervous system via receptors located in the hypothalamus. The leptin receptor is a member of the class I cytokine superfamily (Tartaglia et al., 1995). To date, a total of six different leptin receptors (OB-R) have been identified, termed a-f (Lee et al., 1996). Expression and structure of these isoforms, particularly those of the short (OB-Ra) and long (OB-Rb) forms, have been extensively studied. In rats, ultrastructural localization studies have shown OB-Rb mRNA to be predominantly located in the hypothalamus, a region of the sympathetic nervous system known to be extensively involved in body weight regulation. In particular, OB-Rb has been detected in the arcuate, ventromedial, paraventricular, dorsomedial, and ventral premammillary nuclei, as well as within the lateral hypothalamic area (Fei et al., 1993; Mercer et al., 1996). OB-Rb is considered to be the fully functional form of the receptor. OB-Rb is a transmembrane receptor with an extracellular domain involved in leptin binding and a long intracytoplasmic domain capable of activating the Janus kinase (JAK)/Signal Transducers and Activators of Transcription (STAT) pathways of signal transduction (Baumann et al., 1993; Ghilardi et al., 1997; Tartaglia et al., 1995).

While much is known about the structure and localization of OB-Rb, the precise mechanism by which the receptor regulates the body composition remains to be elucidated. Previous work has identified that both dietary fat type and energy restriction have individual and interactive effects on weight gain and circulating leptin levels, consequently influencing leptin resistance. Energy restriction has been shown to decrease both circulating leptin levels and hypothalamic OB-Rb mRNA (Baskin et al., 1998; Cha and Jones, 1998; Lin and Huang, 1997; Mizuno et al., 1998; Saladin et al., 1995). Both the type and amount of dietary fat have been shown to affect circulating leptin levels, particularly large amounts of saturated fats, which tend to increase plasma

leptin levels (Cha and Jones, 1998; Hun et al., 1999; Raclot et al., 1997). Dietary fat amount also influences factors involved in OB-Rb binding and signaling, such as those found in the JAK/STAT pathway (El-Haschimi et al., 2000; Sengupta et al., 1996; Wang et al., 1999a).

While the fatty acid composition of the brain was once thought to be static, it is now well known that dietary fat composition alters membrane fatty acid composition through de novo membrane phospholipid synthesis and acyl group turnover in membrane phospholipids (Clandinin et al., 1994). Altering the fatty acid make-up of membranes, such as the unsaturated:saturated fat ratio (USFA/SFA), changes membrane morphology and fluidity and could lead to an enhancement or impairment of the structure or function of any membrane-associated complexes (Innis and Clandinin, 1981; Kinsella, 1990). As other researchers have noted (Wang et al., 1999a), the abundance of work on obesity and leptin levels in both animals and humans taken together suggests that obesity is likely caused by a combination of dietary fat type and amount, changes in membrane fluidity, altered expression of hypothalamic neuropeptides, and decreased SNS activity (Bray, 1993; Cha and Jones, 1998; Davidowa and Plagemann, 2000; El Haschimi et al., 2000; Lin et al., 2001; Matsuo et al., 1995; Shiraishi et al., 1999; Watson et al., 2000). However, whether these effects occur at the level of production (adipose tissue) or the level of uptake (hypothalamus), or both, has not been established. The present study addresses the possibility that the effects of dietary fat and energy restriction on weight gain and plasma leptin levels, with subsequent leptin resistance and obesity, are a result of dysregulation of the hypothalamic leptin/OB-Rb system due to changes in the hypothalamic membrane environment and OB-Rb binding affinity. As such, the primary aim of the present work was to examine effects of diets high in saturated, polyunsaturated, or n-3 fatty acids, combined with energy restriction, on leptin binding affinity in the hypothalamus. The secondary aim of the study was to examine the effects of dietary fat and energy restriction on hypothalamic membrane fatty acid composition, circulating leptin levels, and body weight control in rats.

3.3. EXPERIMENTAL PROCEDURES

3.3.1. Study Design, Animals and Diets

Seventy-two male Sprague-Dawley rats (290±12.7g) were purchased from Charles River, Inc. (Montréal, QC) and were housed individually in stainless steel hanging cages in a temperature-controlled environment with a 12h light-dark cycle. After habituation to a commercial rat chow for 10d, rats were stratified according to weight, and then were systematically randomized to one of six dietary fat treatment groups. A completely randomized, 2X3 factorial design was assembled to assess the effects of dietary fat and energy restriction on food intake, body weight, plasma leptin levels, and hypothalamic fatty acid composition. Diets contained either beef tallow (BT), safflower oil (SO), or menhaden (fish) oil (FO) as the fat source (Table 1). The beef tallow diet was supplemented with 1% safflower oil to maintain an adequate intake of 18:2(n-6). The FO diet contained a comparable amount of 18:2(n-6) as the BT diet as well as a total n-3 to n-6 fatty acid ratio of approximately 6.9:1.0, a ratio considered optimal for prevention of essential fatty acid deficiency (Bourre et al., 1989). Animals within each dietary fat group were given either free access (A) to the diets or were restricted (R) to 60% of the *ad libitum* daily intakes. The *ad libitum* diets contained 15% casein and 55% carbohydrate with adequate amounts of vitamins and minerals. Fat comprised 20% of the *ad libitum* diets. Diets consumed by the restricted animals were adjusted to supply equal amounts of all macro- and micronutrients, including fat, with the exception of cornstarch, as compared to the *ad libitum* diets. Food intakes were recorded daily, and body weights were recorded weekly. Animals were monitored daily for overt signs of illness or essential fatty acid deficiency. Ethical approval for the study was obtained from the McGill University Animal Care Committee.

3.3.2. Tissue Preparation and Laboratory Methods

At the end of the 10wk feeding trial and after a 12h fast, rats were anaesthetized with carbon dioxide gas and killed with use of a guillotine. Blood was collected and immediately placed on ice. Plasma was derived from whole blood by centrifugation.

Whole brains were immediately removed. Brains were immediately flash-frozen whole in liquid nitrogen. Brains were stored at -80°C until further analyses.

3.3.2.1. Lipid Extraction and Preparation of Fatty Acid Methyl Esters

Hypothalami from 52 brains were dissected just prior to analyses. Hypothalami were divided in half and each half was placed individually in a vial. One half was flash frozen in liquid nitrogen and stored for further analyses. The other half was used for determination of fatty acid composition of hypothalamic phospholipids. A modified extraction method was used to extract total lipids from hypothalamic tissue samples (Folch et al., 1957). Total phospholipids were isolated using the method according to Juaneda and Rocquelin (1985), and fatty acids were then saponified and methylated using a modified protocol by Metcalfe and Schmitz (1961). Briefly, approximately 15 mg of hypothalamic tissue was placed in a culture tube along with $CHCl_3$/MeOH (2:1, by vol., containing 0.01% BHT) and heptadecanoate as an internal standard. Samples were then quickly homogenized, without heating, in a polytron homogenizer at 3000xg, then weighed. Saline solution was added and samples were vortexed and shaken in a wrist action shaker for 15 mins. Samples were then centrifuged at 1000xg for 10 mins. The supernatant was removed from all tubes, and samples were dried down under nitrogen.

To isolate the phospholipid fraction, samples were resuspended in a few drops of $CHCl_3$. SPE silica gel columns (Supelclean LC Si, 500 mg, 6 mL tubes, Supelco, Bellefonte, PA), preconditioned with $CHCl_3$, were used to separate phospholipids from neutral lipids. Samples were added directly onto the columns and allowed to drain through the column packing. Columns were washed with $CHCl_3$ to remove neutral lipids, which were then discarded. Phospholipids were eluted with MeOH, and dried down under nitrogen.

For subsequent saponification and methylation of the phospholipid samples, 0.5 M NaOH was added to all tubes. Tubes were then heated in a boiling water bath for about 3 mins. Methylating solution (MeOH:BF_3, 14 % solution, Sigma, Oakville, ON) was added to all tubes, which were then heated in a boiling water bath for 2 mins. Hexane was then added to the samples, and tubes were heated in a boiling water bath further for 1 min. The tubes were then cooled to room temperature and a few drops of a

saturated saline solution were added. Tubes were swirled gently, a few more drops of saline were added, and samples were gently mixed. The upper layer from each tube was then transferred to a crimp seal vial. Samples were dried down under nitrogen while in the vials, then resuspended in hexane.

3.3.2.2. Gas Chromatography

Fatty acid methyl ester composition of the hypothalamic phospholipid fractions was determined in duplicate using a Hewlett-Packard 5890 gas liquid chromatograph (GC). (Palo Alto, CA), equipped with a 30 m x 0.2 mm SP 2330 column (Supelco, Bellefonte, PA), flame ionization detectors, and an automated injection system. The injector and detector temperatures were set at 210°C and 250°C, respectively. Helium was used as the carrier gas. The oven temperature was held for 2 mins at 140°C, then increased to 160°C at a rate of 2°C per min. The temperature was held at 160°C for 10 mins, then increased to 220°C at a rate of 3°C per min. Fatty acid methyl esters were identified based on the retention time of known standards (Supelco, Bellefonte, PA).

3.3.2.3. Plasma Leptin Assay

Plasma leptin levels were measured using a Rat Leptin RIA Kit (Linco Research, Inc., St. Charles, MO). Samples were prepared in polystyrene tubes according to the manufacturer's protocol, using 100μL of sample plasma. Radioactivity remaining in the tubes was determined by gamma counting (LKB Wallac, 1282 compugamma CS, Fisher Scientific, Montréal, QC). The amounts of rat leptin (ng/mL) in unknown samples were calculated by independent treatment of raw data using the software package accompanying the gamma counter.

3.3.2.4. Leptin Receptor Binding Assays

Hypothalami were removed from brains as described for the fatty acid analysis. Hypothalamic membranes were prepared for binding assays by suspending approximately 15 mg of hypothalamic tissue in sonication buffer (75 mM Tris-HCl, pH 7.4, 25 mM $MgCl_2$, 1 mM EDTA, and 1 mM phenylmethylsulfonyl flouride) and lysed,

on ice, using a Polytron homogenizer (Belley and Chadee, 1999). The samples were centrifuged at 2000xg for 5 mins using a Beckmann centrifuge (Optima LE-80K Ultracentrifuge, Turku, Finland) to remove cell debris, and the supernatant was then centrifuged at 35,000xg for 45 mins at 4°C. The crude membrane pellet was then resuspended in storage buffer (20 mM HEPES, pH 7.4, 25 mM $MgCl_2$, and 1 mM EDTA), then flash-frozen in liquid nitrogen and stored at -80°C.

Competition studies were conducted using a modified protocol by Golden et al. (1997). Total membrane protein was determined by protein assay using a BCA Protein Assay Kit (Pierce, Rockford, IL). Briefly, equal amounts of standards or unknown samples were pipetted in duplicate into microwell plate wells. Working reagent was added and the samples were mixed on a plate shaker for about 30 sec. The plate was then incubated at 37°C for 30 mins. The plate was cooled to room temperature and the absorbance of the sample was read at 540nm on a microplate reader (KC-4 Microplate Reader, Bio-Tek Instruments, Inc., Winooski, VT). Software accompanying the microplate reader was used to determine the exact protein concentration of the duplicates. The average concentration of the duplicates was used in the binding assay protocol.

Three sets of assays were conducted in duplicate using 10, 50, or 100 μg of total membrane protein. Membranes were incubated in polystyrene tubes with 0.5 nM ^{125}I-labeled recombinant murine leptin (2200 Ci/mmol; NEN Life Science Products, Inc., Boston, MA) and varying concentrations of unlabeled recombinant rat leptin (from 0.5 to 500 nM; R&D Systems, Minneapolis, MN) in Ringer-HEPES buffer with 1% BSA overnight at 4°C. Total incubation volume was 300 μL. The following morning, contents of each tube were combined with ice-cold PBS (0.01 M, pH 7.4). Tubes were filtered immediately by rapid vacuum filtration through preconditioned Whatman GF-D borosilicate glass microfiber filters (0.68 mm thickness, particle retention up to 2.7 μm; Whatman LabSales, Hillsboro, OR). Filters were preconditioned by soaking for 1h in ice-cold PBS with 5% BSA to reduce non-specific binding to the filter. After initial filtration, tubes were washed twice with ice-cold PBS. Radioactivity remaining on the filters was then determined by gamma counting (LKB Wallac, 1282 compugamma CS, Fisher Scientific, Montréal, QC).

3.3.2.5. Hypothalamic Membrane Leptin Assay

Hypothalamic membrane leptin concentrations were determined using a Rat Leptin RIA Kit (Linco Research, Inc.), with modifications (Nagatani et al., 2000). A total of 50 ug membrane protein per tube was used. Where there was insufficient membrane protein per sample, samples within the same group were pooled, but all samples were still prepared in duplicate. As the initial suspension of membrane protein in storage buffer varied in concentration among samples, the amount of kit buffer added was adjusted such that sample and kit buffer totalled 200 μL per tube. In this manner, a new reference value (B_0, or maximum amount of rat leptin antibody that is bound to the labeled leptin) was determined for each sample. Thus, any tracer counts above the reference value represents additional labeled leptin that is binding either to its receptor or to some component with OB-Rb-like immunoreactivity in the membrane. Additionally, non-specific binding was determined for each sample by adding the same volume of storage buffer (minus the membrane protein) corresponding to a given sample, with enough kit buffer added to adjust the volume to 200 μL per tube. In this manner, any non-specific binding to storage buffer components was controlled for. Total leptin binding was then determined to be total binding per sample minus the adjusted non-specific binding for each sample. Excess tracer per sample constituted any extra counts above the reference value established for each sample. Based on this modified protocol, the minimum detection limit of the assay was determined to be 0.05 ng/mL of membrane homogenate for 50 μg of total membrane protein, with inter- and intra-assay coefficients of variation of 2.60% and 8.29%, respectively, at approximately 84.7% of maximum binding.

3.3.3. Statistical Analyses

Data were analyzed by two-way analysis of variance, with initial body weight included in the model as a covariate, using a general linear program in a SAS commercial software package (Version 8.1, SAS Institute, Inc., Cary, NC). Normal distribution of all variables was assured using the Shapiro-Wilk test for normality. Comparisons between individual diets were made by using multiple pairwise t-tests, thereby controlling comparison-wise error, along with Bonferroni adjustments, after treatment effects were

determined by analysis of variance. Differences were considered to be significant at $p<0.05$. Pairwise t-tests were also used to determine the difference between body weights at week eight, where a drop in weight gain was observed in both *ad libitum*-fed BT and FO rats. Using a multivariate mode in SAS, two sets of partial correlation coefficients were calculated for all variables to examine associations between outcome measures. First, coefficients were calculated to examine the relationship between plasma leptin levels and other variables (n=47), excluding excess tracer and brain leptin levels, since data for these two variables were pooled and thus had smaller sample sizes (n=38). Tracer and hypothalamic leptin level data were then added to the analyses and correlation coefficients were calculated to determine specifically whether relationships existed between these and other outcome measures. Data are presented as means ± SEM, except where otherwise noted.

3.4. RESULTS

At the end of the study, all animals were in good health and showed no overt signs of essential fatty acid deficiency. Diet composition and fatty acid profiles of dietary oils are listed in Tables 3.1 and 3.2, respectively. Growth curves of animals fed the 3 diets at *ad libitum* or 60% of *ad libitum* intakes are shown in Figure 3.1. Body weights, weight gain, food intakes (in g/day, averaged for entire feeding trial) and brain weights, adjusted for initial body weight, of rats fed diets varying in dietary fat type and level of energy intake are shown in Table 3.3. All animals gained weight, although the restricted groups gained considerably less ($p<0.0001$) weight than groups given free access to diets. At no point during the feeding trial were weight gains among rats fed the various diets in either the *ad libitum* or restricted groups different from each other. Only the level of energy intake affected food intakes among dietary treatment groups ($p<0.0001$).

Fatty acid composition of hypothalamic membranes is presented in Table 3.4. Energy restriction to 60% of *ad libitum* intakes resulted in increased ($p<0.05$) concentrations of 18:3(n-3). As well, dietary fat type resulted in a trend ($p<0.07$) in 18:3(n-3) levels, with SO- or FO-fed groups having greater concentrations of this acid than animals fed the BT diets. Energy restriction to 60% of free access intakes resulted in increased levels of 20:1(n-9), 20:4(n-6), and 22:4(n-6) ($p<0.02$, $p<0.05$, and $p<0.04$, respectively). While levels of these fatty acids were not affected by fat type, hypothalamic phospholipid concentrations of 20:4(n-6), unlike 20:1(n-9) or 22:4(n-6), were affected by the interaction of fat type and level of energy intake. Animals fed the FO diet at 60% of *ad libitum* intakes had higher ($p<0.05$) levels of 20:4(n-6) as compared to rats fed the restricted BT diet, or either of the free access SO or FO diets.

Interactions ($p<0.006$) between the effects of dietary fat source and energy restriction were observed for levels of 22:5(n-3) in hypothalamic phospholipids, although this fatty acid was detected in only very small amounts in the phospholipids. Separation of means showed that rats fed the restricted SO diet had higher ($p<0.03$) levels of 22:5(n-3) as compared to those fed the restricted FO diet. As well, rats that received the FO diet at *ad libitum* levels had greater amounts of hypothalamic membrane 22:5(n-3) than did

animals receiving either the *ad libitum* SO diet or restricted FO diet ($p<0.04$ and $p<0.02$, respectively). Rats fed the restricted FO diet also had lower concentrations of 22:5(n-3) than did animals fed either of the other restricted diets ($p<0.02$ for BT and $p<0.03$ for SO).

Levels of 22:6(n-3) were not affected individually by either dietary fat type or level of energy intake, but were affected by the interaction of fat source and intake level. Levels of 22:6(n-3) were higher in animals fed the restricted FO diet as compared to those fed either the *ad libitum* SO or restricted BT diets ($p<0.01$ for both).

Although only marginally significant, the interaction of fat source and energy intake level appeared to result in higher ($p=0.08$) levels of 22:5(n-6) in rats with free access to the SO diet, as compared with those animals with free access to the BT diet. Additionally, the interaction of dietary fat type and level of energy intake had a marginal ($p=0.075$) effect on the sum of n-3 fatty acids among treatment groups, with restricted SO and FO-fed rats having higher levels of total n-3 fatty acids than those animals fed the restricted BT diet.

Circulating leptin levels are depicted in Figure 3.2. Energy restriction to 60% resulted in lower ($p<0.0001$) plasma leptin levels. To further examine associations between circulating leptin levels and other outcome measures of the study, partial correlation coefficients were calculated. After adjustment for all other outcome measures, plasma leptin levels were found to be associated with final body weight ($r=0.45$, $p<0.02$), weight gain ($r=1.00$, $p<0.0001$, Figure 3.3), and food intake ($r=0.67$, $p<0.001$). With the exception of a marginal association between leptin levels and 16:0 ($r=0.35$, $p=0.067$, Figure 3.4), no correlations between leptin and any of the fatty acids present in the membranes were identified.

Using ^{125}I-labeled leptin as a ligand, adult rat hypothalamic membrane homogenates were found to be devoid of any measurable specific binding of leptin to its receptor. However, small amounts of leptin could be detected in some hypothalamic membrane homogenates using the modified RIA. Overall, the presence of leptin could be detected in 5 of 6 ABT samples (mean±SEM = 0.49±0.19 ng/mL, excluding samples where leptin was undetected), 7 of 8 ASO samples (0.66±0.18 ng/mL), 4 of 5 AFO samples (0.25±0.17 ng/mL), 3 of 6 RBT samples (0.72±0.21 ng/mL), 4 of 6 RSO

samples (0.29±0.20 ng/mL), and 5 of 7 RFO samples (0.51±0.20 ng/mL). Additionally, large amounts of excess tracer (^{125}I-labeled leptin), well above the reference value for the assay, could be detected in the membrane homogenates. In the ABT, ASO, AFO, RBT, RSO, and RFO groups, excess tracer levels amounted to 964±219, 820±211, 884±291, 492±239, 1149±233, and 1035±238 CPM, respectively (mean±SEM). Neither excess hypothalamic tracer levels nor brain leptin levels were affected by dietary manipulation, and there appeared to be no trends in their levels among groups. However, brain leptin levels were strongly associated with hypothalamic levels of both 18:3(n-3) and 22:5(n-6) ($r=0.99$, $p<0.0003$, Figure 3.5, and $r=0.97$, $p<0.001$, Figure 3.6, respectively). Tracer levels were weakly but positively associated with the sum of monounsaturates ($r=0.77$, $p<0.07$, Figure 3.7).

Table 3.1. Macronutrient composition of experimental diets.[1]

	Energy intake (% of *ad libitum*)	
g/100g diet	**100%**	**60%**
Fat	20	33.3
Casein	15	25
Corn Starch	45	8.5
Sucrose	10	16.7
Cellulose	5	8.3
Vitamin Mix	1	1.7
Mineral Mix	3.5	5.8
L-Cystine	0.18	0.3
Choline Bitartrate	0.25	0.42
Tert-butylhydroquinone	0.004	0.007

[1]All ingredients purchased form ICN (Aurora, OH), except beef tallow which was donated by Laurentien (Montréal, QC) and cornstarch (generic brand, Marché Richelieu, Montréal, QC).

Table 3.2. Major fatty acid composition of the experimental fats.[1]

Fatty Acid (%)	Beef Tallow	Safflower Oil	Menhaden Oil
14:0	4.1	0.1	8.4
16:0	27.7	6.0-7.5	15.2
16:1(n-7)	6.0	-	11.6
18:0	14.0	2.0-2.5	2.7
18:1	45.5	11.0-13.5	9.5
18:2(n-6)	1.9	70.0-80.0	1.8
18:3(n-3)	0.2	Trace-3.3	1.8
20:0	0.6	0.5	0.2
20:1(n-9)	-	-	1.3
20:4(n-6)	-	-	2.3
20:5(n-3)	-	-	16.0
22:6(n-3)	-	-	10.8
Other	-	< 10.4	18.4

[1]Fatty acid composition of safflower and menhaden oils as per manufacturer's analyses (ICN, Aurora, OH); fatty acid composition of beef tallow as previously determined (Cha, 1998).

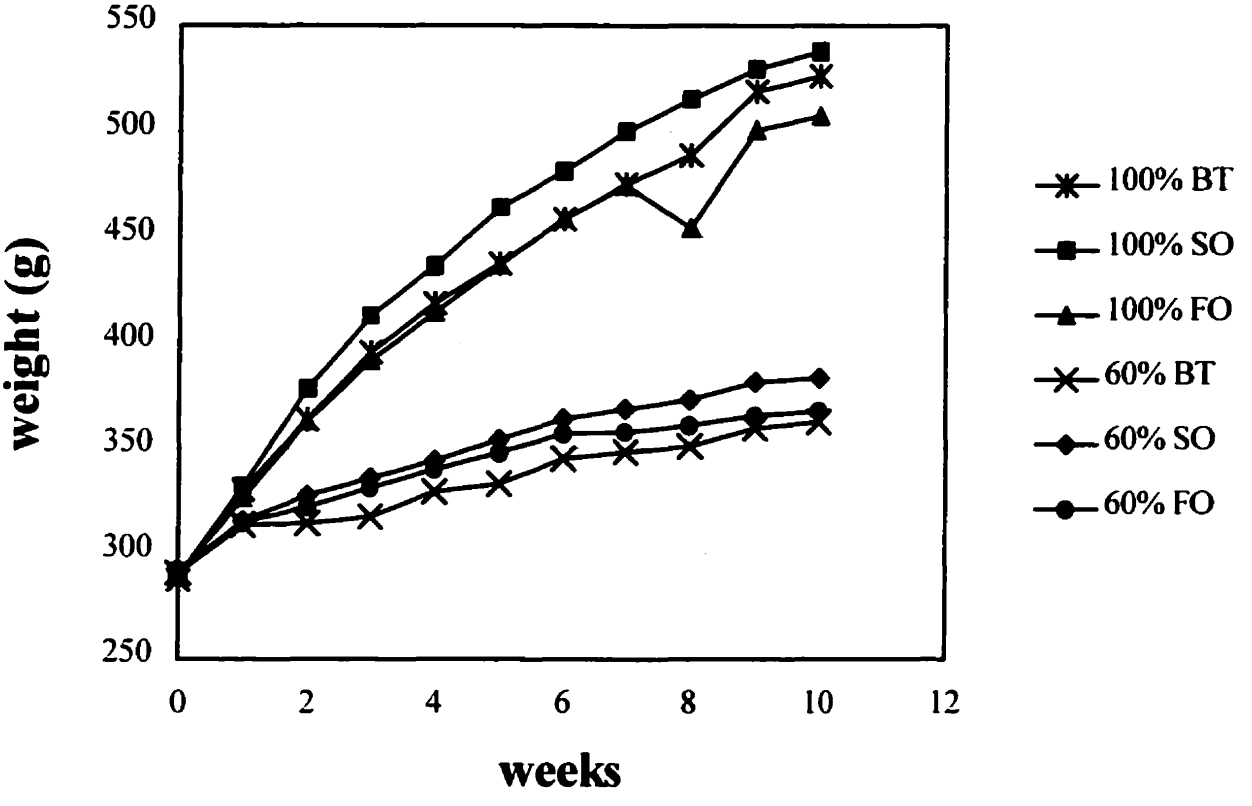

Figure 3.1. Body weights of rats fed diets containing beef tallow (BT), safflower oil (SO), or menhaden (fish) oil (MO) *ad libitum* or restricted to 60% of *ad libitum* intakes for 10 weeks. Values are means, n=12 per group. Restriction was significant ($p<0.0001$) for all groups as compared to animals with free access to diets.

Table 3.3. Body weights, weight gain, food intake, and brain weights of rats fed diets varying in dietary fat type and level of energy restriction.[1]

	Energy intake	Dietary fat consumed		
		Beef tallow	Safflower oil	Menhaden oil
		(g)		
Body weight	100%	539.88±15.30	539.77±14.29	532.28±14.44
	60%	362.25±14.18*	377.14±13.35*	362.25±12.80*
Weight gain	100%	248.73±15.30	248.62±14.29	241.12±14.44
	60%	71.09±14.18*	85.98±13.34*	80.24±12.80*
Food intake (average of all daily intakes)	100%	24.24±0.60	22.64±0.56	22.70±0.57
	60%	13.60±0.56*	13.44±0.52*	12.82±0.50*
Brain weight	100%	2.15±0.03	2.10±0.03	2.13±0.03
	60%	2.09±0.03	2.09±0.03	2.06±0.03

[1]Values are means ± SEM. n=7 (100% beef tallow group); n=7 (60% beef tallow group); n=9 (100% safflower group); n=8 (60% safflower group); n=8 (100% menhaden group); n=8 (60% menhaden group). For each column, means with * superscript are significantly different from the 100% group ($p<0.05$, multiple pair-wise t-tests).

Table 3.4. Hypothalamic membrane fatty acid composition of rats fed diets varying in dietary fat type and level of energy restriction.[1]

Fatty Acid	Energy Intake	Dietary fat consumed		
		Beef tallow	Safflower oil	Menhaden oil
		(% of total fatty acids identified)		
16:0	100%	21.39±1.06	21.33±0.99	22.57±1.00
	60%	21.80±0.98	21.07±0.92	23.10±0.88
18:0	100%	21.79±1.27	23.68±1.19	22.32±1.20
	60%	22.94±1.18	23.98±1.11	24.11±1.06
18:1 (n-9)	100%	20.65±1.28	20.35±1.20	21.65±1.21
	60%	21.34±1.19	22.02±1.11	22.09±1.07
18:2 (n-6)	100%	1.24±0.47	2.88±0.44	2.14±0.44
	60%	1.67±0.43	1.40±0.41	1.36±0.39
18:3 (n-3)	100%	0.19±0.09	0.32±0.09	0.33±0.09
	60%	0.28±0.09*	0.53±0.08*	0.53±0.08*
20:1 (n-9)	100%	1.65±0.23	1.71±0.22	1.68±0.22
	60%	1.86±0.22*	2.43±0.20*	2.23±0.20*
20:4 (n-6)	100%	10.44±0.72	9.53±0.67^{a}	9.56±0.67^{b}
	60%	9.70±0.66*^{c}	11.28±0.62*	12.10±0.60*^{abc}
20:5 (n-3)	100%	0.35±0.19	0.41±0.18	0.39±0.18
	60%	0.40±0.18	0.76±0.17	0.80±0.16
22:4 (n-6)	100%	4.82±0.40	4.39±0.38	4.58±0.38
	60%	4.67±0.37*	5.65±0.35*	5.64±0.34*
22:5 (n-6)	100%	0.29±0.08	0.34±0.08	0.49±0.08
	60%	0.45±0.08	0.38±0.07	0.32±0.07
22:5 (n-3)	100%	0.12±0.05	0.07±0.04^{df}	0.19±0.04^{de}
	60%	0.20±0.04^{cf}	0.16±0.04^{b}	0.04±0.04^{bce}
22:6 (n-3)	100%	15.06±0.95	13.21±0.88^{a}	14.28±0.89
	60%	13.27±0.88^{c}	15.06±0.83	16.63±0.79^{ac}
ΣSFA[2]	100%	44.30±1.53	43.44±1.54	43.92±1.44
	60%	45.71±1.41	43.93±1.33	44.48±1.39
ΣMUFA[2]	100%	22.95±0.97	22.61±0.91	23.14±0.91
	60%	23.66±0.90	23.89±0.85	22.40±0.88
ΣPUFA[2]	100%	33.33±1.63	31.81±1.52	31.72±1.53
	60%	31.21±1.51	34.54±1.42	35.04±1.48
ΣUSFA[2]	100%	54.85±2.00	53.84±1.71	56.03±1.79
	60%	54.89±2.02	58.79±1.64	60.82±1.82
Σ(n-6)	100%	17.12±0.79	18.26±0.79	16.90±0.75
	60%	16.66±0.73	18.50±0.69	18.30±0.72
Σ(n-3)	100%	15.93±0.82	15.28±0.82	15.41±0.77
	60%	14.21±0.76	16.40±0.71	17.13±0.74
18:0/	100%	1.06±0.08	1.19±0.07	1.05±0.07
18:1(n-9)	60%	1.08±0.07	1.09±0.07	1.09±0.07
18:2(n-6)/	100%	0.13±0.06	0.33±0.05	0.24±0.05
20:4(n-6)	60%	0.18±0.05	0.13±0.05	0.11±0.05

18:3(n-3)/	100%	0.78±0.24	0.94±0.21	0.81±0.22
20:5(n-3)	60%	0.95±0.21	0.82±0.20	0.95±0.20
UI[2]	100%	180.62±10.41	167.76±9.72	176.19±9.82
	60%	169.7±9.6	193.44±9.08	205.16±8.70
USFA/SFA ratio	100%	1.26±0.06	1.30±0.06	1.27±0.05
	60%	1.22±0.05	1.35±0.05	1.30±0.05
(n-6)/(n-3) ratio	100%	1.08±0.06	1.22±0.06	1.10±0.06
	60%	1.21±0.06	1.13±0.05	1.08±0.06

[1]Values are means ± SEM. n=7 (100% beef tallow group); n=7 (60% beef tallow group); n=9 (100% safflower group); n=8 (60% safflower group); n=8 (100% menhaden group); n=8 (60% menhaden group). For each column, means with * superscript represent significant differences in main effects between the 60% and 100% groups ($p<0.05$, ANOVA). Means with different letter superscripts in the same row represent interactive effects that are significantly different from each other, where *a*=RFO vs ASO; *b*=RFO vs RSO; *c*=RFO vs RBT; *d*=ASO vs AFO; *e*=RFO vs AFO; *f*=ASO vs RBT; and A = *ad libitum*, R = restricted, BT = beef tallow, SO = safflower oil, and FO = fish (menhaden) oil ($p<0.05$, multiple pairwise t-tests).

[2]SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; USFA = unsaturated fatty acids; UI = unsaturation index.

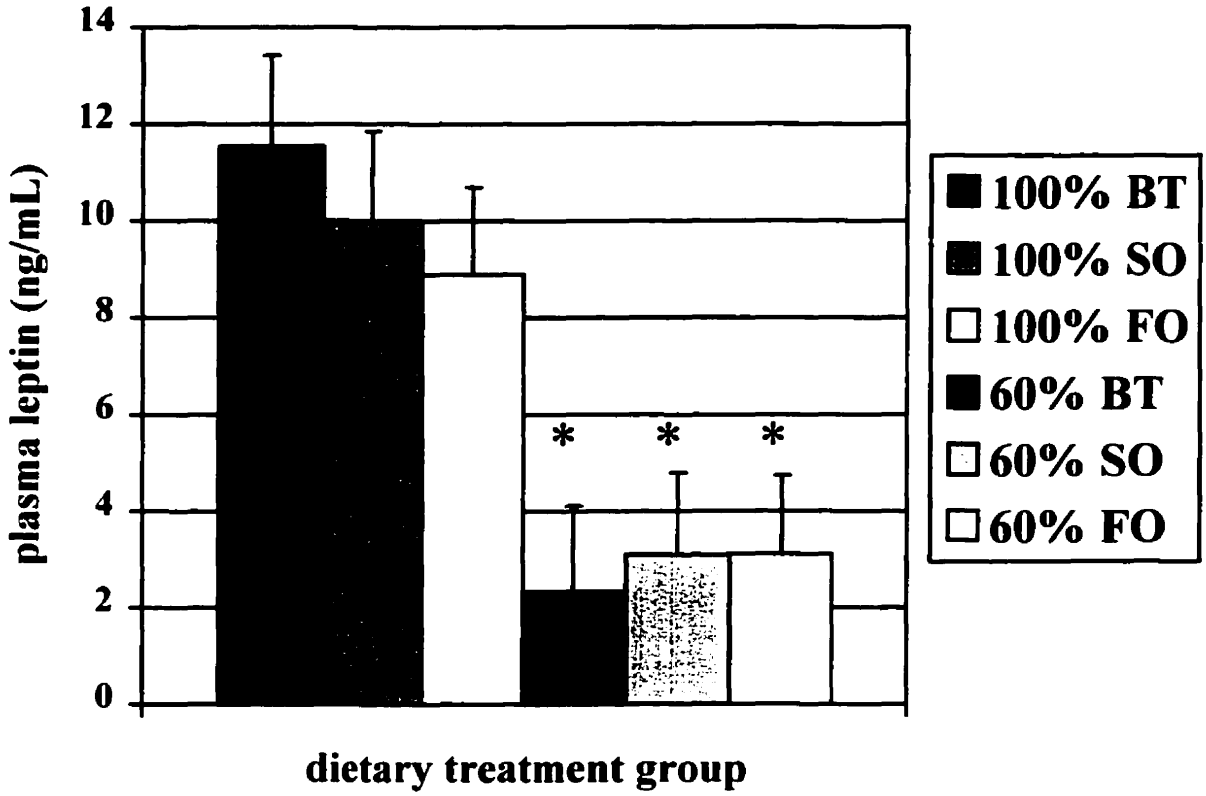

Figure 3.2. Plasma leptin concentrations of rats fed diets containing beef tallow, safflower oil, or menhaden (fish) oil *ad libitum* or restricted to 60% of *ad libitum* intakes for 10 weeks. Values are means ± SEM. n=7 (100% beef tallow group); n=7 (60% beef tallow group); n=9 (100% safflower oil group); n=8 (60% safflower oil group); n=8 (100% menhaden/fish oil group); n=8 (60% menhaden/fish oil group). Restriction was significant ($p<0.0001$) for all groups as compared to animals with free access to diets, as indicated by *.

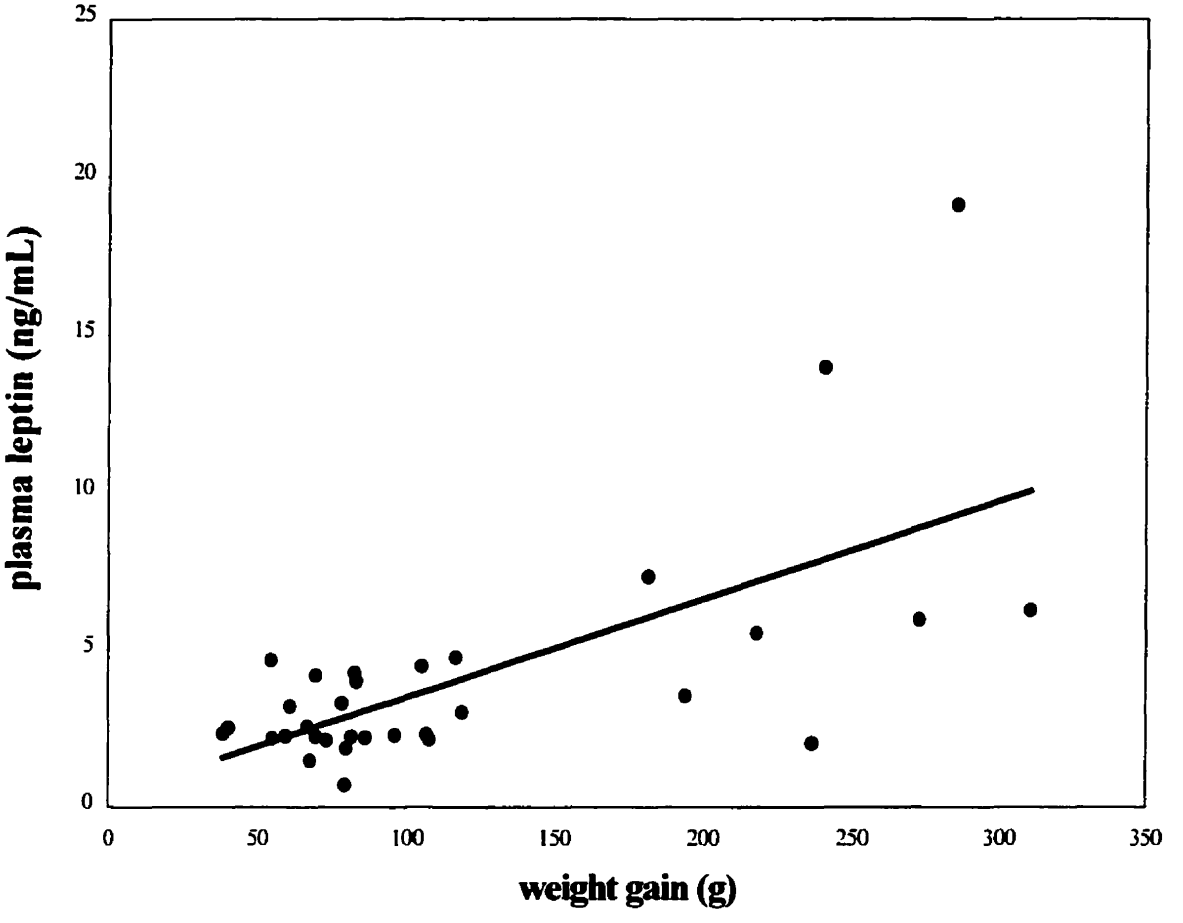

Figure 3.3. Correlation between body weight gain and plasma leptin concentrations, r = 1.00, $p<0.0001$.

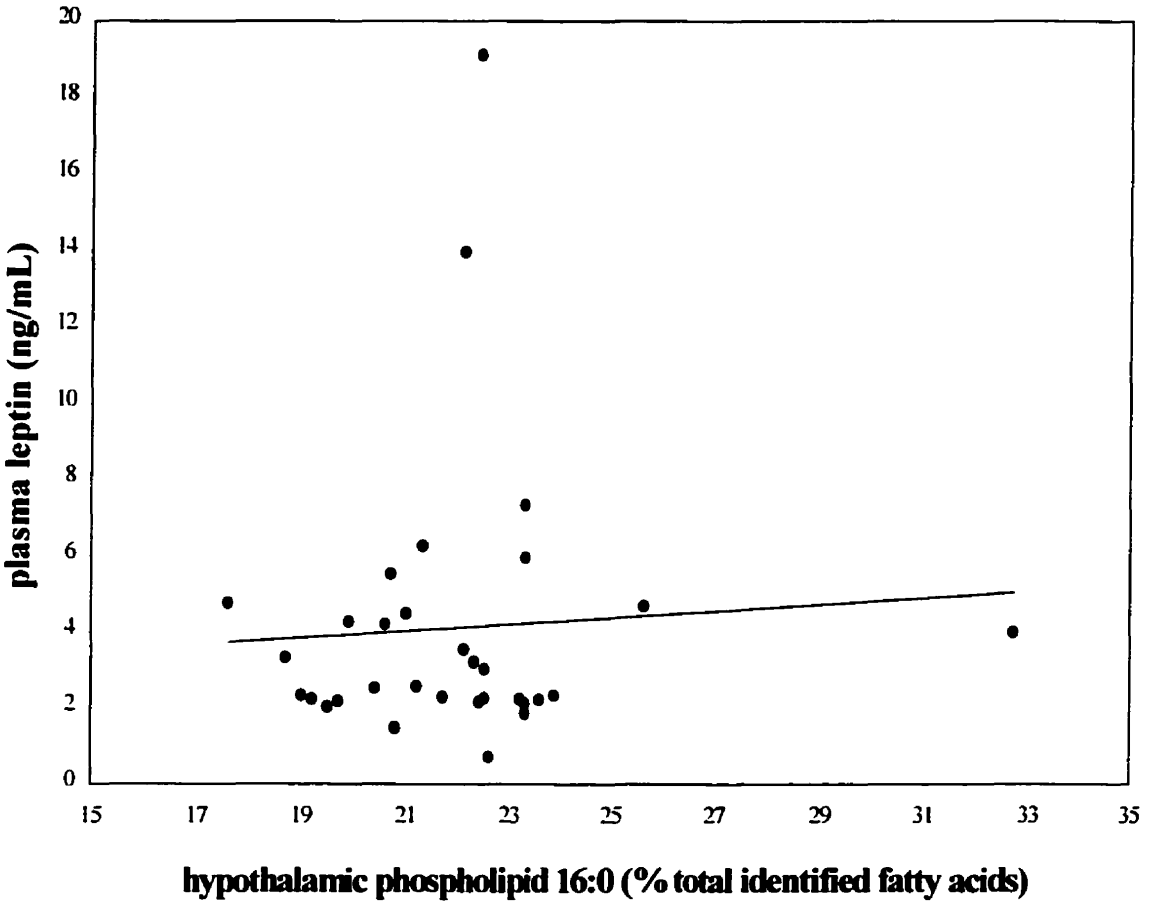

Figure 3.4. Correlation between hypothalamic phospholipid 16:0 and plasma leptin concentrations, $r = 0.35$, $p<0.067$.

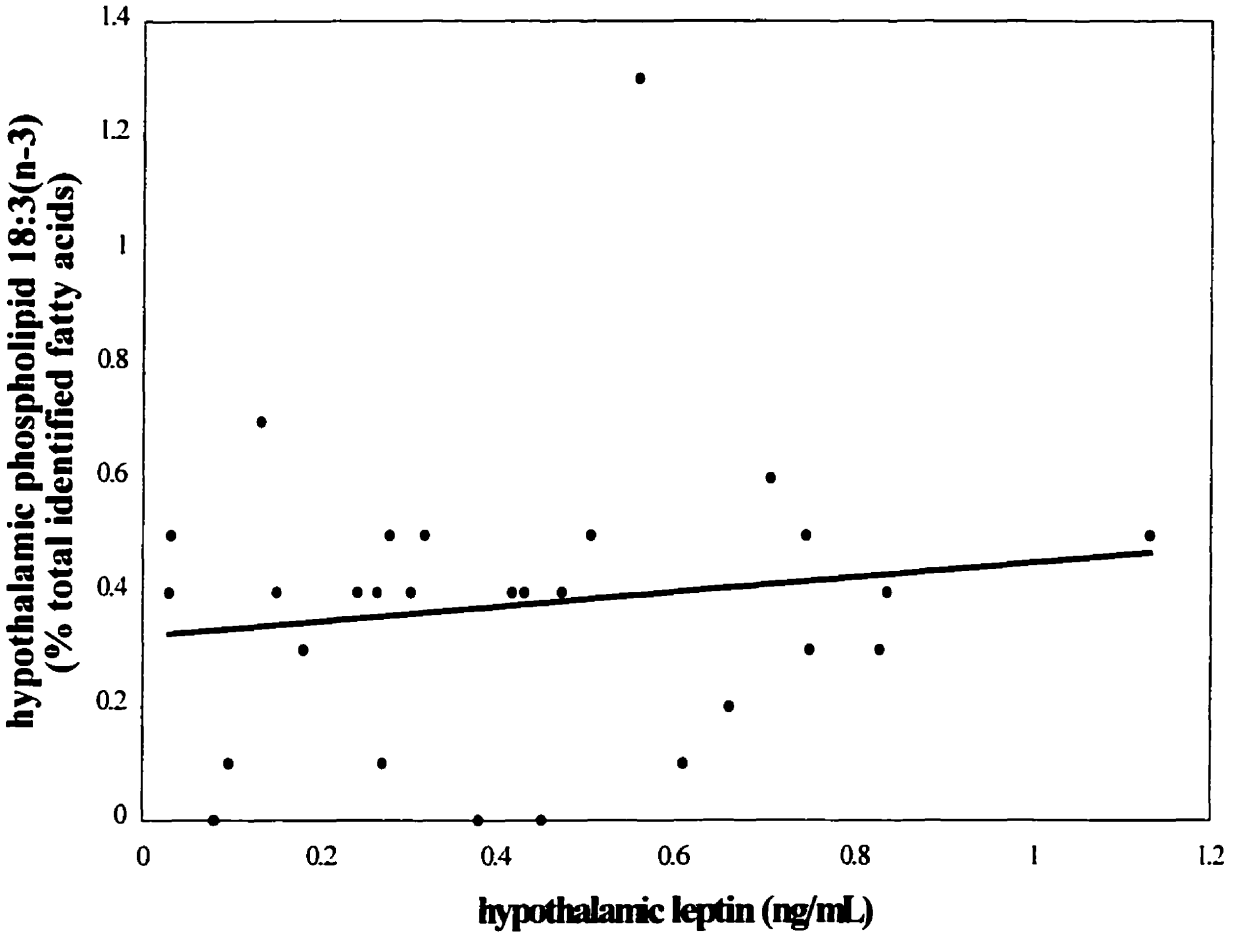

Figure 3.5. Relationship between hypothalamic leptin and 18:3(n-3) concentrations, r = 0.99, $p<0.0003$.

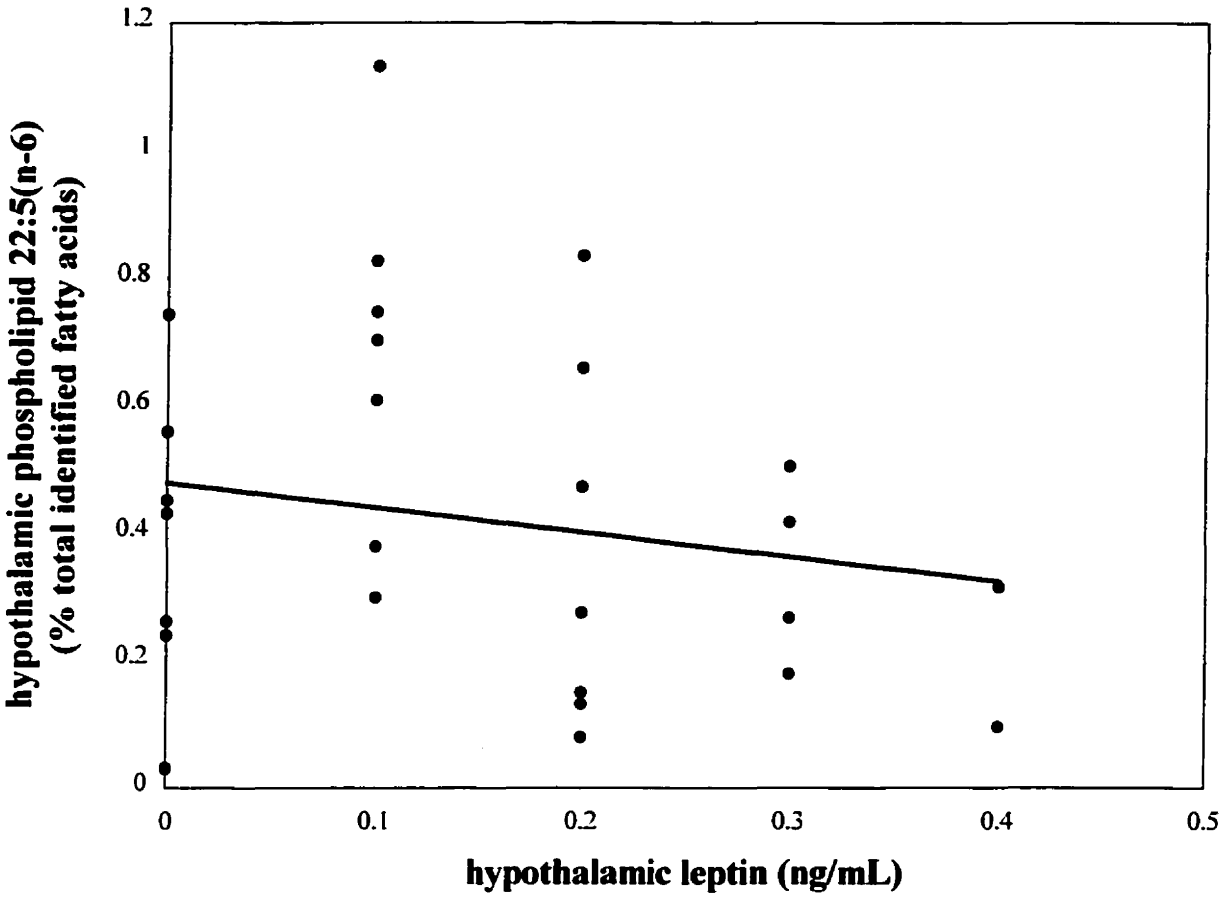

Figure 3.6. Relationship between hypothalamic leptin and 22:5(n-6) concentrations, r = 0.97, $p<0.001$.

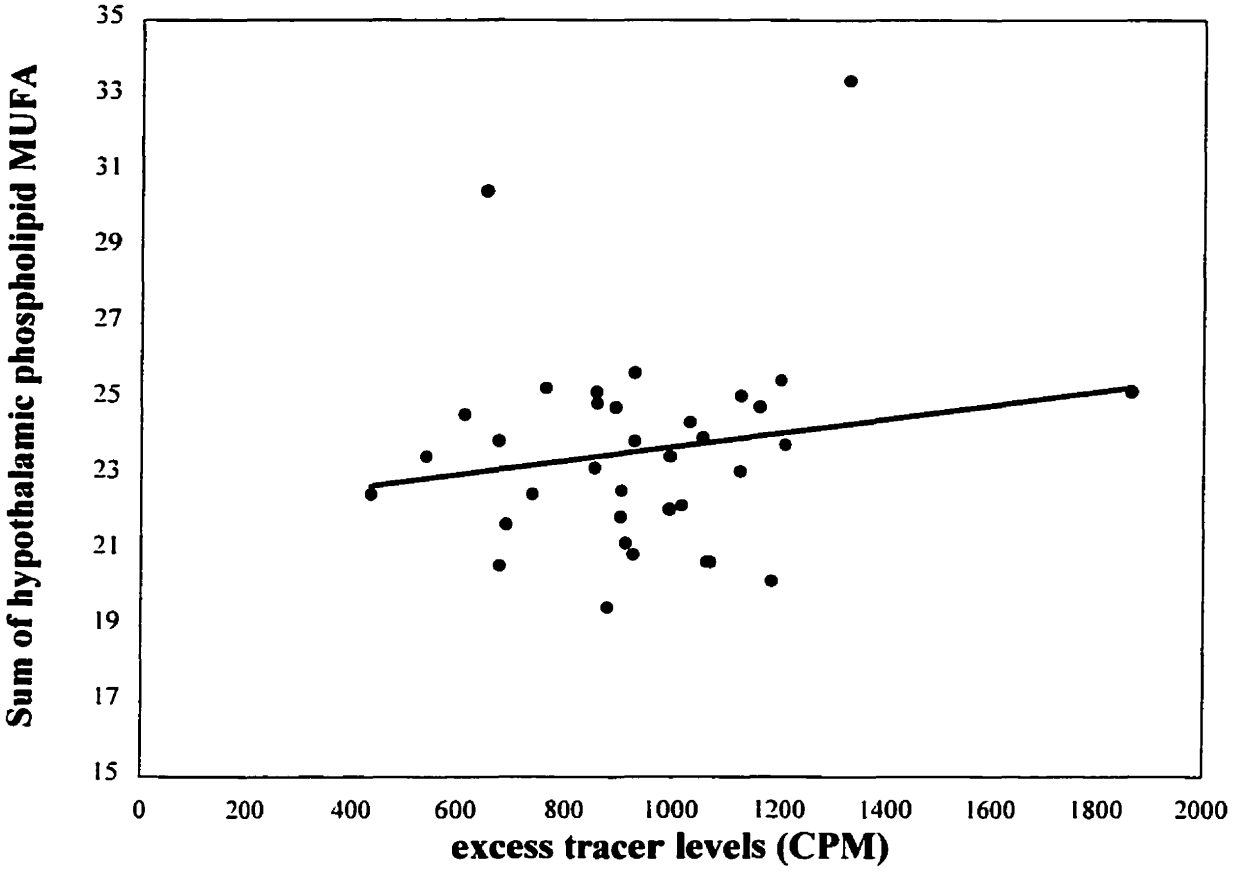

Figure 3.7. Relationship between excess tracer levels and the sum of hypothalamic phospholipid MUFA, $r = 0.77$, $p<0.07$.

3.5. DISCUSSION

The present results focused on aspects of leptin resistance at the level of leptin uptake in the hypothalamus during a period of feeding high-fat diets varying in fat type with or without energy restriction. Our findings show that specific changes occur in hypothalamic membrane phospholipid fatty acid composition induced by the different fat types and levels of energy intake. Our results also suggest a link between increased membrane fluidity, increased binding affinity, and, consequently, lower circulating leptin levels. Taken together, these findings stress the importance of dietary fat type and level of energy intake in the development of leptin resistance in rats.

The increased food consumption, lower rates of energy expenditure, and hyperleptinemia characteristic of leptin resistance may be caused by reduced peripheral or central leptin sensitivity, or both. Recent evidence exists to suggest three stages of leptin resistance (Lin et al., 2000a and b). Early leptin resistance occurs after 1 wk of a high-fat diet and is characterized by no differences in weight gain, circulating leptin levels or OB-Rb expression, as compared to low fat-fed controls. The middle stage of leptin resistance occurs after 8 wks of high-fat feeding and is characterized by peripheral leptin insensitivity and hyperleptinemia, but increased OB-Rb mRNA in the arcuate nucleus of ob/ob (Huang et al., 1997) and C57B1/6J mice (Lin et al., 2000b) and central leptin sensitivity. Late stage leptin resistance occurs after 19 wks on a high-fat diet and is characterized by decreased central leptin sensitivity, pronounced hyperleptinemia, and decreased arcuate nucleus and choroid plexus OB-Rb mRNA expression, as compared to controls, consistent with the findings of others (Chang et al. 1999). Thus, as observed by Lin et al. (2000a), some degree of food intake and body composition homeostasis may be possible in the middle stage of leptin resistance. However, with long-term exposure to high-fat diets, a complete breakdown in the regulation of body weight occurs, resulting in decreased OB-Rb mRNA expression, pronounced hyperphagia, and extreme obesity (Lin et al., 2000a and b).

In the present study, we believed that rats restricted to 60% of *ad libitum* intakes would be able to maintain more constant body weights over the course of the feeding trial, thus avoiding leptin resistance. Certainly, the restricted animals in our study gained

only small amounts of weight. Consistent with the theory of early leptin resistance, body weights of all groups during wk 1 appeared to be very similar, although the *ad libitum* groups were beginning to show higher weight gains as compared to the restricted groups. By the end of the feeding trial, rats with free access to diets considerably increased their body weight, resulting in diet-induced obesity. Using the same model and design, Cha and Jones (1998) demonstrated that this type and extent of high-fat feeding does result in increased body weight, fat pad size, and hyperleptinemia, and, consequently, diet-induced obesity in animals with free access feeding. Since results from the present study demonstrate that only the level of energy intake, and not dietary fat source, affected weight gain in rats, BT, SO, and FO may have the same effects on the development of obesity and leptin resistance.

Although the hyperleptinemia seen in the *ad libitum*-fed animals could be due to greater size and fat mass alone, the excessive weight gain and higher circulating leptin levels noted in the *ad libitum*-fed rats are also consistent with the theory of leptin resistance. At this stage of high-fat feeding, the animals likely had full peripheral leptin resistance, and were progressing to central leptin insensitivity. Animals with diet-induced obesity were characterized by plasma leptin levels approximately 3 times higher than those of the restricted rats. Restricted animals had plasma leptin levels consistent with those for fasted, lean adult Sprague-Dawley rats. The normal plasma leptin range for adult fasted Sprague-Dawley rats is 0.8-3.0 ng/mL, with an average of 1.5 ng/mL (Linco Research, Inc., St. Charles, MO). The hyperleptinemic effects of dietary polyunsaturates were not detected in the present study.

Few studies have examined the specific effects of dietary fat type on hypothalamic membrane phospholipid composition. Carrié et al. (2000) studied a number of brain regions and found that n-3 polyunsaturated fatty acid deficiency and supplementation with egg yolk or brain phospholipids resulted in an extreme increase in 22:5(n-6) with n-3 fatty acid deficiency, but also a decrease in 22:6(n-3) levels in hypothalamic phospholipids. Similar results have been shown to occur in rat brain cells when rats are fed 18:3(n-3) deficient diets (Bourre et al., 1989). Unlike Carrié et al. and Bourre et al., we found that RFO-fed rats had significantly higher levels of 22:6(n-3) than ASO- and RBT-fed animals, with no differences noted in any other groups. Our results

cannot be explained by low levels of 18:3(n-3) in either the BT or SO diets alone, since all groups fed either BT or SO diets should have displayed lower hypothalamic 22:6(n-3) concentrations than those fed FO diets. Rather, 22:6(n-3) levels appear to depend, at least in part, on total amounts of dietary n-3 fatty acids as well as the level of energy intake, since neither aforementioned studies on n-3 deficient diets in rats involved energy restriction. Likewise, 22:5(n-6) levels appear to depend on the interaction between total dietary n-6 levels and energy intake.

Marteinsdottir et al. (1998) found that feeding rats diets containing fish oil (6% by wt) increased 20:3(n-6) levels and decreased 22:4(n-6) levels in hypothalamic phospholipids. Diets consisting of a combination of primrose (4.5% by wt) and fish (1.5% by wt) oils decreased 22:5(n-6) concentrations. In both studies, no other hypothalamic phospholipid fatty acid levels were affected by dietary treatment. Our results support what these studies have suggested; that the hypothalamus, in comparison with other brain regions, is highly resistant to diet-induced changes in membrane fatty acid composition. The fact that 20:4(n-6) and 22:4(n-6) were most affected by dietary treatment is also consistent with the research of others, although the researchers studied anterior pituitary, and not hypothalamic, membranes (Alessio et al., 1992). Generally, in the hypothalamus, diets consisting of 6% wt/wt fish oil, as compared to primrose oil, soybean oil, or a combination of primrose and fish oil, decrease 20:4(n-6) and 22:4(n-6) concentrations, likely due to inhibition of delta-5-desaturase activity by 20:5(n-3) and 22:6(n-3) (Brenner et al., 1984; Nassar et al., 1986). However, the fact that we found increased levels of 20:4(n-6) and 22:4(n-6) as well as reversed treatment effects on 22:5(n-3) for FO-fed rats with energy restriction suggests that for particular fatty acids, the influence of dietary fat type on hypothalamic membrane struction may depend on the state of energy balance.

In the binding assays, no specific leptin binding could be detected. This finding is consistent with that of Dal Farra et al. (2000) who employed a pure ^{125}I-monoiodoleptin analog to quantify leptin binding in a variety of mouse tissues. The absence of any detectable specific brain leptin binding in our study supports the conclusion of Dal Farra and colleagues; that OB-Rb in rats is located primarily in the Golgi apparatus and not at the membrane level, as shown by ultrastructural localization studies (Dal Farra et al.,

2000; De Matteis and Saverio, 1998; Diano et al., 1998; Miller et al., 1999). However, other researchers have used similar techniques and have successfully detected leptin binding at both the Golgi levels and the plasma membrane (Funahashi et al., 2000). Possibly, with high-fat feeding for 10 wks, rats began to enter the late stage of leptin resistance where OB-Rb mRNA levels had decreased to a point where binding affinity could not be detected. The fact that we were able to detect small amounts of leptin in the membrane homogenates supports this possibility. It has been suggested (Wilkinson et al., 2000) that the brain is capable of producing its own leptin, since leptin gene expression has been detected in areas of the hypothalamus where OB-Rb is concentrated, although this theory remains controversial. The RIA used to detect brain leptin levels does not differentiate between bound and unbound leptin; as such, any leptin present may be either that which is endogenously produced, that which is bound to OB-Rb or other neurons with OB-Rb-like immunoreactivity, or that which is taken up from peripheral circulation but remains in the homogenates unbound due to decreased binding affinity. There is also the possibility that, if OB-Rb is concentrated in a specific area in the hypothalamus, such as the arcuate nucleus, then homogenization of the membrane itself may result in dilution of receptor concentrations to levels that are undetectable by traditional binding assays. The fact that hypothalamic leptin levels could only be detected randomly in some samples suggests that any of the above explanations for its presence could hold true. Given that the restricted rats should have retained more sensitivity to leptin, this response should have been noted in the leptin binding data. However, the absence of this response emphasizes that interpretation of the results of the brain leptin assays must be performed with caution, due to the low number of samples that had detectable levels of brain leptin.

Diet-induced changes in membrane fluidity have been implicated as having a role in the development of leptin resistance. Certainly, the recent finding that lean subjects adapted to high-fat diets had higher leptin levels than subjects consuming low-fat diets, even though both groups of subjects had similar body mass indices and fat masses, suggests an effect of dietary fat on leptin levels independent of the amount of body fat (Cooling et al., 1998). Fluidity of the membrane bilayer at any given temperature is typically increased with (1) decreased saturation of membrane fatty acids, (2) increased concentration of essential fatty acids, (3) a lower n-6/n-3 ratio, and (4) lower

concentrations of cholesterol, and can modulate the functions of membrane-bound proteins, such as receptors (Kinsella, 1990). In the brain, fluidity is particularly increased by a greater presence of its predominant long-chain fatty acids, these being 20:4(n-6), 22:4(n-6), and 22:6(n-3) (Holub and Kuksis, 1978; Lynch and Thompson, 1984; Marteinsdottir et al., 1998). While the precise effects of membrane fluidity on OB-Rb function remain to be elucidated, it is generally assumed that an increase in membrane fluidity would enhance receptor function, while a more rigid membrane environment would inhibit receptor activity.

In terms of decreased saturation of membrane fatty acids, correlation analyses support the theory that reduced membrane fluidity decreases receptor function in that 16:0 was weakly associated with increased circulating leptin levels. Furthermore, the ABT diet had the highest levels of 16:0 as compared to the other diets, and the ABT-fed animals possessed the highest circulating leptin levels. The correlation of excess tracer levels, though only marginally significant, with the sum of monounsaturates also supports the membrane fluidity theory. The excess tracer levels detected with the modified RIA suggest intra-membrane binding, although whether the ligand was binding to OB-Rb itself or neurons with OB-Rb-like immunoreactivity could not be determined.

The increased concentrations of 18:3(n-3), both an essential and n-3 fatty acid, in the restricted animals provide stronger evidence that increased membrane fluidity helped to enhance OB-Rb binding affinity, since these rats had significantly lower levels of circulating leptin. As well, the strong correlations seen between hypothalamic leptin levels and amounts of 18:3(n-3) and 22:5(n-6) suggest that as more of these fatty acids are incorporated into hypothalamic phospholipids, either more circulating leptin is taken up by the brain or more endogenous brain leptin is produced, if the brain is actually able to do so. In either case, more leptin would be available for binding in the hypothalamus and then the ability of leptin to regulate body weight via the hypothalamus would then depend on the receptor's binding and signaling abilities at that point. Ultimately, as more of these fatty acids are incorporated into the membrane phospholipids, there is the possibility that, via elongase and desaturase enzymes, they can be converted in the brain to longer-chain fatty acids, particularly those influencing membrane fluidity.

In comparing the animals with free access to diets with those that were restricted, the results further suggest that decreased membrane fluidity in the *ad libitum*-fed groups contributed to the development of hyperleptinemia and leptin resistance, possibly explaining the large difference in circulating leptin levels between the *ad libitum* and restricted groups. Specifically, the restricted groups had overall significantly higher levels of 20:4(n-6) and 22:4(n-6), two of the long-chain fatty acids known to promote fluidity in the brain. Interestingly, levels of 20:4(n-6) were lower in ASO-, AFO-, and RBT-fed animals, which were the groups that had the lowest circulating leptin levels within their respective energy intake level groups, although plasma leptin levels were not found to be different from one another among intake level groups. While only the RFO-fed rats were found to have significantly higher levels of 22:6(n-3) as compared to ASO- and RBT-fed animals, who had the lowest 22:6(n-3) levels, restriction in the SO-fed animals did increase 22:6(n-3) levels, though not significantly. However, research has shown that obese animals have lower levels of delta-5-desaturase activity, which would result in not only in lower levels of 20:4(n-6) and consequently 22:4(n-6), but also lower levels of 20:5(n-3) and consequently 22:6(n-3) (Blond et al., 1989). This evidence supports not only our findings but the suggestion that decreases in levels of these fatty acids reduce membrane fluidity, thus contributing to decreased leptin binding and obesity. Possibly, it is the overall amount of the predominant long-chain fatty acids in the hypothalamus that results in the promotion of membrane fluidity and leptin receptor activity, as reflected by the significantly lower plasma leptin levels in the restricted animals. Since hypothalamic phospholipid levels of cholesterol were not examined in the present study, it is not possible to speculate on the effects cholesterol levels had on circulating leptin levels.

The results found in rats in the present study are applicable to humans and the condition of human obesity. Previous work has shown that, when fed similar diets in the same study design, weight gain in rats is primarily due to body fat accumulation (Cha and Jones, 1998). The extent of weight gain noted in the *ad libitum*-fed rats is not indifferent to that commonly seen in obese to morbidly obese human subjects. Likewise, the relatively small amount of weight gained by restricted rats may be easily compared to the gradual amount of modest weight gain that occurs with age in humans. Given the high

degree of similarity between rat and human forms of OB-R (Masuzaki et al., 1995), as well as the relative similarities in the metabolisms of humans and rats, their rates of development, their brain/body ratios, and the fatty acid composition of neural structures, as noted by others (Bourre et al., 1989), it is very possible to propose that the present results obtained in the rat could also be valid for humans subject to similar dietary conditions or extent of obesity.

The effects of dietary fat on other factors not examined in the present study but implicated in leptin resistance are known. The reduction in SNS activity caused by highly saturated diets has been well documented (Bray, 1993; Matsuo et al., 1995). Leptin resistance caused by a diet fat-induced decrease in blood-brain barrier leptin transport, with consequent decreases in OB-Rb-leptin binding, has also been suggested (Burgeura et al., 2000; Wang et al., 1999b). Both c-fos and STAT3, intermediates in leptin signal transmission, are also known to be affected by dietary fat (El-Haschimi et al., 2000; Wang et al., 1999b). The roles of other neuropeptides under OB-Rb's control, such as NPY or POMC, were also not explored. In addition to these factors, there is the possibility that the ratio of fat to carbohydrate, the latter being reduced in dietary treatments to accomplish restriction, may have also contributed to the results noted in our study. It is well known that leptin and insulin action are closely linked, and that obese animals tend to be not only hyperleptinemic but also hyperinsulinemic. Furthermore, effects of dietary fat and energy restriction at the level of leptin production on energy homeostasis cannot be ruled out. Certainly, more research is needed to further elucidate the effects of dietary fat and energy level on the OB-Rb/leptin system.

In summary, results from the current study present a number of salient findings. The results of the study demonstrate that dietary fat has no effect on circulating leptin levels with energy restriction. As well, hypothalamic accretion of certain fatty acids, such as 20:4(n-6), 22:4(n-6), and 22:5(n-3) may depend on the state of energy balance. A novel finding is the specific diet fat-induced change in hypothalamic membrane phospholipid fatty acid composition in rats in the context of a high-fat diet with different fat types and levels of energy intake. Our results also support previous findings suggesting that leptin binding at the level of the hypothalamic membrane may not be detectable, at least in traditional radioligand binding experiments. However, the use of a

modified RIA method to detect leptin binding may be achieved by examining excess tracer levels. The present study provides evidence for the addition of the hypothalamus to the list of tissues where the standard RIA kit, with modifications to increase sensitivity, may be used to quantify leptin levels. The findings of our study also support a link between increased membrane fluidity, increased binding affinity, and, consequently, lower circulating leptin levels. The possibility that the biological actions of leptin can be controlled through dietary effects on OB-Rb or its signaling has important implications for the control of obesity and obesity-related disorders.

3.6. ACKNOWLEDGMENTS

This research was supported by a grant from the Natural Sciences and Engineering Research Council of Canada. The authors would like to acknowledge Dr. Louise Thibault for performing the brain dissections, as well as Drs. Shoji Nagatani and Brahim Selmaoui for their assistance with the hypothalamic membrane leptin assay. The authors also thank Mr. Gordon Bingham for assisting with the animal care.

CHAPTER IV. GENERAL DISCUSSION AND CONCLUSIONS

Despite the ever-growing body of research regarding leptin metabolism, the mechanisms by which leptin acts in the hypothalamus to control body weight homeostasis, thereby preventing leptin resistance, remain generally unknown. Evidence in the literature suggests a "three stage" progression of leptin resistance, and results from the present study are consistent with this theory. Whether this leptin resistance is due to dysfunctional leptin uptake or production remains to be elucidated. However, the results from the present study provide evidence suggesting that diet-induced changes in hypothalamic membrane fluidity may play a role in the mediation of leptin's biological actions, thus influencing the progression of leptin resistance in animals fed long-term high-fat diets.

The specific structure and function of the long form of the leptin receptor, OB-Rb, are presented in Chapter II, Manuscript I. Clearly, there are a number of levels in the binding mechanism and signaling pathways of the receptor that can be influenced by dietary fat type and amount. Diet-induced alterations in the fatty acid content of brain membrane phospholipids, and thus membrane fluidity, can affect the action of membrane-receptor complexes, such as OB-Rb. Dietary fat can also play a major role in the expression or action of other factors critical to OB-Rb function, such as cAMP, MAPK, c-fos, or STAT3, or even affect other weight-regulating neuropeptides under OB-Rb's control, such as NPY or POMC.

Ultrastructural localization studies have identified the existence of the long form of the leptin receptor at the hypothalamic membrane level. Previous studies have also demonstrated that diet-induced changes in hypothalamic phospholipid fatty acid profile are possible, although perhaps to a lesser extent than in other brain regions. Modifications to the hypothalamic membrane may affect the binding or signaling acitvities of OB-Rb, thus contributing to the development of leptin resistance. The results obtained in Chapter III, Manuscript II show that, consistent with the findings of others, we were unable to detect specific binding in our assays using hypothalamic membrane homogenates. However, the fact that we could detect both small amounts of leptin in the homogenates as well as levels of tracer vastly exceeding that of the reference

values suggest that intra-membrane binding does occur. With the small sample size, we were unable to determine whether our treatments had any effect on these measurements. However, the weak correlations between hypothalamic levels of 16:0 and circulating leptin levels and excess tracer levels and sum of monounsaturated fatty acids in the membranes suggest that dietary fat can alter leptin binding in the hypothalamus. The stronger associations between brain leptin levels and both 18:3(n-3) and 22:5(n-6) further support this theory.

Previous work has identified that both dietary fat type and level of energy intake have both individual and interactive effects on weight gain and circulating leptin levels, consequently influencing leptin resistance. The results obtained in the present study support previous findings that the levels of energy intake and body weight gain, and not dietary fat source, increase circulating leptin levels. As such, our results support previous research suggesting that there is no effect of fat type on circulating leptin levels with energy restriction. Consistent with the theory of late stage leptin resistance, upon conclusion of our feeding trial rats fed *ad libitum* demonstrated extreme obesity and plasma leptin levels that were approximately 3 times higher that those in restricted animals. Rats restricted to 60% of *ad libitum* intakes gained only small amounts of weight and showed no signs of hyperleptinemia.

We were able to demonstrate, for the first time, the specific changes that occur in hypothalamic phospholipid fatty acid composition with various fats, in the context of a high-fat diet with and without energy restriction. This is important, as literature on the effects of diet and energy restriction on hypothalamic membrane composition alone is scarce. Using these data, the introduction of energy restriction was shown to alter the effect of diet on certain hypothalamic phospholipid fatty acids. Furthermore, we were able to show that animals with higher circulating leptin levels tended to have lower levels of fatty acids known to promote membrane fluidity.

In summary, the findings of our study suggest a link between diet-induced increased membrane fluidity, increased binding affinity, and, consequently, lower circulating leptin levels. Based on our results, modifications in the amount and type of dietary fat may provide protection against leptin resistance and subsequent obesity and hyperleptinemia.

BIBLIOGRAPHY

Alessio ML, Wandscheer DE, Soares MCF, Clauser H, Enjalbert A, Kordon C, Léger CL. Effect of an essential fatty acid deficiency on the phospholipid composition in anterior pituitary membranes. *Biochem Biophys Res Commun* 1992;183:1047.

Banks AS, Davis SM, Bates SH, Myers, Jr. MG. Activation of downstream signals by the long form of the leptin receptor. *J Biol Chem* 2000;275:14563.

Baskin DG, Hahn TM, Schwartz MW. Leptin sensitive neurons in the hypothalamus. *Horm Metab Res* 1999a;31:345.

Baskin DG, Schwartz MW, Seeley RJ, Woods SC, Porte DJ, Breininger JF, Jonak Z, Schafer J, Krouse M, Burghardt C, Campfield LA, Burn P, Kochan JP. Leptin receptor long-form splice-variant protein expression in neuron cell bodies of the brain and co-localization with neuropeptide Y mRNA in the arcuate nucleus. *J Histochem Cytochem* 1999b;47:353.

Baskin DG, Seeley RJ, Kuijper JL, Lok S, Weigle DS, Erickson JC, Palmiter RD, Schwartz MW. Increased expression of mRNA for the long form of the leptin receptor in the hypothalamus is associated with leptin hypersensitivity and fasting. *Diabetes* 1998;47:538.

Baumann H, Morella KK, White DW, Dembski M, Bailon PS, Kim H, Lai CF, Tartaglia LA. The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. *Proc Natl Acad Sci USA* 1993;93:8374.

Bazan JF. A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erythropoeitin and IL-6 receptors, and the p75 IL-2 receptor beta-chain. *Biochem Biophys Res Commun* 1989;164:788.

Bazan JF. Structural design and molecular evolution of a cytokine receptor superfamily. *Proc Natl Acad Sci USA* 1990;87:6934.

Belley A, Chadee K. Prostaglandin E_2 stimulates rat and human colonic mucin exocytosis via the EP_4 receptor. *Gastroeneterol* 1999;117:1352.

Bjorbaek C, El-Haschimi K, Frantz JD, Flier JS. The role of SOCS-3 in leptin signaling and leptin resistance. *J Biol Chem* 1999;274:30059.

Bjorbaek C, Elmquist JK, Frantz JD, Shoelson SE, Flier JS. Identification of SOCS-3 as a potential mediator of central leptin resistance. *Mol Cell* 1998;1:619.

Bjorbaek C, Uotani S, Da Silva B, Flier JS. Divergent signaling capabilities of the long and short isoforms of the leptin receptor. *J Biol Chem* 1997;272:32686.

Blenis J. Signal transduction via the MAP kinases: proceed at your own RSK. *Proc Natl Acad Sci USA* 1993;90:5889.

Blond JP, Henchiri C, Bezard J. Delta 6 and delta 5 desaturase activities in liver from obese Zucker rats at different ages. *Lipids* 1989;24:389.

Boado RJ, Golden PL, Levin N, Pardridge WM. Up-regulation of blood-brain barrier short-form leptin receptor gene products in rats fed a high fat diet. *J Neurochem* 1998;71:1761.

Bourre J-M, Francois M, Youyou A, Dumont O, Piciotti M, Pascal G, Durand G. The effects of dietary α-linolenic acid on the composition of nerve membranes, enzymatic activity, amplitude of electrophysiological parameters, resistance to poisons, and performance of learning tasks in rats. *Lipids* 1989;119:1880.

Bray GA. Hypothalamic and genetic obesity: an appraisal of the autonomic hypothesis and the endocrine hypothesis. *Int J Obes* 1984;8:119.

Bray GA. Obesity, a disorder of nutrient partitioning: the MONA LISA hypothesis. *J Nutr* 1991;121:1146.

Bray, GA. The nutrient balance hypothesis: peptides, sympathetic activity, and food intake. *Ann N Y Acad Sci* 1993;676:223.

Bray GA, Inoue S, Nishizawa Y. Hypothalamic obesity. The autonomic hypothesis and the lateral hypothalamus. *Diabetologia* 1981;20:366.

Brenner RR. Effect of unsaturated acids on membrane structure and enzyme kinetics. *Prog Lipid Res* 1984;23:69.

Broberger C, Holmberg K, Kuhar MJ, Hokfelt T. Cocaine- and amphetamine-regulated transcript in the rat vagus nerve: a putative mediator of cholecystokininb-induced satiety. *Proc Natl Acad Sci USA* 1999;96:13506.

Burgeura B, Couce ME, Curran GL, Jensen MD, Lloyd RV, Cleary MP, Poduslo JF. Obesity is associated with a decreased leptin transport across the blood-brain barrier in rats. *Diabetes* 2000;49:1219.

Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity in central neural networks. *Science* 1995;269:546.

Carrié I, Clément M, de Javel D, Francés H, Bourre, JM. Specific phospholipid fatty acid composition of brain regions in mice: effects of n-3 polyunsaturated fatty acid deficiency and phospholipid supplementation. *J Lipid Res* 2000;41:465.

Cha MC. 1998. Effects of Dietary Fat Selection and Energy Restriction on Tissue Lipid Metabolism: Structure, Function, and Regulation. Ph. D. Thesis. McGill University, Montrèal, QC.

Cha MC, Jones PJ. Dietary fat type and energy restriction interactively influence plasma leptin concentration in rats. *J Lipid Res* 1998;39:1655.

Chang GQ, Benyaminova L, Leibowitz SF. Dietary fat reduces expression of the leptin receptor, ob-R, in hypothalamic paraventricular (PVN) and arcuate (ARC) nuclei. *Soc Neurosci* 1999;25:414.

Chen H, Charlat O, Tartaglia LA, Woolf EA, Weng X, Ellis SJ, Lakey ND, Culpepper J, Moore KJ, Breitbart RE, Duyk GM, Tepper RI, Morgenstern JP. Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell* 1996;84:491.

Cheung CC, Clifton DK, Steiner RA. Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. *Endocrinol* 1997;138:4489.

Clandinin MT, Jumpsen J, Suh M. Relationship between fatty acid accretion, membrane composition, and biological functions. *J Peds* 1994;125:S25.

Collins S, Kuhn CM, Petro AE, Swick AG, Chrunyk BA, Surwit RS. Role of leptin in fat regulation. *Nature* 1996;380:677.

Cooling J, Barth J, Blundell J. The high-fat phenotype: is leptin involved in the adaptive response to a high fat (high energy) diet? *Int J Obes Relat Metab Disord* 1998;22:1606.

Dal Farra C, Zshrger N, Vincent JP, Cupo A. Binding of a pure ^{125}I-monoiodoleptin analog to mouse tissues: a developmental study. *Peptides* 2000;21:577.

Davidowa H, Plagemann A. Decreased inhibition by leptin of hypothalamic arcuate neurons in neonatally overfed young rats. *Neuroreport* 2000; 11:2795.

De Matteis R, Saverio C. Ultrastructural immunolocalization of leptin receptor in mouse brain. *Neuroendocrinol* 1998;68:412.

Devos R, Richards JG, Campfield LA, Tartaglia LA, Guisez Y, Van Der Heyden J, Travernier J, Plaetinck G, Burn P. OB protein binds specifically to the choroid plexus of mice and rats. *Proc Natl Acad Sci USA* 1996;93:5668.

Devos R, Guisez Y, Van Der Heyden J, White DW, Kalai M, Fountoulakis M, Plaetinck G. Ligand-independent dimerization of the extracellular domain of the leptin receptor and determination of the stoichiometry of leptin binding. *J Biol Chem* 1997;272:18304.

Diano S, Kalra SP, Horvath TL. Leptin receptor immunoreactivity is associated with the golgi apparatus of hypothalamic neurons and glial cells. *J Neuroendocrinol* 1998;10:647.

Ebihara K, Ogawa Y, Katsuura G, Numata Y, Masuzaki H, Satoh N, Tamaki M, Yoshioka T, Hayase M, Matsuoka N, Aizawa-Abe M, Yoshimasa Y, Nakao K. Involvement of agouti-related protein, an endogenous antagonist of hypothalamic melanocortin receptor, in leptin action. *Diabetes* 1999;48:2028.

El-Haschimi K, Pierroz DD, Hileman SM, Bjorbaek C, Flier JS. Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. *J Clin Invest* 2000;105:1827.

Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature* 1997;385:165.

Fei H, Okano HJ, Li C, Lee GH, Zhao C, Darnell R, Friedman JM. Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues. *Proc Natl Acad of Sci USA* 1997;94:7001.

Flier JS, Maratos-Flier E. Obesity and the hypothalamus: novel peptides for new pathways. *Cell* 1998;92:437.

Folch J, Lee M, Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 1957;226:479.

Fong TM, Huang RRC, Tota MR, Mao C, Smith T, Varnerin J, Karpitskiy VV, Krause JE, Van Der Ploeg LH. Localization of leptin binding domain in the leptin receptor. *Mol Pharmacol* 1998;53:234.

Funahashi H, Ryushi T, Mizushima H, Katoh S, Shioda S. Ultrastructural localization of the receptor for leptin in the rat hypothalamus. *Horm Behav* 2000;37:327.

Gavrilova NJ, Setchenska MS, Markovska TT, Momchilova-Pankova AB, Koumanov KS. Effect of membrane phospholipid composition and fluidity on rat liver plasma membrane tyrosine kinase activity. *Int J Biochem* 1993;25:1309.

Gearing DP, King JA, Gough NM, Nicola NA. Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor. *Embo J* 1989;8:3667.

Ghilardi N, Ziegler S, Wiestner A, Stoffel R, Heim MH, Skoda RC. Defective STAT signaling by the leptin receptor in diabetic mice. *Proc Natl Acad Sci USA* 1996;93:6231.

Ghilardi N, Skoda RC. The leptin receptor activates janus kinase 2 and signals for proliferation in a factor-dependent cell line. *Mol Endocrinol* 1997;11:393.

Gibson RA, McMurchie EJ, Charnock JS, Kneebone GM. Homeostatic control of membrane fatty acid composition in the rat after dietary lipid treatment. *Lipids* 1984;19:942.

Golden PL, Maccagnan TJ, Pardridge WM. Human blood-brain-barrier leptin receptor. *J Clin Invest* 1997;99:14.

Goodwin RG, Friend D, Ziegler SF, Jerzy R, Falk BA, Gimpel S, Cosman D, Dower SK, March CJ, Namen AE, Park LS. Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. *Cell* 1990;60:941.

Hakansson ML, Meister B. Transcription factor STAT3 in leptin target neurons of the rat hypothalamus. *Neuroendocrinol* 1998;68:420.

Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM. Weight regulation effects of the plasma protein encoding the obese gene. *Science* 1995;269:543.

Haynes WG, Sivitz W, Morgan DA, Walsh SA, Mark AL. Sympathetic and cardiorenal actions of leptin. *Hypertension* 1997;30:619.

Haynes WG, Morgan DA, Djalali A, Sivitz WI, Mark AL. Interactions between the melanocortin system and leptin in control of sympathetic nerve traffic. *Hypertension* 1999;33:542.

Hill CS, Treisman R. Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell* 1995;80:199.

Hiroike T, Higo J, Jingami H, Toh H. Homology modeling of human leptin/leptin receptor complex. *Biochem Biophys Res Commun* 2000;275:154.

Holub BJ and Kuksis A. Metabolism of molecular species of diacylglycerophospholipids. *Lipids Res* 1978;16:1.

Huang XF, Lin S, Zhang R. Upregulation of leptin receptor mRNA expression in obese mouse brain. *Neuroreport* 1997;8:1035.

Huang Q, Viale A, Picard F, Nahon J, Richard D. Effects of leptin on melanin-concentrating hormone expression in the brain of lean and obese Lep(ob)/Lep(ob) mice. *Neuroendocrinol* 1999;69:145.

Hun CS, Hasegawa K, Kawabata T, Kato M, Shimokawa T, Kagawa Y. Increased uncoupling protein 2 mRNA in white adipose tissue, and decrease in leptin, visceral fat, blood glucose, and cholesterol in KK-A^y mice fed with eiscosapentanoic and

docosahexanoic acids in addition to linoleic acid. *Biochem Biophys Res Commun* 1999;259:85.

Idzerda RL, March CJ, Mosley B, Lyman SD, Vanden Bos T, Gimpel SD, Din WS, Grabstein KH, Widmer MB, Park LS, Cosman D, Beckmann MP. Human interleukin 4 receptor confers biological responsiveness and defines a novel receptor superfamily. *J Exp Med* 1990;171:861.

Innis SM, Clandinin MT. Dynamic modulation of mitochondrial inner-membrane lipids in rat heart by dietary fat. *Biochem J* 1981;193:155.

Itoh N, Yohehara S, Schreurs J, Gorman DM, Maruyama K, Ishii A, Yahara I, Arai, K. Cloning of an interleukin-3 receptor gene: a member of a distinct receptor gene family. *Science* 1990;247:324.

Juaneda P, Rocquelin G. Rapid and convenient separation of phospholipids and non phosphorus lipids from rat heart using silica cartridges. *Lipids* 1985;20:40.

Kapitonov VV, Jurka J. Letters to the editor - The long terminal repeat of an endogenous retrovirus induces alternative splicing and encodes an additional carboxy-terminal sequence in the human leptin receptor. *J Mol Evol* 1999;48:248.

Kinsella JE. Lipids, membrane receptors, and enzymes: effects of dietary fatty acids. *JPEN* 1990;14:200S.

Koch CA, Andersen DJ, Moran MF, Ellis CA, Pawson T. SH2 and SH3 domains: elements that control interactions of the cytoplasmic signaling proteins. *Science* 1991;252:668.

Koylu EO, Couceyro PR, Lambert PD, Ling NC, DeSouza EB, Kuhar MJ. Immunohistochemical localization of novel CART peptides in rat hypothalamus, pituitary and adrenal gland. *J Neuroendocrinol* 1997;9:823.

Kristensen P, Judge ME, Thim L, Ribeke V, Christiansen KN, Wulff BS, Clausen JT, Jensen PB, Madsen OD, Vrang N, Larsen PJ, Hastrup S. Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature* 1998;393:72.

Lambert PD, Couceyro PR, McGirr KM, Dall Vechia SE, Kuhar MJ. CART peptides in the central control of feeding and interactions with neuropeptide Y. *Synapse* 1998;29:293.

Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, Friedman JM. Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 1996;379:632.

Li C, Friedman JM. Leptin receptor activation of SH2 domain containing protein tyrosine phosphatase 2 modulates Ob receptor signal transduction. *Proc Natl Acad Sci USA* 1999;96:9677.

Lin L, Martin R, Schaffhauser AO, York DA. Acute changes in the response to peripheral leptin with alteration in the diet composition. *Am J Physiol Regul Integr Comp Physiol* 2001;280:R504.

Lin S, Huang XF. Fasting increases leptin receptor mRNA expression in lean but not obese (ob/ob) mouse brain. *Neuroreport* 1997;8:3625.

Lin S, Storlien LH, Huang X. Leptin receptor, NPY, POMC mRNA expression in the diet-induced obese mouse brain. *Brain Res* 2000a;875:89.

Lin S, Thomas TC, Storlien LH, Huang XF. Development of high fat diet-induced obesity and leptin resistance in C57B1/6J mice. *Int J Obes Rel Metab Disord* 2000b;24:639.

Lynch DV, Thompson GA. Retailored lipid molcular species: a tactical mechanism for modulating membrane proteins. *Trans Biochem Sci* 1984;9:442.

Marteinsdottir I, Horrobin DF, Stenfors C, Theodorsson E, Math AA. Changes in dietary fatty acids alter phospholipid fatty acid composition in selected regions of rat brain. *Prog Neuro-Psychopharmacol & Biol Psychiat* 1998;22:1007.

Masuzaki M, Ogawa Y, Isse N, Satoh N, Okazaki I, Tamura N, Yoshimasa Y. Human obese gene expression. Adipocyte-specific expression and regional differences in adipose tissue. *Diabetes* 1995;44:855.

Matsuo T, Sumida H, Suzuki M. Beef tallow diet decreases norepinephrine turnover rates in rat hypothalamus and cerebral cortex. *Metabolism* 1995;44:1377.

Mercer JG, Hoggard N, Williams LM, Lawrence CB, Hannah LT, Trayhurn P. Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybrization. *FEBS Lett* 1996;387:113.

Metcalfe LD, Schmitz AA. The rapid preparation of fatty acid esters for gas chromatography analysis. *Anal Chem* 1961;33:363.

Meydani SN. Effect of (n-3) polyunsaturated fatty acids on cytokine production and their biological function. *Nutrition* 1996;12:S8.

Miller SM, Schmalz PF, Benarroch EE, Szurszewski JH. Leptin receptor immunoreactivity in sympathetic prevertebral ganglion neurons of mouse and rat. *Neurosci Lett* 1999;265:26194.

Misra A, Garg A. Leptin, its receptor and obesity. *J Invest Med* 1996;44:540.

Mizuno TM, Kleopoulos SP, Bergen HT, Roberts JL, Priest CA, Mobbs CV. Hypothalamic proopiomelanocortin mRNA is reduced by fasting and [corrected] in ob/ob and db/db mice, but is stimulated by leptin. *Diabetes* 1998;47:294.

Mobbs C, Mizuno T. Leptin regulation of proopiomelanocortin. *Front Horm Res* 2000;26:57.

Murakami T, Yamashita T, Iida M, Kuwajima, Shima K. A short form of leptin receptor performs signal transduction. *Biochem Biophys Res Commun* 1997;231:26.

Nagatani S, Guthikonda P, Foster DL. Appearance of a nocturnal peak of leptin secretion in the pubertal rat. *Horm Behav* 2000;37:345.

Nassar BA, Huang YS, Manku MS, Das UN, Morse N, Horrobin DF. The influence of dietary manipulation with n-3 and n-6 on liver and plasma phospholipid fatty acids in rats. *Lipids* 1986;21:652.

Patthy L. Homology of a domain of the growth hormone/prolactin receptor family with type III modules of fibronectin. *Cell* 1990;61:13.

Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F. Effects of the obese gene product on body weight regulation in *ob/ob* mice. *Science* 1995;269:540.

Raclot T, Groscolas R, Langin D, Ferre P. Site-specific regulation of gene expression by n-3 polyunsaturated fatty acids in rat white adipose tissues. *J Lipid Res* 1997;38:1963.

Saladin R, De Vos P, Guerre-Millo M, Leturque A, Girard J, Staels B, Auwerx J. Transient increase in obese gene expression after food intake or insulin administration. *Nature* 1995;377:527.

Schwartz MW, Baskin DG, Bukowski TR, Kuijper JL, Foster D, Lasser G, Prunkard DE, Porte, Jr. D, Woods SC, Seeley RJ, Weigle DS. Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in ob/ob mice. *Diabetes* 1996a;45:531.

Schwartz MW, Seeley RJ, Campfield LA, Burn P, Baskin DG. Identification of targets of leptin action in rat hypothalamus. *J Clin Invest* 1996b;98:1101.

Schwartz MW, Seeley RJ, Woods SC, Weigle DS, Campfield LA, Burn P, Baskin DG. Leptin increases hypothalamic proopiomelanocortin mRNA expression in the rostral arcuate nucleus. *Diabetes* 1998;46:2119.

Sengupta TK, Schmitt EM, Ivashkiv LB. Inhibition of cytokines and JAK-STAT activation by distinct signaling pathways. *Proc Natl Acad Sci USA* 1996;93:9499.

Sevetson BR, Kong X, Lawrence, Jr. JC. Increasing cAMP attenuates activation of mitogen-activated protein kinase. *Proc Natl Acad Sci USA* 1993;90:10305.

Shimizu Y, Tanishita T, Minokoshi Y, Shimazu T. Activation of mitogen-activated protein kinase by norepinephrine in brown adipocytes from rats. *Endocrinology* 1997;138:248.

Shiraishi T, Sasaki K, Niijima A, Oomura Y. Leptin effects on feeding-related hypothalamic and peripheral neuronal activities in normal and obese rats. *Nutrition* 1999;15:576.

Smith FJ, Campfield LA, Moschera JA, Bailon P, Burn P. Feeding inhibition by neuropeptide Y. *Nature* 1996;382:307.

Songyang Z, Shoelson SE, Chaudhuri M, Gish GD, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, Lechleider RJ, Neel BG, Birge RB, Fajoudo JE, Chou MM, Hanafusa H, Schaffhausen B, Cantley LC. SH2 domains recognize specific phosphopeptide sequences. *Cell* 1993;72:767.

Stanley BG, Magdalin W, Seirafi A, Nguyen MM, Leibowitz SF. Evidence for neuropeptide Y mediation of eating produced by food deprivation and for a variant of the Y1 receptor mediating this peptide's effect. *Peptides* 1992;13:581.

Stromberg H, Svenson SPS, Hermanson O. Distribution of the transcription factor signal transducer and activator of transcription 3 in the rat central nervous system and dorsal root ganglia. *Brain Res* 2000;853:105.

Takahashi Y, Okimura Y, Mizuno I, Iida K, Takahashi T, Kaji H, Abe H, Chihara K. Leptin induces mitogen-activated protein kinase-dependent proliferation of C3H10T1/2 cells. *J Biol Chem* 1997;272:12897.

Tappia PS, Ladha S, Clark DC, Grimble RF. The influence of membrane fluidity, TNF receptor binding, cAMP production and GTPase activity on macrophage cytokine production in rats fed a variety of fat diets. *Mol Cell Biochem* 1997;166:135.

Tartaglia LA. The leptin receptor. *J Biol Chem* 1997;272:6093.

Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore KJ, Smutko JS, Mays GG, Woolf EA, Monroe CA, Tepper RI. Identification and expression cloning of a leptin receptor, OB-R. *Cell* 1995;83:1263.

Thornton JE, Cheung CC, Clifton DK, Steiner RA. Regulation of hypothalamic proopiomelanocortin mRNA by leptin in ob/ob mice. *Endocrinology* 1997;138:5063.

Ur E, Grossman A, Depres JP. Obesity results as a consequence of glucocorticoid induced leptin resistance. *Horm Metab Res* 1996;28:744.

Vaisse C, Halaas JL, Horvath CM, Darnell, Jr. JE, Stoffel M, Friedman JM. Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. *Nature Gen* 1996;14:95.

Vrang N, Larsen PJ, Kristensen P, Tang-Christensen M. Central administration of cocaine-amphetamine-regulated transcript activates hypothalamic neuroendocrine neurons in the rat. *Endocrinology* 2000;141:794.

Wang H, Storlien LH, Huang X. Influence of dietary fats on c-Fos-like immunoreactivity in mouse hypothalamus. *Brain Res* 1999a;843:184.

Wang ZW, Zhou YT, Kakuma T, Lee Y, Higa M, Kalra SP, Dube MG, Kalra PS, Unger RH. Comparing the hypothalamic and extrahypothalamic actions of endogenous hyperleptinemia. *Proc Natl Acad Sci USA* 1999b;96:10373.

Wang Q, Bing C, Al-Barazanji K, Mossakowaska DE, Wang XM, McBay DL, Neville WA, Taddayon M, Pickavance L, Dryden S, Thomas ME, McHale MT, Glover IS, Wilson S, Buckingham R, Arch JR, Trayhurn P, Williams G. Interactions between leptin and hypothalamic neuropeptide Y neurons in the control of food intake and energy homeostasis in the rat. *Diabetes* 1997;46:335.

Watson PM, Commins SP, Beiler RJ, Hatcher HC, Gettys TW. Differential regulation of leptin expression and function in A/J vs. C57BL/6J mice during diet-induced obesity. *Am J Physiol Endocrinol Metab* 2000;279:E356.

Widdowson PS, Wilding JPH. Hypothalamic neuropeptide Y and its neuroendocrine regulation by leptin. *Front Horm Res* 2000;26:71.

Widdowson PS, Upton R, Buckingham R, Arch J, Williams G. Inhibition of food response to intracerebroventricular injection of leptin is attenuated in rats with diet-induced obesity. *Diabetes* 1997;46:1782.

Wilkinson M, Morash B, Ur E. The Brain is a source of leptin. *Front Horm Res* 2000;26:105.

Wolf G. Neuropeptides responding to leptin. *Nutr Rev* 1997;55:85.

Yamashita Y, Murakami T, Otani S, Kuwajima M, Shima K. Leptin receptor signal transduction: OBRa and OBRb of fa type. *Biochem Biophys Res Commun* 1998;246:752.

Yeagle PL. Lipid regulation of cell membrane structure and function. *FASEB J* 1989;3:1833.

Zhang Y, Proenca M, Maffei M, Barone M, Leopold L, Friedman J. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994;372:425.

Zhang Y, Olbort M, Schwarzer K, Nuesslein-Hildesheim B, Nicolson M, Murphy E, Kowalski TJ, Schmidt I, Leibel RL. The leptin receptor mediates apparent autocrine regulation of leptin gene expression. *Biochem Biophys Res Commun* 1997;240:492.

Zhang Y, Hufnagel C, Eiden S, Guo KY, Diaz PA, Leibel R, Schmidt I. Mechanisms for LEPR-mediated regulation of leptin expression in brown and white adipocytes in rat pups. *Physiol Genomics* 2001;4:189.