The Role of Hypothalamic Neuropeptides in the Regulation of Food Intake and Body Weight

BY

Jason James Taylor

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ABSTRACT

Hypothalamic neuropeptides, such as neuropeptide Y (NPY) produced by neurons in the arcuate nucleus (ARC) and alpha-melanocyte stimulating hormone (α-MSH, a post-translational cleavage product of proopiomelanocortin, POMC) produced by neurons in both the ARC and the ventromedial nucleus (VMN), appear to be important in regulating food intake and body weight. Specifically, NPY (acting via NPY receptors) stimulates food intake and increases body weight gain while α-MSH (acting via melanocortin receptors) inhibits food intake and decreases body weight gain. These observations have led to the hypothesis that obesity may be a result of an increase in the activity of hypothalamic NPY neurons and/or a decrease in the activity of hypothalamic POMC neurons. It has been hypothesized that altered hypothalamic neuropeptide signaling may play a role in both genetic obesity (e.g., *ob/ob* mouse) and hypothalamic obesity, which occurs as a result of cellular destruction in the VMN (e.g. gold thioglucose (GTG)-induced obesity). Leptin is a protein produced by fat cells that plays a critical role in regulating food intake and body weight (e.g., leptin deficient *ob/ob* mice are obese). It has been proposed that leptin acts on the hypothalamus to decrease food intake and decrease body weight gain and that these effects are a result of inhibition of hypothalamic NPY neurons and/or stimulation of hypothalamic POMC neurons. Therefore, three studies, employing different mouse models of obesity, were undertaken to examine the roles of NPY neurons, POMC neurons, and leptin in the regulation of food intake and weight gain. Study I assessed the hypothesis that NPY neurons are important in promoting GTG-induced hypothalamic obesity. C57Bl/6J mice (n=10) were treated

neonatally with monosodium glutamate (MSG), a compound that destroys hypothalamic NPY neurons, and subsequently treated with GTG (as adults) to induce hypothalamic obesity. Destruction of NPY neurons by MSG did not prevent GTG-induced hyperphagia and weight gain. The results strongly suggest that NPY does not play a role in mediating GTG-induced obesity. Study II assessed the hypotheses that: 1) the VMN is particularly sensitive to the effects of leptin on food intake and body weight, and 2) GTG-induced obesity is associated with destruction of leptin-sensitive VMN neurons. The C57Bl/6J mice (n=16) received low-dose infusions of leptin (0.01μg) directly into the VMN which produced decreases in food intake and body weight comparable to that observed in mice treated with higher intracerebroventricular doses of leptin. Treatment of the mice (n=10) with GTG destroyed VMN neurons and subsequent infusions of leptin failed to produce changes in food intake and body weight. These results suggest that the VMN is particularly sensitive to leptin and the hypothalamic obesity induced by GTG is due to leptin resistance. Study III assessed the hypotheses that: 1) melanocortinergic receptors mediate the effects leptin on food intake and body weight, and 2) upregulation of melanocortinergic receptors mediates the enhanced sensitivity of *ob/ob* mice to leptin. The results of Study III suggest that melanocortinergic neurons are important mediators of leptin action in both lean and *ob/ob* mice and that the increased leptin-sensitivity observed in *ob/ob* mice is not a result of upregulation of melanocortinergic receptors.

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LIST OF ABBREVIATIONS

AGRP	Agouti related peptide
α-MSH	Alpha-melanocyte stimulating hormone
ANOVA	Analysis of variance
ARC	Arcuate nucleus
ATP	Adenosine triphosphate
BW	Body weight
DMN	Dorsomedial nucleus
EtOH	Ethyl alcohol
GTG	goldthioglucose
ICV	intracerebroventricular
i.p.	intraperitoneal
Lep	Leptin
LH	Lateral hypothalamus
LSD	Least squares difference
MBH	Mediobasal hypothalamus
mRNA	Messenger ribonucleic acid
MSG	Monosodium glutamate
NPY	Neuropeptide Y
POMC	Proopiomelanocortin
PVN	Paraventricular nucleus
Sal	Saline
SSC	Standard saline citrate
VMH	Ventromedial hypothalamus
VMN	Ventromedial nucleus
2-DG	2-deoxy-glucose

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INTRODUCTION

1. HOMEOSTATIC MECHANISMS REGULATE BODY WEIGHT.

1.1 Homeostatic mechanisms regulate physiological processes.

Physiological processes carried out within cells and tissues within the body appear to be closely monitored and regulated. In 1878, Claude Bernard proposed that an organism lives not only in an external environment, but its cells and tissues also live in an internal environment [1]. Almost fifty years later, Cannon coined the term "homeostasis" to describe the mechanisms regulating Bernard's "internal environment" [2]. Homeostasis is maintained through a control system that maintains the value of a measurable variable within a certain range. The hypothetical framework of a control system generally consists of a detector and "controlling elements" or "effectors". The detector compares the value of a variable to a pre-determined "set-point". If the detector determines that the value of the variable does not equal the set-point, the detector generates a signal that alters the activity of the effectors in such a way as to return the value of the variable to the set-point.

1.2. Body weight is defended around a set-point.

Homeostatic mechanisms may regulate body weight since it appears to be maintained around a set point. For example, body weight in adult humans is stable or increases slowly at an average rate of 0.2 kilograms per year, representing an excess of energy consumption over energy expenditure of less than 0.2% of total energy intake [3]. This small deviation between energy intake and energy expenditure is clearly suggestive of a regulatory system that maintains body weight around a set-point. In animals, after

fasting and the consequent decrease in body weight, food intake increases until the original body weights are restored [4]. Conversely, after force feeding and the consequent increase in body weight, food intake decreases until the original body weights are restored [5]. Thus, it appears that food intake is one homeostatic mechanism involved in regulating body weight around a set point.

However, control of food intake does not fully account for body weight stability. Fasting does not produce weight loss commensurate with the decrease in caloric intake and force feeding fails to produce weight gain commensurate with the increase in caloric intake [5]. In addition, increasing caloric intake (e.g., force feeding) does not yield the same degree of obesity between different strains of rats or mice, or even between members of the same strain [6, 7, 8]. Moreover, weight gain can occur without hyperphagia, and weight loss can occur without hypophagia [9]. Thus, changes in body weight are not solely a result of changes in food intake.

Several lines of evidence indicate that control of energy expenditure is another important component of body weight regulation. For example, in genetically obese mice, obesity precedes hyperphagia, and occurs even if food intake is experimentally decreased [10, 11]. Genetically obese mice exhibit an increased rate of weight gain as compared to their lean littermates even when the effects of hyperphagia are factored out; the obese mice seem to be more energy efficient [12].

It is hypothesized that coordination of food intake and energy expenditure accounts for the regulation of body weight around a set-point. This hypothesis predicts that deviations from the body weight set-point will effect changes in food intake and energy expenditure that will have the net effect of returning body weight to the original

set-point. For example, in response to a decrease in body weight, the prediction is that food intake will be increased and energy expenditure will be decreased until body weight is restored. Similarly in response to an increase in body weight, the prediction is that food intake will be decreased and energy expenditure will be increased until body weight is restored. Numerous studies have provided evidence that body weight is regulated at a particular set-point and that this occurs through coordinated homeostatic mechanisms involving food intake and energy expenditure [3].

1.3. The hypothalamus coordinates homeostatic mechanisms.

The hypothalamus, a small area of the diencephalon comprising less than 1% of brain volume, appears to contain the neural circuitry critical for maintaining homeostasis [1, 13, 14]. It is generally considered that the hypothalamus plays a critical role in regulating the activity of three separate systems. These three functional systems are 1) the endocrine system, 2) the autonomic nervous system, and 3) a poorly understood neural system concerned with motivation and behavior [1]. The hypothalamus acts directly on the internal environment through the endocrine and autonomic systems, and indirectly through motivation and behavior. For example, when an organism's energy stores are depleted, endocrine and autonomic mechanisms inhibit non-vital activities and the organism seeks out food (i.e., there is a change in motivation and behavior) to replenish its energy stores. Since the regulation of body weight appears to involve the 3 functional systems that are regulated by the hypothalamus, it seems likely that the hypothalamus is involved in regulating body weight.

2. HYPOTHALAMIC ELEMENTS REGULATE BODY WEIGHT.

2.1. Early work discovers hypothalamic "satiety" and "feeding" centers.

The importance of the hypothalamus in regulating body weight was clearly established in 1940 by Hetherington and Ranson using stereotaxic surgery to place electrolytic lesions in the hypothalamus of the rat [15]. Lesions of the mediobasal hypothalamus (MBH) resulted in hyperphagia and obesity, and lesions of the lateral hypothalamus (LH) resulted in hypophagia and weight loss [15, 16]. These observations led to the construction of the "dual center" model of body weight regulation [16, 17]. In this model, the MBH, termed the "satiety center", contains neurons that mediate satiety while the LH, termed the "feeding center" contains neurons that mediate feeding. Lesions in the MBH result in hyperphagia, electrical stimulation of the MBH suppresses food intake [18]. In contrast, lesions in the LH result in hypophagia, but electrical stimulation of the LH can induce food intake [19]. Therefore, in the case of an MBH lesion, satiety is decreased and inhibitory tone to the LH is removed. Together, these effects may lead to hyperphagia and obesity.

The hypothalamic obesity that results from damage to the MBH displays a characteristic biphasic pattern of weight gain. Following damage to the MBH, there is a short period of rapid gain in body weight associated with hyperphagia. After a period of time, the rate of gain in body weight slows until it plateaus, and food intake returns to near pre-lesion levels. Thus, hypothalamic obesity progresses from a dynamic phase characterized by a rapid gain in body weight to a static phase characterized by maintenance of body weight around a newly established set-point [4].

The techniques used by early researchers such as Hetherington and Ranson did not target the specific neurons that may be involved in food intake and body weight regulation. For example, mechanical and electrolytic lesions destroy neurons within the target area as well as fibers of passage originating from distant sites that pass through the targeted area. Thus, with mechanical or electrolytic lesions, both cell bodies and axons are damaged and it is difficult to determine which is responsible for the effects observed after the lesion. In order to distinguish between these 2 possibilities, neurotoxic excitatory amino acids (ibotenic acid and kainic acid) were used to selectively destroy cell bodies in the MBH while they spared fibers of passage [20, 21]. Similar to electrolytic lesions of the MBH, destruction of cell bodies intrinsic to the MBH resulted in hyperphagia and obesity [20, 21]. In addition, chemical agents such as gold thioglucose (GTG) and monosodium glutamate (MSG), which also selectively lesion neuronal cell bodies within the MBH but not fibers passing through, have been used be used to determine the role of neurons in the MBH in the regulation of energy balance.

2.2. GTG lesions the ventromedial nucleus, causing hypothalamic obesity.

Gold thioglucose is a toxic chemical agent that lesions neurons in the ventromedial nucleus (VMN) of the mediobasal hypothalamus when it is injected intraperitoneally in mice. The chemical lesion, like an electrolytic lesion of the VMN, causes hyperphagia and obesity. Among gold thio-compounds of closely related structure (e.g., gold thiogalactose and gold-thiosorbitol) only GTG causes a necrotic lesion, suggesting that the glucose moiety of GTG is essential for production of the lesion [22]. In support of this finding, it has been shown that pre-treatment with glucose analogues

such as sodium thioglucose and 2-Deoxy-D-glucose (2-DG) prevent the induction of a VMN lesion by GTG [22]. Infusion of phlorizin, which, like 2-DG, has been shown to inhibit glucose uptake in cells, also prevents GTG-induced necrosis of the VMN [22]. These observations suggest that GTG enters cells of the VMN through a pathway normally used for glucose entry. Whereas infusion of a glucose uptake inhibitor increases food intake, acute elevation of plasma glucose decreases food intake [22, 23]. These findings are consistent with the hypothesis that glucose utilization by glucose-responsive cells of the VMN is involved in the regulation of food intake [22]. In addition, GTG-treated mice are insensitive to glucose and glucopenia [24]. Taken together, these studies suggest that GTG targets glucose-responsive neurons of the VMN. Destruction of these glucose-responsive neurons (as with GTG) not only results in hyperphagia, but also obesity suggesting that they play an important role in short-term food intake and long-term body weight regulation. [24].

The most striking consequence of a single intraperitoneal (i.p.) injection of GTG is an initial rapid increase in body weight and food intake. Like other treatments that damage the MBH and cause hypothalamic obesity, GTG induces a biphasic pattern of body weight gain. A dynamic phase of rapid weight gain and hyperphagia lasts 10-15 days after treatment and is followed by a static phase of slower weight gain and declining food intake, which plateau 30-60 days later [25]. During the dynamic phase, GTG treatment produces increased body fat through increases in adipocyte size [26]. Since GTG does not seem to affect adipocytes directly, changes in fat cell metabolism appear to be due to hypothalamic damage induced by GTG [27, 28]. The changes in the hypothalamus following GTG treatment appear to be long-term since the body weights of

GTG-obese mice return to post-GTG levels following a fast [25]. These observations suggest that the patterns of food intake and body weight gain characteristic of hypothalamic obesity following GTG treatment are due to lasting changes in the hypothalamus, resulting in the establishment of a new body weight set point. Thus, GTG is a useful tool for examining the role of the VMN in body weight regulation. In addition to the VMN, the arcuate nucleus (ARC) of the MBH also plays an important role in body weight regulation. As with GTG and the VMN, MSG appears to be a useful tool for examining the role of the ARC in body weight regulation.

2.3. Monosodium glutamate lesions the arcuate nucleus.

MSG is the monosodium salt of L-glutamate, a straight chain amino acid that can excite neuronal cell membranes. After binding to specific membrane receptors, glutamate, administered systemically, can act as an excitotoxin, causing membrane depolarization, increased intracellular sodium and water, and decreased ATP levels [29b]. Intracellular potassium leaves the cell to become concentrated in the extracellular matrix, further depolarizing the cell until the cell eventually dies [29b]. Studies demonstrate that glutamate accumulates in the arcuate nucleus and arcuate neurons are susceptible to its neurotoxic effects presumably because they express the appropriate glutamate receptor [29c].

Like glutamate, MSG's neurotoxicity is a result of membrane receptor binding after systemic administration, and subsequent depolarization of neuronal cell membranes leading to cell death [29]. Membrane depolarization increases the influx of sodium and water which depletes intracellular potassium stores and further depolarizes the cell and

disrupts its ionic regulatory mechanisms [29d]. Consequently, the cell eventually dies and necrosis begins, followed by macrophage infiltration and neuronophagia [29,30, 31]. Glial cells and axons, which lack the appropriate receptor, are spared from the toxic effects of MSG [29e].

The MSG lesion induced by systemic administration to neonates is restricted to the ARC, a hypothalamic site previously implicated in the regulation of body weight [29c]. MSG sensitivity disappears after 10 days of age, and adult rats are resistant to i.p. injection of MSG [30]. The blood brain barrier, which is thought to be poorly developed until post-natal day 10 in rats, prevents entry of peripheral substances into the central nervous system. Thus, it appears that MSG can cross the immature blood-brain barrier and enter the hypothalamus to lesion the arcuate nucleus [30, 31, 32].

3. LEPTIN REGULATES BODY WEIGHT VIA THE HYPOTHALAMUS.

3.1. A circulating satiety signal acts at the level of the hypothalamus.

In 1952, Hervey predicted the existence of a circulating satiety signal through his observation that, in a parabiotic rat pair that share a common circulatory system, a VMH-lesioned rat becomes hyperphagic and obese, while its unlesioned parabiotic control becomes hypophagic and loses body weight [33]. The lesioned rat is insensitive to the increasing levels of satiety signal it produces as it becomes obese. However, the unlesioned rat remains sensitive to the increased signal delivered to it through the parabiotic union and becomes hypophagic. Coleman's study of two mutant mouse strains, obese (*ob/ob*) and diabetic (*db/db*), provided further evidence of a circulating satiety signal [34, 35]. Both strains exhibit hyperphagia and obesity, decreased body

temperature, increased energy efficiency, and infertility [36]. A parabiotic union of a *db/db* and normal mouse results in hypophagia in the normal; whereas parabiosis of two *db/db* mice has no effect [34]. It was proposed that, a *db/db* mouse produces, but cannot respond to, a circulating satiety signal that inhibits food intake in a normal mouse. Union of an *ob/ob* and *db/db* mouse results in hypophagia in the *ob/ob* mouse, suggesting that the *db/db* mouse provides a circulating satiety signal to the *ob/ob* parabiont [35]. Coleman hypothesized that *ob/ob* mice do not produce a circulating satiety signal, whereas *db/db* mice produce the signal but cannot respond to it [36]. The recent discovery of a circulating protein encoded by the *ob* gene that induces satiety in *ob/ob* but not *db/db* mice suggests that Coleman's predictions were correct.

3.2. The ob gene encodes the body weight regulating protein leptin.

In 1994, Zhang and colleagues cloned and sequenced the *ob* gene expressed in mouse and human adipose tissue, finding 84% homology between species [37]. The *ob* gene transcribes a 4.5 kb mRNA sequence encoding a 167 amino-acid protein [37]. A 21 amino acid N-terminal sequence directs the protein into the secretory pathway where it is subsequently removed, leaving a 146 amino acid protein named leptin (leptos (Greek): thin) [37, 38]. Mutation of the *ob* gene in *ob/ob* mice produces an inactive protein and leads to obesity, suggesting that leptin is Coleman's "satiety signal" [37]. Consistent with this suggestion, it was demonstrated that in *ob/ob* and normal mice, leptin treatment decreases food intake and body weight in a dose- and time-dependent manner [38, 39, 40, 41]. In addition to its effects on food intake, leptin treatment is also associated with increased thermogenesis and a higher metabolic rate [38, 39, 42]. As predicted, leptin

treatment has no effect in *db/db* mice suggesting that the *db/db* phenotype is due to mutation of the leptin receptor [38, 40]. This was subsequently confirmed when the genetic defect of the *db/db* mouse was identified as a mutation in the gene encoding the leptin receptor [43].

Leptin mRNA is expressed at high levels in adipocytes, and at much lower levels in other tissues including muscle and placenta [44, 45, 46, 47]. Leptin circulates in the blood at levels proportional to adipose tissue mass [38]. Serum leptin levels fall in people and mice during weight loss and rise during weight gain [45]. If leptin levels rise above a set-point, further weight gain may be inhibited by decreased food intake and increased energy expenditure. This dynamic mechanism accounts for the stability of body weight and its defense normally observed in people and mice. Serum leptin levels are elevated in most obese people and several animal models of obesity (e.g. *db/db*, GTG- and MSG-treated mice) suggesting that some obesities are due to leptin resistance [45, 48, 49]. Taken together, these observations suggest that leptin may be an important signal for coordinating energy balance. In support of this hypothesis, leptin appears to play an important role in the neuroendocrine response to starvation.

3.3. Leptin coordinates the neuroendocrine response to starvation.

In response to starvation, an animal exhibits neuroendocrine abnormalities such as hypercorticosteronemia, hypothyroidism, infertility, decreased thermogenesis and energy expenditure, and decreased immune function. Genetically obese *ob/ob* mice also exhibit these abnormalities [50]. These neuroendocrine abnormalities in *ob/ob* mice are reversed by leptin treatment suggesting that the absence of leptin is responsible for these effects

[38, 39, 51, 52]. In addition, leptin treatment during a fast blunts the neuroendocrine response to starvation [49, 53, 54, 55]. Taken together, these observations suggest that when leptin levels fall (as occurs in starvation) physiological responses are initiated to conserve energy and promote food-seeking behaviors.

3.4. The brain is an important target of leptin action.

Initial experiments demonstrated that central administration of leptin requires a much lower dose than systemic administration to produce the same effects on food intake, energy expenditure, and body weight [40]. Thus, the brain is a likely target of leptin action, and researchers have quickly searched for and isolated the leptin receptor (Ob-R) from mouse choroid plexus [61]. The effects of leptin are mediated by interactions with a receptor encoded by the Ob-R gene that is alternatively spliced, resulting in at least five different forms: Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, and Ob-Re [43, 61, 62]. All five are cytokine receptors and have extracellular leptin-binding domains [43, 61, 62]. Three (Ob-Ra, Ob-Rc, and Ob-Rd), have intracellular domains, but lack all the protein motifs necessary for signal transduction [61, 62]. Ob-Re has no intracellular domain and may be a soluble protein [62]. Only Ob-Rb encodes all the protein motifs necessary for signal transduction in the hypothalamus [43]. Ob-Rb is expressed at high levels in the hypothalamus of mice, rats, and people [63, 64, 65, 66]. Two animal models of obesity (*db/db* mouse and *fa/fa* rat) have mutations in the Ob-Rb gene and are insensitive to leptin treatment suggesting that hypothalamic nuclei expressing OB-Rb receptors are necessary for the regulation of food intake and body weight by leptin [67, 68].

A number of studies have demonstrated that Ob-Rb receptors are expressed at high levels by hypothalamic nuclei such as the ARC and VMN [63, 64, 65, 69, 70, 71]. Peripheral administration of leptin induces c-fos expression at these sites as well [72, 73]. Lesioning these nuclei leads to leptin resistance and hypothalamic obesity [74]. These observations suggest that hypothalamic nuclei such as the ARC and VMN are important sites of the effects of leptin on food intake and body weight regulation. Both of these sites contain cells that produce neuropeptides that have been implicated in food intake and body weight regulation [57]. For example, the ARC contains cells that express neuropeptide Y (NPY), a potent stimulator of food intake, whereas the VMN contains cells that express proopiomelanocortin (POMC), a polypeptide precursor implicated in inhibition of food intake [57]. The understanding of neuropeptide regulation of food intake and body weight has been greatly advanced by the discovery of leptin. It appears that neuropeptidergic cells, such as those that produce NPY and POMC, are major targets through which leptin exerts its regulatory effects on food intake and body weight.

4. HYPOTHALAMIC NEUROPEPTIDES MEDIATE THE EFFECTS OF LEPTIN.

4.1. Hypothalamic neuropeptide Y neurons may mediate the effects of leptin.

Neuropeptide Y is a 36 amino acid peptide that has been implicated as a key neurotransmitter for the regulation of food intake and body weight [57]. It is synthesized throughout the brain but is particularly abundant in the hypothalamus. Within the hypothalamus, NPY is synthesized largely in neurons within the ARC. These neurons send projections into surrounding hypothalamic structures including the paraventricular nucleus (PVN), where NPY is released from nerve terminals. NPY is considered to be an

important orexigenic component of the hypothalamic control of food intake and body weight. Evidence for this comes from experiments where central infusion of NPY into the PVN stimulated food intake and, when infused chronically, produced obesity [75, 78]. Central infusion of NPY decreases sympathetic outflow to brown adipose tissue and increases lipogenic enzyme activity in white adipose tissue [76, 77]. Thus, NPY increases food intake, decreases energy expenditure, and increases lipogenesis, which may promote obesity.

NPY has been proposed to mediate the effects of leptin on food intake and body weight [79, 80]. This hypothesis is supported by the observation that 1) arcuate NPY mRNA is elevated in *ob/ob* and *db/db* mice [81, 82]; 2) leptin inhibits NPY mRNA in the arcuate nucleus [83]; and 3) obesity is attenuated in *ob/ob* mice that lack NPY [84].

The ARC contains the largest population of NPY neurons in the brain, many of which send axonal projections to the PVN, a major integration site implicated in body weight regulation [85, 86]. NPY mRNA expression in the ARC is elevated during fasting and periods of body weight loss, and is associated with increased NPY release into the PVN [87, 88, 89]. In addition, arcuate NPY mRNA is overexpressed in *ob/ob* and *db/db* mice [81, 82]. Overexpression of arcuate NPY during a fast and in *ob/ob* mice is blunted by leptin treatment [53, 64, 83]. Taken together with the observation that leptin receptors are expressed in arcuate NPY neurons, it appears that leptin may regulate body weight through inhibition of NPY neurons [71].

In view of the evidence that NPY is an important orexigenic component of the hypothalamic control of food intake and body weight, it is surprising that NPY deficient mice do not exhibit decreased food intake or body weight [90]. In fact, they are

phenotypically normal except for an increase in susceptibility to seizures [90]. Similarly, targeted deletion of the NPY-5 receptor (which is highly expressed in the PVN and LH) does not result in decreased food intake, but actually causes obesity [91, 92]. In light of the evidence that NPY mediates the effects of leptin on food intake and body weight, these observations indicate that leptin acts through pathways in addition to those involving NPY. Experiments using leptin deficient *ob/ob* mice lacking NPY demonstrated that the double-mutant mice are halfway between normal lean and *ob/ob* mice in terms of body weight and adiposity [84]. Neuropeptide Y was required for full expression of the *ob* phenotype, indicating that reduced leptin signaling elicits hypothalamic responses involving NPY. Because the NPY defect did not completely counteract the leptin defect in the double-mutants, it further suggests that NPY is not the only neuropeptide regulator of food intake and body weight that responds to leptin.

4.2. Hypothalamic proopiomelanocortinergic neurons may mediate the effects of leptin.

Characterization of the agouti mouse model of obesity has led to the study of the role of the central melanocortin system in the leptin pathway. Agouti mice are hyperphagic and obese, and have a yellow colored coat [93]. The yellow-colored coat is due to ectopic expression of agouti protein, which acts as an antagonist of melanocortin receptors. Normally, expression of the agouti protein is limited to melanocytes within hair follicles [94]. In agouti mice the agouti protein is ectopically expressed in numerous tissues including the brain [94]. The obesity in these mice appears to be due to antagonism of central melanocortin receptors by ectopically expressed agouti protein [95]. In support of this hypothesis it was found that transgenic mice that ectopically express an agouti cDNA clone are obese [96]. In addition, targeted deletion of the

hypothalamic melanocortin receptor (MC-4) gene results in obesity [97]. Mutations in the human MC-4 receptor also induce obesity [98, 99, 100]. Thus, MC-4 receptors and melanocortinergic neurons may play an important role in body weight regulation.

The MC-4 receptor is highly expressed by neurons in the VMN and the ARC [101]. Hypothalamic MC-4 receptors bind alpha-melanocyte stimulating hormone (α-MSH), a product of the polypeptide precursor POMC [95]. Proopiomelanocortinergic neurons expressing α-MSH are localized to the ARC and are regulated by nutritional status [102, 103]. For example, in fasted animals, POMC mRNA is markedly reduced [104, 105]. Central infusion of α-MSH or an MC-4 receptor agonist decreases food intake and body weight gain, while an MC-4 receptor antagonist SHU9119 increases food intake and body weight gain [95]. Taken together, these observations suggest that POMC neurons in the ARC play an important role in the regulation of food intake and body weight.

Furthermore, hypothalamic proopiomelanocortinergic neurons appear to be direct targets of leptin action. Proopiomelanocortin and leptin receptor mRNA are co-expressed in ARC neurons [106]. Leptin also modulates POMC gene expression. In situations of low leptin levels (e.g., *ob* mice and fasted rodents) POMC mRNA expression is reduced in the ARC, but this can be prevented by leptin treatment [104, 105, 107]. Furthermore, the central effects of leptin treatment are blunted by co-administration of an MC-4 antagonist [108, 109]. Thus, leptin may stimulate POMC neurons in the ARC that may, in turn, integrate the leptin signal between other controlling hypothalamic elements such as the PVN and LH.

HYPOTHESES

Study I. The role of neuropeptide Y in mediating GTG-induced obesity.

Goldthioglucose destroys cells in the VMN, producing hyperphagia and hypothalamic obesity. Elevated synthesis of NPY in the ARC has been proposed to play an important role in mediating hypothalamic obesity because 1) NPY produces hyperphagia and obesity, 2) *ob/ob* mice are hyperphagic, obese, and display elevated NPY in the ARC, and 3) leptin treatment inhibits NPY mRNA in the ARC. *Study I assessed the hypothesis that an increase in the activity of NPY neurons play a role in GTG-induced hypothalamic obesity.* If the hypothesis is correct (i.e., NPY promotes GTG-induced obesity) then elimination of NPY neurons in the hypothalamus by neonatal MSG treatment should attenuate the increased food intake and body weight produced by GTG.

Study II. Hypothalamic sensitivity to leptin and the effect of GTG.

Peripheral or central administration of leptin reduces food intake and body weight. Central administration of leptin requires a much lower dose to reduce food intake and body weight than peripheral administration. Leptin receptors are expressed in the VMN and arcuate nucleus. Previous studies have demonstrated that the arcuate nucleus is an important target of leptin but the relative importance of the VMN has not been examined. *Study II assessed the hypothesis that leptin acts directly in the VMN to inhibit food intake and body weight.* If the hypothesis is correct (i.e., leptin acts through the VMN) then microinfusion of leptin into the VMN will reduce food intake and body weight.

GTG produces a well defined lesion in the VMN together with hyperphagia and obesity. In addition, GTG treatment results in elevated plasma leptin concentrations, suggestive of leptin insensitivity in the VMN. *Study II assessed the hypothesis that GTG-induced obesity is associated with destruction of leptin-sensitive VMN neurons.* If the hypothesis is correct (i.e., GTG destroys leptin-sensitive neurons) then microinfusion of leptin into the VMN after GTG treatment will not affect food intake or body weight.

Study III. The role of melanocortinergic receptors in mediating leptin action.

Neurons which synthesize POMC (the precursor to α-MSH) may mediate the effects of leptin because 1) leptin receptor mRNA is co-localized with POMC mRNA in hypothalamic neurons, 2) leptin infusion increases hypothalamic POMC mRNA, 3) α-MSH (the ligand for the MC4 receptor) reduces food intake and body weight, and 4) MC4 receptor knockout mice are obese. *Study III assessed the hypothesis that MC4 receptors mediate the effects of leptin on food intake and body weight.* If the hypothesis is correct (i.e., the MC4 receptor does mediate the effects of leptin) then an MC4 antagonist should block the effects of leptin on food intake and body weight.

Genetically obese mice display increased leptin sensitivity. Compared to lean mice, *ob/ob* mice require lower doses of leptin to produce similar decreases in food intake and body weight. *Study III assessed the hypothesis that 1) enhanced sensitivity of ob/ob mice to leptin mat be mediated by upregulation of melanocortinergic receptors or 2) the increased sensitivity is mediated by upregulation of non-melanocortinergic receptors.* If melanocortinergic receptors assume a greater role in mediating the effects of leptin in *ob/ob* mice then an MC4 receptor agonist should be more effective in

reducing food intake in *ob/ob* mice than lean controls. If non-melanocortinergic receptors assume a greater role in mediating the effects of leptin in *ob/ob* mice then an MC4 receptor antagonist should be less effective in blocking the effects of leptin in *o/ob* mice than lean controls.

METHODS

STUDY 1. *EXPERIMENT 1.*

1.1. Animals and treatments

Adult C57BL/6J mice were bred in the institutional animal facility and each dam and her pups were housed together in shoebox cages. On postnatal day 4, male and female pups received a subcutaneous intrascapular injection of either saline (Sal) or 4 mg/g BW of MSG (Sigma, St. Louis, USA). Pups treated with MSG were identified by a small hole-punch in the left ear. The mice were weaned at 3 weeks and were individually housed in hanging steel cages with wire mesh floors in a room with a 12/12 hour light-dark cycle, and given rodent chow and water *ad libitum*. Two months later the mice were weighed and then injected i.p. with either Sal or GTG (0.8 mg/g BW; Sigma, St. Louis, USA). The experimental design resulted in four treatment groups; Sal/Sal, Sal/GTG, MSG/Sal, and MSG/GTG. Groups were balanced to have approximately equal distributions of males and females in case there was an effect of sex on treatment. After injection of GTG or Sal, daily food intake was determined by placing a pre-measured quantity of pelleted rodent chow (Purina) on the bottom of each hanging cage and slipping a piece of paper underneath each cage to catch any crumbs that might fall through the wire mesh floor. At 24 hour intervals the uneaten chow pellets and crumbs were re-weighed using an electronic scale (Sartorius, USA) accurate to 1/100 of a gram and daily food intake was calculated. Mice were weighed at 24 hour intervals and any that showed toxic effects in response to GTG (e.g. body weight loss that persisted for more than 4 days after GTG injection; n=9) were excluded from the study.

1.2. Tissues and histology

Mice were killed 2 weeks after GTG or Sal injection by carbon dioxide asphyxiation and decapitated, and their brains were rapidly removed and frozen on powdered dry ice. The brains were kept frozen at -70 °C until sectioned (10 μm) on a cryostat (Slee, London, England). Rnase free conditions were used at all times to prevent RNA degradation. Frozen coronal sections through the hypothalamus were thaw-mounted onto subbed slides (2 sections per slide) and dried on a slide warmer set at 40 °C. The sections were rehydrated using a graded series of ethanols; 100% ethanol (EtOH), 95% EtOH, 70% EtOH for 2minutes each followed by two 5 minute washes of phosphate buffered Sal (PBS; 0.1 M at pH 7.0). After rehydration the sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0), dehydrated (by running the sections through the rehydration set-up in the opposite order), and stored in slide boxes with DRI RITE (Sigma, St. Louis, USA) at -20 °C. Every 10^{th} slide was stained with cresyl violet to produce a reference library of representative sections for each mouse. Prior to the *in situ* hybridization procedure the reference slides were used to sort the remaining frozen slides to ensure that the hypothalamic sections used during *in situ* hybridization were matched for anterior-posterior level. The reference slides were also used to verify the presence or absence of GTG or MSG lesions (in the VMN and ARC respectively) in each of the four groups.

1.3. *In situ* hybridization

In situ hybridization for NPY mRNA was performed using a single stranded DNA probe complementary to NPY mRNA. The radiolabeled probe was synthesized by PCR with tritiated nucleotides (supplied by Dr. C.V. Mobbs, New York, USA). Matched

sections of the arcuate nucleus were brought to room temperature and pre-hybridized in 2X standard saline citrate (SSC), 5 mM EDTA, 2.5X Denhardt's solution, 5 mM dithiothreitol, 100 mg/ml herring sperm DNA, 100 mg/ml yeast transfer RNA, 5 mg/ml single stranded calf thymus DNA, and 50% deionized formamide for 2 hours at 42 °C. Hybridization was carried out in the same buffer containing 10% dextran sulfate and labeled probe ($2X10^6$ dpm/20 µl/section) at 42 °C overnight. Sections were washed twice in 1X SSC for 15 minutes each time, and in 0.1X SSC overnight at room temperature, followed by a final wash in 0.1X SSC for 1 h at 55 °C. Slides were dehydrated, air-dried, and apposed to autoradiography film. After several exposures of the slides to film (2, 4, and 7 days), signal was quantified digitally. A digital camera lens (Cohu) and the computer program JAVA (Jandel Scientific, Corte Madera, USA) were used to magnify and digitally capture the image of each brain section. Camera magnification and lighting were kept identical for the entire study. Using the computer program NIH Image (NIH, Bathesda, USA) the optical density of the region in the hypothalamus exhibiting signal was determined. NIH image measured the optical density of an area of constant size within the region in the hypothalamus exhibiting signal. To standardize optical density from section to section a corrected optical density was determined by subtracting the optical density of the hypothalamus not exhibiting signal from the original value. This corrected optical density was taken to reflect the amount of hypothalamic NPY mRNA within the section.

STUDY II. *EXPERIMENT 2.*

2.1. Animals and Surgery

Adult male C57BL/6J mice were obtained from Charles River Laboratories (Quebec, Canada) and housed individually as in experiment 1. Immediately before stereotaxic surgery, each mouse was anaesthetized i.p. with ketamine (150 mg/kg BW) and xylazine (10 mg/kg BW). After the mouse was fully anaesthetized, its scalp was sterilized with a 70 % EtOH wash and shaved. A rostral-caudal scalp incision (approximately 1.5 cm long) was made to visualize lambda, bregma, and the sagittal suture of the skull. The mouse's head was placed in a stereotaxic apparatus (Kopf, Tujunga, USA) and fixed using ear bars inserted into the external auditory meati. The upper jaw of the mouse was supported by an incisor bar and secured with a nose bar. The coordinates for lambda and bregma were determined, and the skull was carefully aligned such that lambda and bregma were on the same horizontal plane to ensure correct placement of the cannula. This horizontal plane is the horizontal line used by Slotnick and Leonard in *A stereotaxic Atlas of the Albino-Mouse Forebrain* from which the coordinates were obtained [110]. Two small burr holes (approximately 1 mm in diameter) were drilled through the skull with a dental drill at the following coordinates: 0.9 mm posterior to bregma, and +/- 0.5 mm lateral to the sagittal suture to expose the dura directly above each VMN [110]. In addition, two small burr holes were drilled through the skull just rostral to the lambdoid suture and a stainless steel screw was inserted into each. A small puncture was made through the exposed dura above each VMN with a sharp needle, being careful not to disturb the midsagittal sinus, in order to allow entry of a guide cannula. A 13 mm long stainless steel 26 gauge double guide

cannula (Plastics One, Roanoke, USA) was inserted through each burr hole and implanted 4.2 mm ventral to the skull surface and 1 mm above both VMN. The infusion stylette to be used extended 1mm below the guide cannula. The cannula was fixed to the skull using dental cement and the two screws previously inserted into the skull served as anchors. A 5 mm long stainless steel double dummy cannula (Plastics One, Roanoke, USA) was inserted into the double guide cannula to reduce the incidence of occlusion. Sutures anterior and posterior to the cannula were placed into the scalp, if required, to assist wound healing. The mice received topical 2% xylocaine (Astra, Mississauga, Canada) for post-operative topical anesthesia. Mice were allowed to recover from the surgical procedure for 10 days prior to intrahypothalamic infusions.

2.2. Microinfusion procedure

Prior to the start of the microinfusion procedure, the infusion syringes and stylettes were prepared as described below. Two 1 µl Hamilton syringes were connected to a 14 mm long 33 gauge bilateral infusion stylette (Plastics One, Roanoke, USA) with 20 gauge polyethylene tubing. The stylette, tubing and syringes were filled with saline, and a very small air bubble was introduced into the tubing to act as an indicator of fluid movement. Immediately before microinfusion, the stylettes were filled with either recombinant human leptin (Lep; R&D Systems, Minneapolis, USA) or fresh saline. The solution (Sal or Lep; 0.1 µl/side) was infused over a 2-minute period using an electric syringe pump (Sage Instruments, Cambridge, USA). Before insertion and after removal of the infusion stylette, the infusion pump was allowed to run until a small drop of solution (Sal or Lep) was observed at the tip of each cannula, assuring that the microinfusion system was delivering solution before, during, and after the injection.

Immediately after the infusion stylette was removed from the guide cannula, the dummy cannula was reinserted.

Initially, the mice received bilateral intrahypothalamic infusions of Sal (0.1 μl)at 24-hour intervals over a 72-hour period. Subsequently, the mice received bilateral intrahypothalamic infusions of Lep at 24-hour intervals (0.01 μg dissolved in 0.1 μl of Sal) over a 72-hour period. During the course of the experiment food intake and daily body weight change were measured as in Experiment 1.

Following a 3- to 4-day recovery period, the mice were injected i.p. with GTG (0.7 mg/g BW) as described in Experiment 1. Approximately 10-14 days later the mice received 2 bilateral intrahypothalamic infusions of Sal (0.1 μl/side) at 24-hour intervals followed by 2 bilateral intrahypothalamic infusions of Lep (0.01 μg dissolved in 0.1 μl of Sal) at 24-hour intervals using the same infusion protocol as described above. Finally, the mice were infused with Sal twice at 24-hour intervals. Daily food intake and body weight were measured as in Experiment 1.

2.3. Tissues and histology

Following completion of the infusion protocol, brain tissue was collected and prepared as in Experiment 1 except that every section was stained with cresyl violet. The sections were examined under a microscope to verify correct cannula placement and to confirm the presence of a GTG-induced VMN lesion.

STUDY III. *EXPERIMENT 3.*

3.1. Animals and Surgery

Adult male CBA mice were obtained from Jackson Laboratories, (Barr Harbor, USA) and individually housed as in Experiment 1. A switch from C57BL/6J mice to CBA mice was made in this experiment because, in our experience, CBA mice recover better after the stereotaxic procedure. Stereotaxic surgery was performed using the same protocol as in Experiment 2 but with the following difference. The right lateral ventricle was targeted with a 11 mm long stainless steel 26 gauge single guide cannula using the following coordinates : 2.9 mm posterior from bregma, 0.8 mm right of the sagittal suture, and 1.3 mm ventral to the skull [110]. These coordinates were selected so that the infusion stylette used extended 1mm below the guide cannula. Post-operative care was provided as described in experiment 2.

3.2. Microinfusion procedure

The mice were randomly assigned to one of four groups: 1) pretreatment with Sal followed by treatment with Sal (Sal-Sal), 2) pretreatment with Sal followed by Lep (Sal-Lep), 3) pretreatment with the melanocortin receptor antagonist SHU9119 followed by Sal (SHU-Sal), or 4) pretreatment with SHU followed by Lep (SHU-Lep). The infusion stylettes were prepared in the same manner as Experiment 2 but with the following difference. The infusion stylettes used were 12 mm long and 33 gauge and an additional stylette was filled with SHU9119 (Phoenix Pharmaceuticals, Mountain View, USA). Initially, the mice received an intracerebroventricular infusion of Sal (2 µl) or SHU (1 nmol dissolved in 2 µl Sal). One hour later, the mice were infused with Sal (2 µl) or leptin (1 µg dissolved in 2 µl Sal). Food intake and body weight were measured using

the same protocol as experiment 2. The experiment was repeated after 48 hours four times such that each mouse received each of four possible treatments once.

3.3. Tissues and histology

Following completion of the infusion protocol, brain tissue was collected and prepared as in Experiment 2. The sections were examined histologically to verify cannula placement within the right lateral ventricle.

EXPERIMENT 4.

4.1. Animals and Surgery

Male and female genetically obese adult mice (*ob/ob*; n=10) and their lean controls (+/?; n=14) were obtained from Jackson Laboratories (Barr Harbor, USA) . At the beginning of the experiment the mice were divided into two groups on the basis of body weight and the overall appearance of obesity. The initial mean body weights (± sem) of the lean and obese mice were 32.4 ± 1.8g and 68.5 ± 1.5 g, respectively. The mice were individually housed as described in Experiment 1. The temperature of the room was maintained closely around 23 °C and the relative humidity was between 40-60%. Stereotaxic surgery was performed using the same protocol as in Experiment 3.

4.2. Microinfusion procedure

Following 10 days of recovery, daily food intake and body weight change were measured every 24 hours for 3 days (*ob/ob*; n=10 and lean; n=14) to establish baseline values. The infusion stylettes were prepared in the same manner as Experiment 3, except an additional stylette was filled with the melanocortinergic receptor agonist MTII (0.3 nmol dissolved in 2 µl Sal; Phoenix Pharmaceuticals, Mountain View, USA).

Initially, the mice were infused centrally with either Sal (2 μl) or Lep. Lean mice (n=13) received 1 μg of Lep dissolved in 2 μl Sal while the leptin sensitive *ob/ob* mice (n=7) received a much lower Lep dose (0.2 μg dissolved in 2 μl Sal). 48 hours later, the mice received the opposite infusion treatment such that each mouse was treated with Sal and Lep. Food intake and body weight change during this and subsequent parts of Experiment 4 were measured as in Experiment 1.

Forty-eight hours after Sal/Lep treatment the mice were infused with 2 μl Sal (*ob/ob*, n=7; control, n=7) or 0.3 nmol MTII (*ob/ob*, n=7; control, n=5). 48 hours later, the mice received the opposite infusion treatment such that each mouse was treated with Sal and MTII.

Forty-eight hours after Sal/MTII treatment, the mice (*ob/ob*, n=7; control, n=5) were pretreated with 3 nmol SHU or 2μl Sal. One hour later the mice were infused with leptin. The lean mice (n=4) received 1μg of leptin dissolved in 2 μl Sal while the leptin sensitive *ob/ob* mice (n=8) received 0.2μg of leptin dissolved in 2 μl Sal 48 hours later, the mice received the opposite infusion treatment such that each mouse received a SHU-Lep treatment and a Sal and Lep treatment.

In order to determine the effects of a lower dose of MTII, a second group of *ob/ob* mice (n=6) and their lean controls (n=6) were implanted with a chronically indwelling cannula into the lateral ventricle in the same manner as the first group. Ten days later, the mice were infused with 2 μl Sal (*ob/ob*, n=6; control, n=5) or 0.1 nmol of MTII (*ob/ob*, n=6; control, n=6). Food intake and body weight were measured as in Experiment 1.

4.3. Tissues and histology

Following completion of the infusion protocol, brain tissue was collected and prepared as in Experiment 2.

STATISTICAL ANALYSIS

In all studies, statistical analysis was performed with "DataDesk[4]", a statistical software program for the Macintosh. Analysis of Variance (ANOVA) was performed to detect differences between groups. The least squares difference (LSD) post-hoc test was used to determine significant differences between groups. The alpha level was set at $p<0.05$.

In Experiment 1 a two-way ANOVA was performed on the NPY expression, food intake, and body weight change following the experimental treatments. Neonatal treatment (MSG or Sal) and adult treatment (Sal or GTG) were between-subjects factors and food intake and body weight were within-subjects variables.

In Experiment 2 a one way ANOVA was performed on the mean daily food intake and body weight change in the 24 hour period following the experimental treatments. For the second part of the experiment a repeated measures multivariate analysis of variance (rMANOVA) was performed with treatment (saline1, leptin, saline2) as between subjects factors and food intake and body weight change over time as within-subjects repeated measures variables.

In Experiment 3 and 4, rMANOVAs were performed. In Experiment 3, pretreatment (Sal or SHU) and treatment (Sal or Lep) were between-subjects factors and food intake and body weight change over time were within-subjects repeated measures

variables. In Experiment 4, genotype (ob/ob and +/?), pretreatment (Sal and SHU) and treatment (Sal and Lep) were between-subjects factors and food intake and body weight change over time were within-subjects repeated measures variables.

RESULTS

STUDY 1. ***EXPERIMENT 1.***

A) GTG decreases hypothalamic NPY mRNA and increases food intake and body weight.

GTG injection in adult mice produced a hypothalamic lesion that extended from the lateral aspect of the ARC through the ventrolateral aspect of the VMN (Figure 1b). The lesion overlapped with the medial aspect of the distribution of NPY-producing neurons (Figure 2b) leading to a significant decrease in NPY mRNA (Figure 3a). GTG produced a significant increase in food intake and body weight gain over the 2-week period following its injection (Figure 3b and 3c). Specifically, GTG-injected mice consumed 48% more food between days 10 and 14 than Sal-injected controls (30.0 ± 2.1 g vs. 20.3 ± 0.9 g). Furthermore, over the 14 days following treatment, GTG-injected mice gained approximately 3 times more weight than Sal-injected mice (6.05 ± 0.83 g vs. 2.10 ± 0.83 g).

B) MSG decreases hypothalamic NPY mRNA and does not affect food intake or body weight.

MSG injection in neonatal mice (MSG-Sal group) resulted in a lesion medial to that produced by GTG (Sal-GTG group) that was characterized by a loss of neurons in the ARC (Figure 1c). The lesion entirely overlapped NPY mRNA distribution (Figure 2c), thus essentially eliminating NPY mRNA in the hypothalamus. The amount of NPY mRNA detected was not significantly greater than background (Figure 3a). In contrast to GTG, MSG had no significant effect on food intake or body weight gain (Figure 3b and 3c).

Figure 1. Photomicrographs of representative coronal sections through the ventromedial nucleus and arcuate nucleus of the hypothalamus in mice. These photomicrographs illustrate the effects of GTG treatment (Panel-b), MSG treatment (Panel-c), and MSG and GTG treatments combined (Panel-d), on the hypothalamus. The hypothalamus of a "control mouse" (i.e., injected with saline only) is shown in Panel-a. In all 4 panels the third ventricle is indicated by the letter "v". In Panel-a the locations of the arcuate nucleus (ARC) and ventromedial nucleus (VMN) are indicated. In Panel-b, an asterisk marks the approximate centre of the GTG-induced lesion, which is characterized by a dense accumulation of glial cells in the ventromedial nucleus. In Panel-c, an asterisk marks the approximate center of the MSG-induced lesion, which is characterized by a notable depopulation of cells within the arcuate nucleus. In Panel-d, both the arcuate nucleus and ventromedial nucleus contain relatively few cells as compared to the same nuclei in Panel-a.

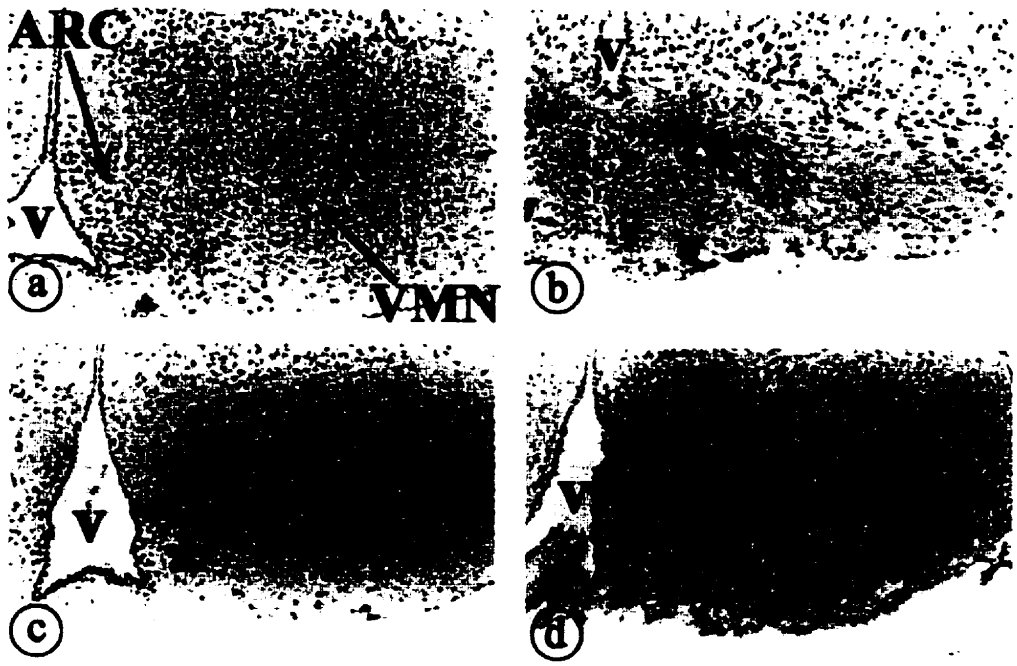

ARC
V
a
VMN
V
b
V
c
V
d

Figure 2. Representative autoradiograms of *in situ* hybridization of neuropeptide Y (NPY) mRNA in sections through the ventromedial nucleus and arcuate nucleus exposed to film. These autoradiograms illustrate the effects of GTG treatment (Panel-b), MSG treatment (Panel-c), and MSG and GTG treatments combined (Panel-d) on NPY mRNA levels in the hypothalamus. The level of expression of NPY in a control mouse (i.e., injected with saline only) is shown in Panel-a. In all 4 panels the third ventricle bisects the figure vertically and the base of the brain is along the bottom of the panel. The NPY-producing neurons are centred in the arcuate nucleus, which produces a pattern of expression that appears as 2 dense circles with the third ventricle running vertically between them (see Panel-a). In Panel-b it can be seen that GTG treatment appeared to produced a decrease in NPY mRNA levels within the hypothalamus since the signal is less than that observed in Panel-a. In Panel-c, it can be seen that MSG treatment resulted in a loss of NPY mRNA in the hypothalamus as the dense accumulations of silver grains are not present. Similarly, in mice treated with MSG and GTG, NPY mRNA was not detected (Panel-d).

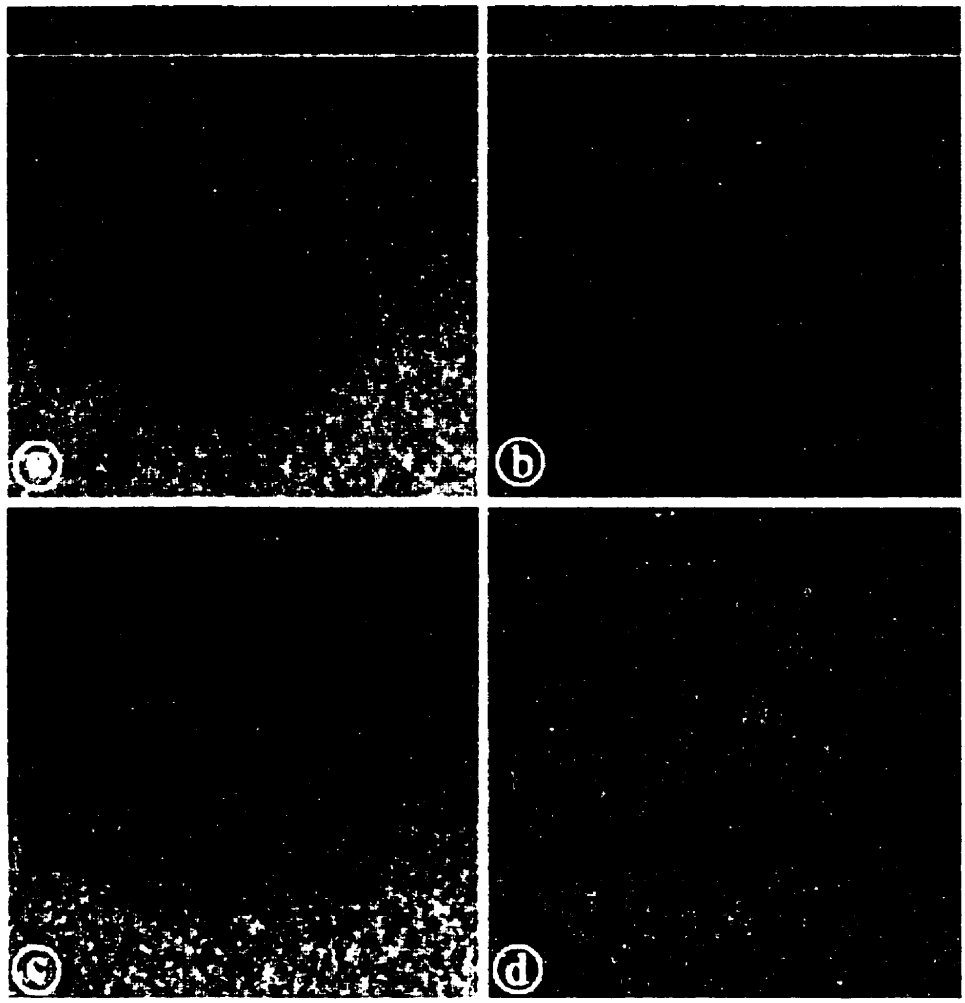
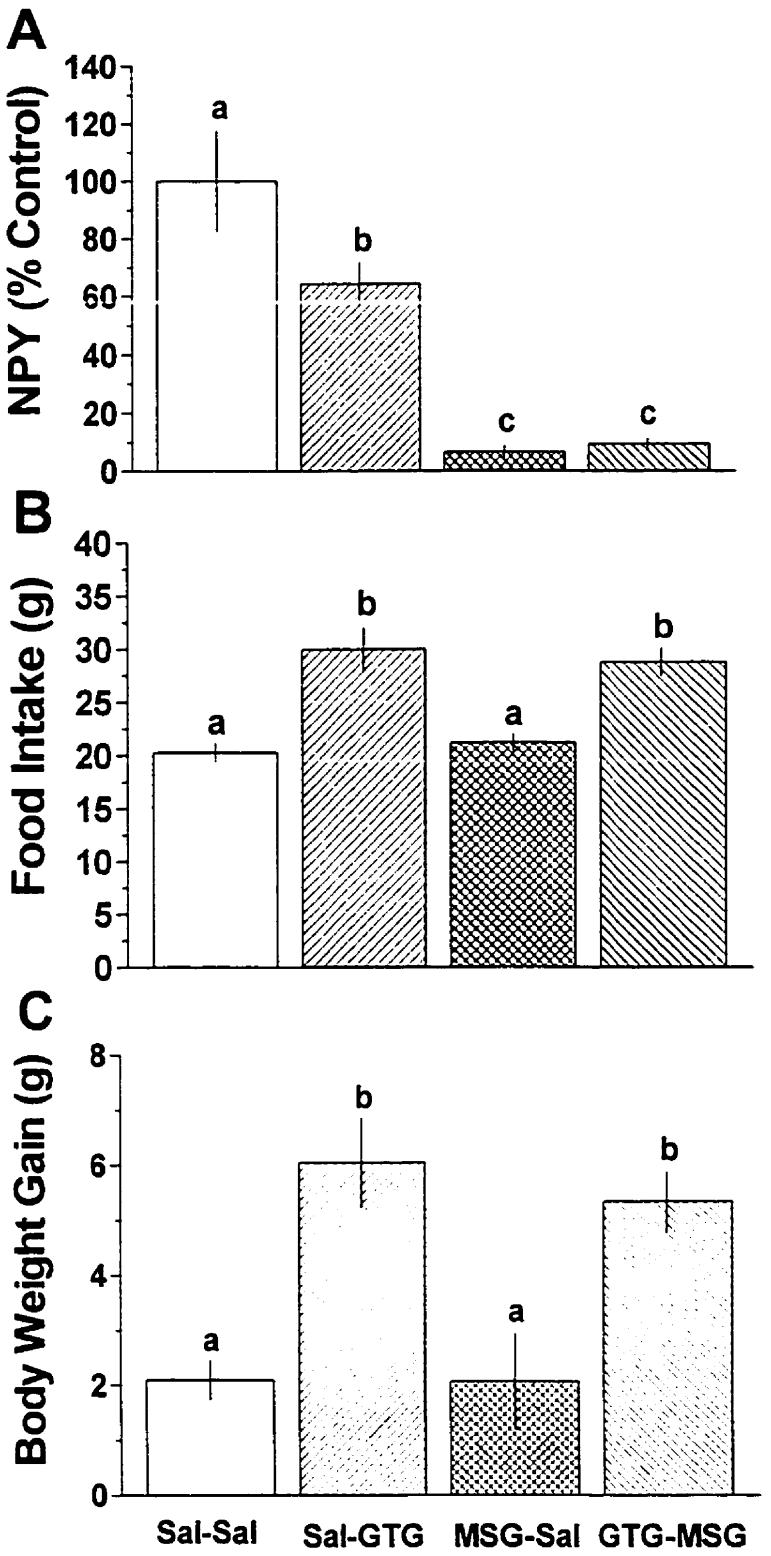

a
b
c
d

Figure 3. (A) Effects of GTG alone (Sal/GTG; n=8), MSG alone (MSG/Sal; n=7), or MSG and GTG (MSG/GTG; n=10), compared with controls injected with saline as neonates and adults (Sal/Sal; n=9), on hypothalamic NPY mRNA levels. GTG treatment led to a decrease in detectable NPY mRNA and MSG treatment virtually eliminated NPY mRNA in the hypothalamus. As with MSG alone, the amount of NPY mRNA detected in mice treated with both MSG and NPY was not significantly greater than background. (B) Total food intake per mouse on Days 10-14 after GTG (or saline) injection. (C) Body weight gain over the 14-day period following GTG (or saline) injection. GTG treatment produced a significant increase in food intake and body weight over the 2-week period following its injection. MSG had no significant effect on food intake or body weight gain. In mice treated with both MSG and GTG food intake and body weight gain increased to similar levels observed during GTG treatment alone. Bars with different letters are significantly different ($p<0.05$). Values are expressed as the mean ± sem.


A
NPY (% Control)
140
120
100
80
60
40
20
0
a
b
c
c
B
Food Intake (g)
40
35
30
25
20
15
10
5
0
a
b
a
b
C
Body Weight Gain (g)
8
6
4
2
0
a
b
a
b
Sal-Sal
Sal-GTG
MSG-Sal
GTG-MSG

C) Pretreatment with MSG does not prevent GTG-induced increased feeding and body weight.

GTG injection in adult mice previously treated with MSG as neonates (MSG-GTG group) resulted in a lesion through the VMN that was also associated with a loss of neurons in the ARC (Figure 1d). As with MSG treatment alone, the amount of NPY mRNA detected in mice treated with MSG and GTG was not significantly greater than background since NPY mRNA in the arcuate nucleus was eliminated (Figure 2d and 3a). Despite virtual elimination of NPY mRNA by pretreatment with MSG, GTG treatment produced hyperphagia and weight gain similar to that produced by GTG alone (Figure 3b and 3c).

STUDY II. ***EXPERIMENT 2***

A) The VMN is sensitive to the effects of leptin.

Figure 4 shows the stereotaxic placement of a cannula bilaterally on either side of the third ventricle and directly above the VMN. Three consecutive days of low-dose infusion of leptin (0.01 μg/side) directly into the VMN produced a significant decrease in food intake and body weight gain (Figure 5a and 5b). Specifically, leptin-treated mice consumed 20% less food per day than Sal-treated mice (3.62 ± 0.14 g/day vs. 4.54 ± 0.13 g/day). Furthermore, whereas Sal-treated mice gained weight (0.185 ± 0.10 g/day), leptin-treated mice lost weight (-0.39 ± 0.08 g/day).

B) GTG lesions block the effects of leptin on food intake and body weight.

GTG injected into adult mice produced lesions that extended from the lateral ARC through the ventrolateral aspect of the VMN as was observed in Experiment 1

Figure 4. A photomicrograph demonstrating tracks that result from the stereotaxic placement of a bilateral cannula terminating directly above the VMN. The arcuate nucleus (ARC) and ventromedial nucleus (VMN) and third ventricle (V) are indicated by the arrows. The tips of the cannulae are indicated by the asterisks.

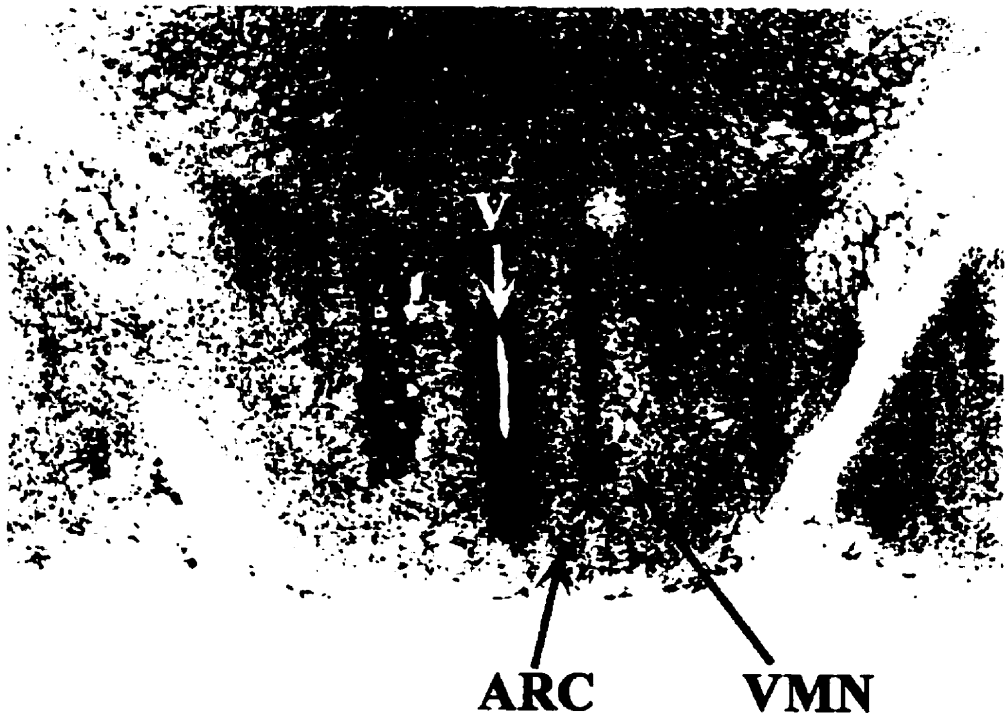

V
ARC
VMN

Figure 5. Effect of bilateral leptin infusion directly into the VMN of mice on daily food intake (A) and daily body weight change (B). Saline was infused at 24 hour intervals over a period of 3 days followed by leptin infusion on 3 successive days (0.01 μg/side/day) in 16 mice. Three consecutive days of leptin infusion directly into the ventromedial nucleus produced a significant decrease in food intake and body weight gain. ** is significantly different ($P<0.01$). Values are expressed as the mean ± sem.

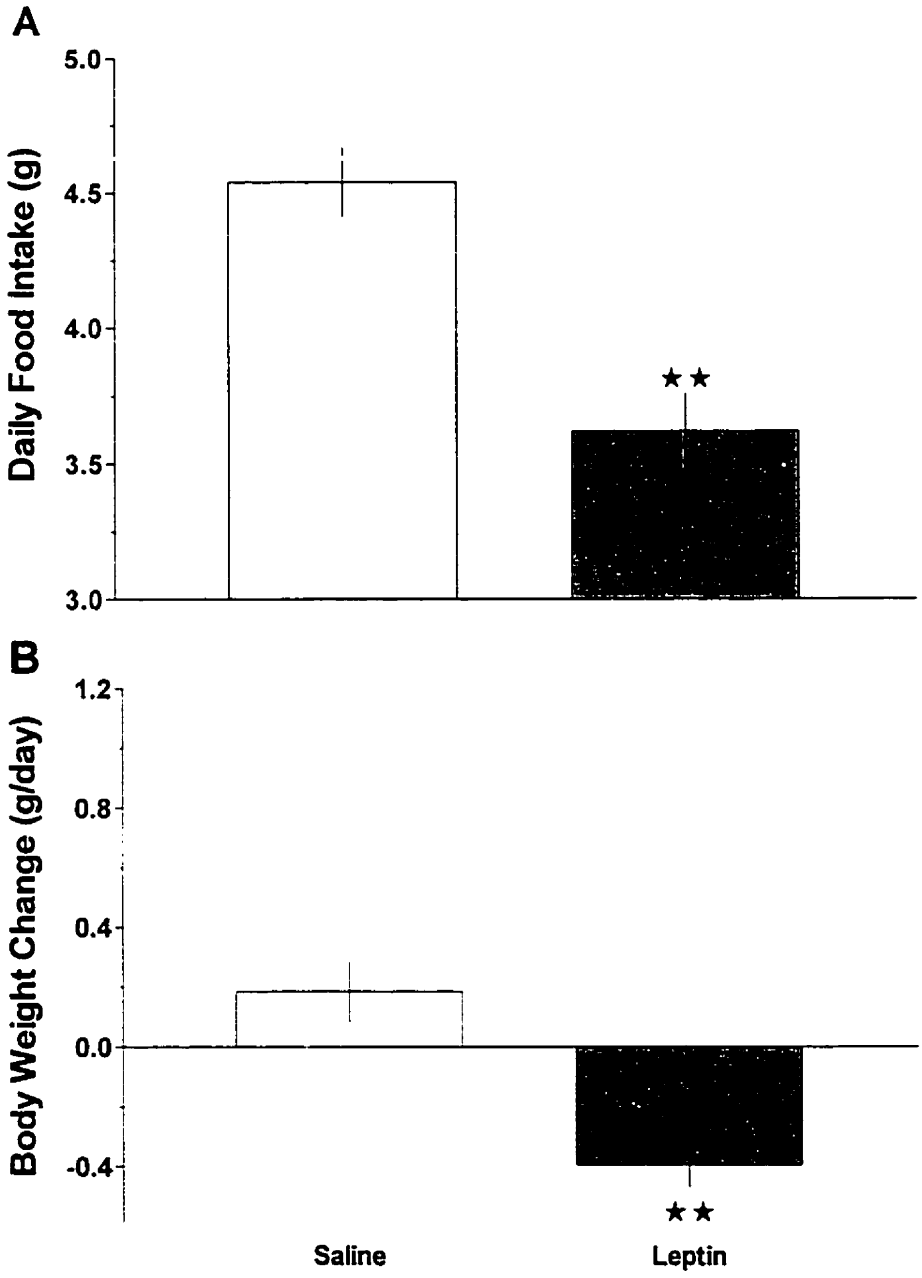
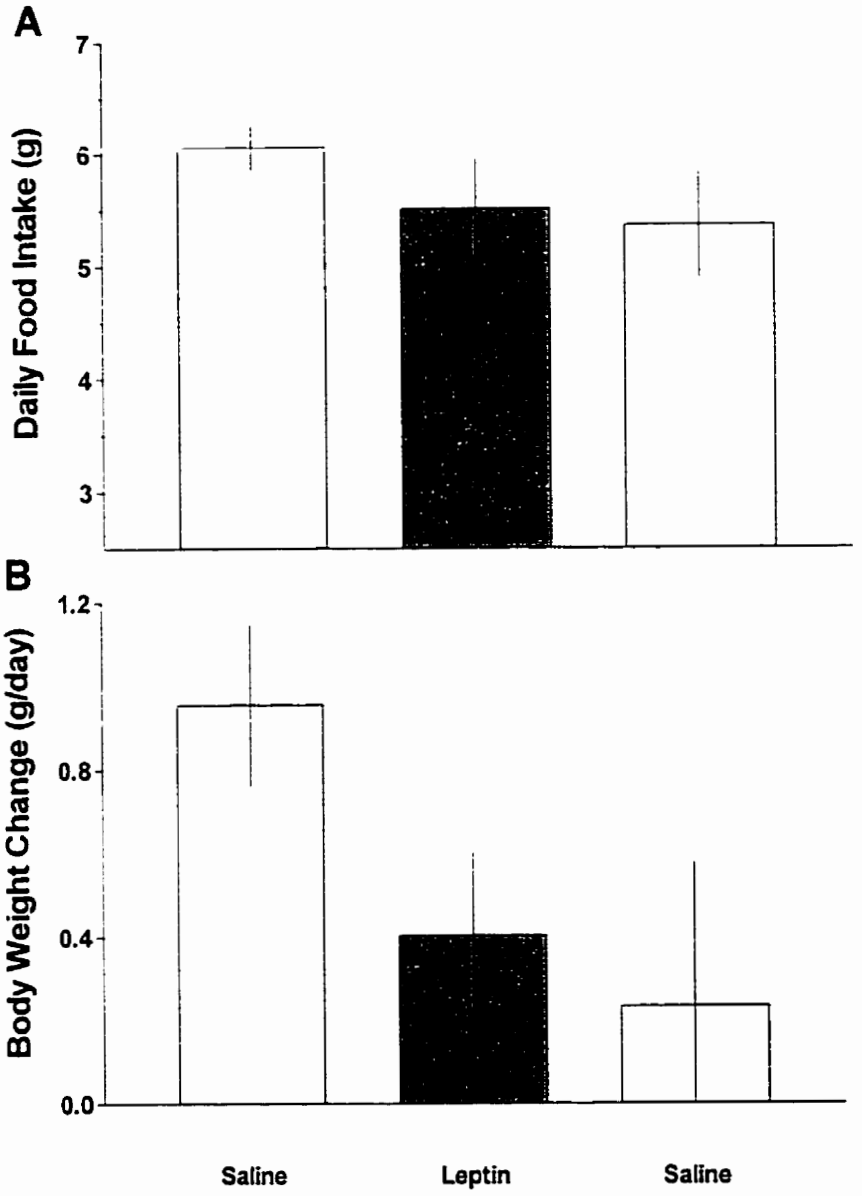

A
5.0
4.5
4.0
3.5
3.0
Daily Food Intake (g)
★★
B
1.2
0.8
0.4
0.0
-0.4
Body Weight Change (g/day)
★★
Saline
Leptin

Figure 6. Effect of leptin infusion bilaterally into the ventromedial nucleus of mice treated 10-14 days earlier with goldthioglucose (0.7 mg/g b.w.) on daily food intake (A) and daily body weight change (B). Saline was infused on 2 successive days followed by leptin infusion on 2 successive days (0.01 g/side/day) followed by saline infusion on 2 successive days in 10 mice. Two consecutive days of leptin infusion did not produce a statistically significant decrease in food intake or body weight gain in mice treated with goldthioglucose 14 days earlier ($P>0.05$). Values are expressed as the mean ± sem.


A
Daily Food Intake (g)
7
6
5
4
3
B
Body Weight Change (g/day)
1.2
0.8
0.4
0.0
Saline
Leptin
Saline

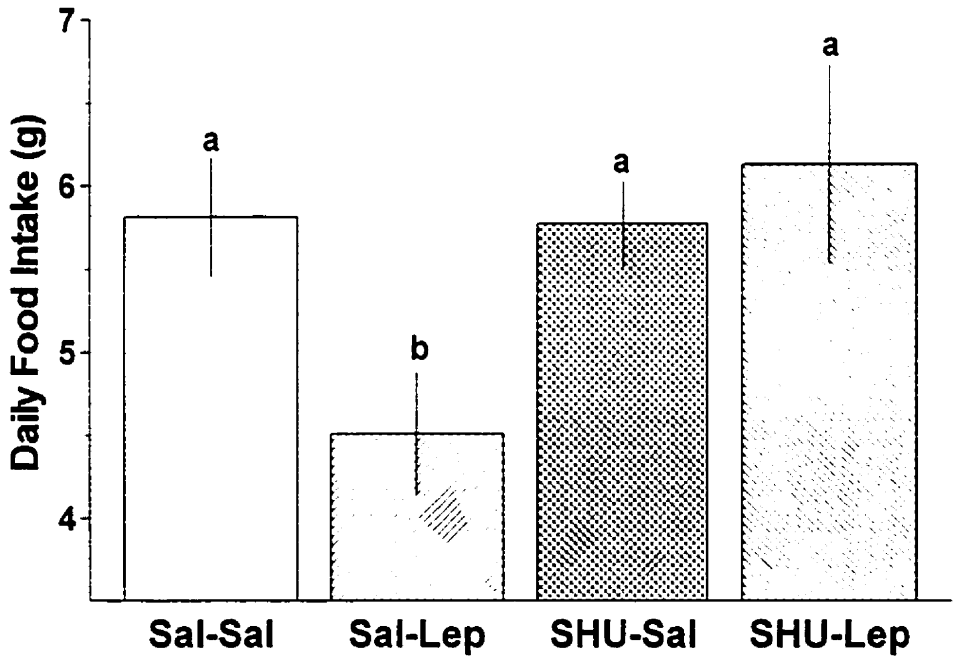
Two consecutive days of leptin infusion (0.01 μg/side) 10-14 days after GTG treatment did not produce a significant decline in food intake or body weight gain (Figure 6a and 6b). There was a tendency for food intake to decline during the infusion protocol of two days Sal (6.06 ± 0.20 g/day), two days leptin (5.51 ± 0.45 g/day) and two days Sal (5.36 ± 0.48 g/day), but it was not significant. Furthermore, throughout the infusion protocol daily food intake was higher than before GTG treatment (compare Figure 5a and 6a). A gradual decrease in body weight gain during the infusion protocol was also noted, but there was no significant difference between body weight change during leptin infusion (0.40 ± 0.20 g/day) when compared to saline infusion before (0.95 ● 0.19 g/day) or after leptin (0.34. ± 0.23 g/day).

STUDY III. *EXPERIMENT 3*

A) A melanocortinergic receptor antagonist blocks leptin-induced decreases in food intake.

Intracerebroventricular infusion of leptin (1 μg) did not significantly inhibit daily food intake in mice pre-treated with 1 nmol of the melanocortinergic antagonist SHU 9119 (Figure 7). Specifically, mice pre-treated with Sal and subsequently infused with leptin consumed 22% less food than Sal/Sal controls (4.51 ± 0.37 g/day vs. 5.81 ± 0.36 g/day). However, mice pre-treated with SHU 9119 and subsequently infused with leptin did not consume more or less food than Sal/Sal controls. Whereas SHU 9119 treatment blocked leptin-induced decreases in daily food intake, it did not significantly block leptin-induced decreases in body weight.

Figure 7. Effect of intracerebroventricular pretreatment with saline or the α-MSH receptor antagonist SHU9119 (1 nmol) on daily food intake following intracerebroventricular infusion of either saline or leptin (1 μg) in 10 CBA mice. The infusion protocol resulted in 4 groups; Sal-Sal, Sal-Lep, SHU-Sal, and SHU-Lep. Leptin did not significantly inhibit daily food intake in mice pretreated with SHU9119. Bars with different letters are significantly different ($P<0.05$). Values are expressed as the mean ± sem.


Daily Food Intake (g)
7
6
5
4
a
b
a
a
Sal-Sal
Sal-Lep
SHU-Sal
SHU-Lep

EXPERIMENT 4

A) Ob/ob mice are hypersensitive to the effects of leptin on food intake and body weight.

It was initially demonstrated that the obese *ob/ob* mice were hyperphagic relative to their lean littermate controls (+/?) as shown in Figure 8. A single intracerebroventricular infusion of leptin into the right lateral ventricle of lean controls (1.0 μg) and *ob/ob* mice (0.2μg) produced a significant decrease in food intake and body weight (Figure 9A and 9B). Lean controls treated with leptin consumed 46% less food per day than when infused with Sal (2.44 ± 0.16 g/day vs. 4.51 ± 0.18 g/day). In addition, Sal treatment resulted in modest weight gain (0.35 ± 0.43 g/day) in +/? mice but leptin treatment produced marked weight loss (-1.26 ± 0.3 g/day). Obese (*ob/ob*) mice treated with leptin consumed 59% less food per day than when infused with Sal (2.15 ± 0.29 g/day vs. 5.28 ± 0.32 g/day). Sal treatment resulted in modest weight gain (0.4 ± 0.27 g/day) in *ob/ob* mice, but leptin treatment produced marked weight loss (-2.55 ± 0.37 g/day). Despite a lower dose of leptin (0.2 μg vs. 1 μg in +/?), *ob/ob* mice displayed decreases in food intake similar to that of +/? mice. Furthermore, *ob/ob* mice treated with 0.2 μg leptin lost significantly more weight than lean controls treated with 1 μg leptin.

B) Ob/ob mice do not exhibit increased sensitivity to an MC4 receptor agonist.

Intracerebroventricular infusion of MTII (0.1 nmol) in lean and *ob/ob* mice was equally ineffective in altering food intake, although comparable decreases in body weight were detected (1.7 g and 1.4 g respectively) as shown in Figure 10A and 10B. A higher dose of MTII (0.3 nmol) did produce significant (and comparable) decreases in food

Figure 8. Effect of the *ob/ob* genotype on daily food intake compared to lean (+/?) littermate controls (n=10-14). Daily food intake was significantly higher in the *ob/ob* group. ** is significantly different ($P<0.05$). Values are expressed as the mean ± sem.

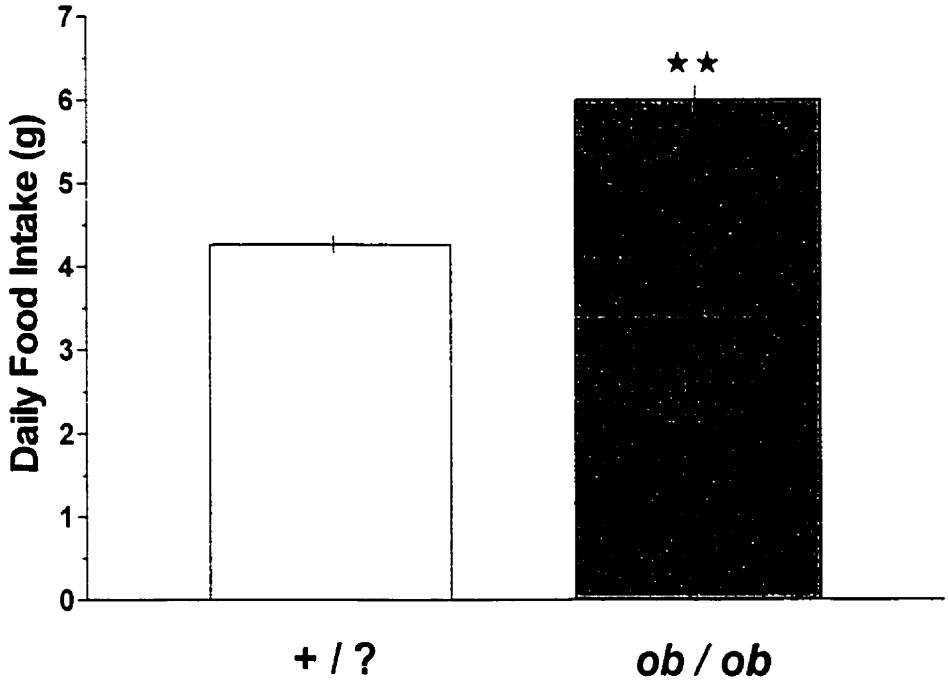
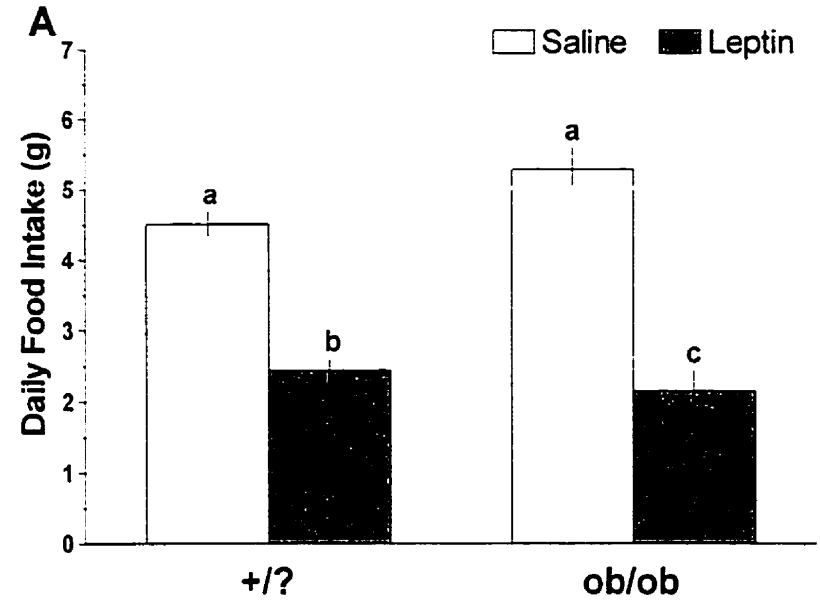

Daily Food Intake (g)
7
6
5
4
3
2
1
0
★★
+ / ?
ob / ob

Figure 9. Effect of intracerebroventricular infusion of leptin on daily food intake and daily body weight change in *ob/ob* mice and their lean controls (+/?). Note that the *ob/ob* mice (n=14) received 0.2 μg of leptin while their lean controls (n=10) received 1.0 μg of leptin. Leptin infusion produced a significant decrease in food intake and body weight in both controls and *ob/ob* mice. Despite a lower dose of leptin, *ob/ob* mice displayed decreases in food intake similar to that of controls, and lost significantly more weight than controls. Bars with different letters are significantly different ($P < 0.05$). Values are expressed as the mean ± sem.


A
Saline
Leptin
Daily Food Intake (g)
7
6
5
4
3
2
1
0
a
b
a
c
+/?
ob/ob

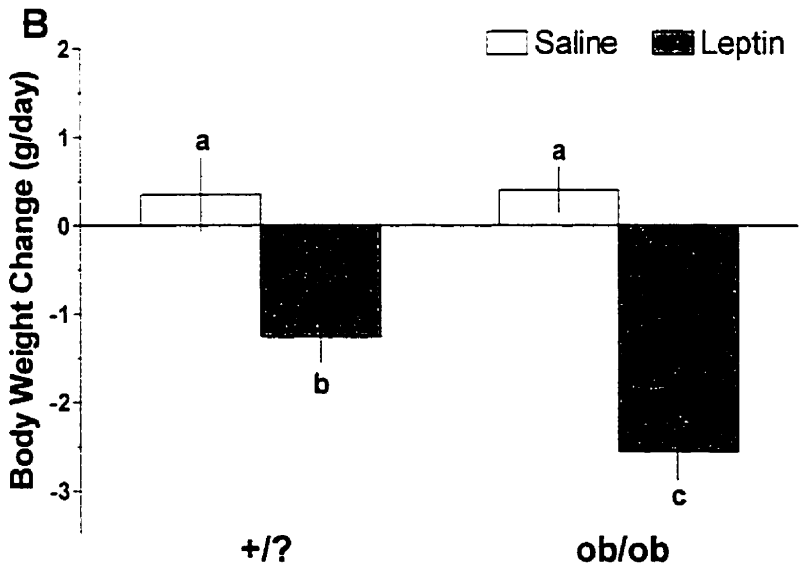
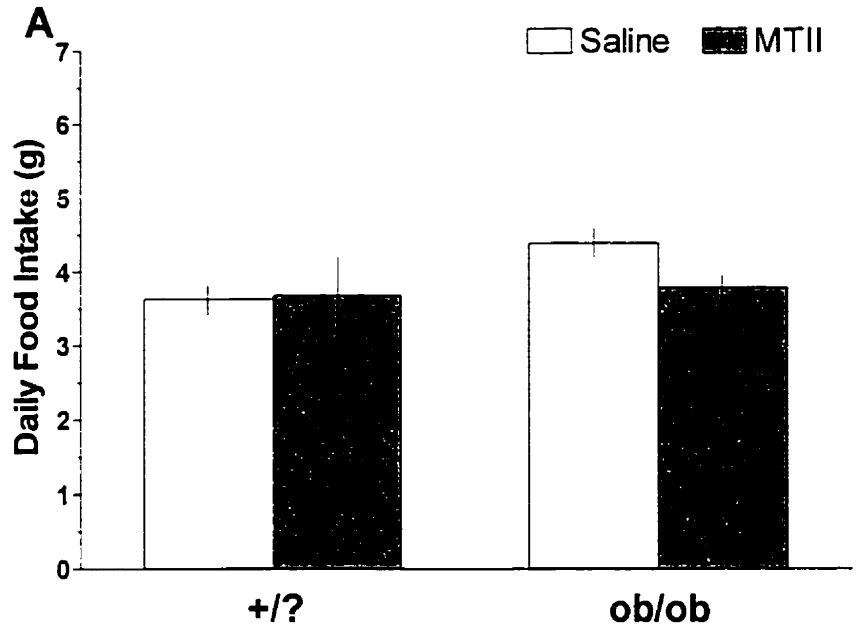
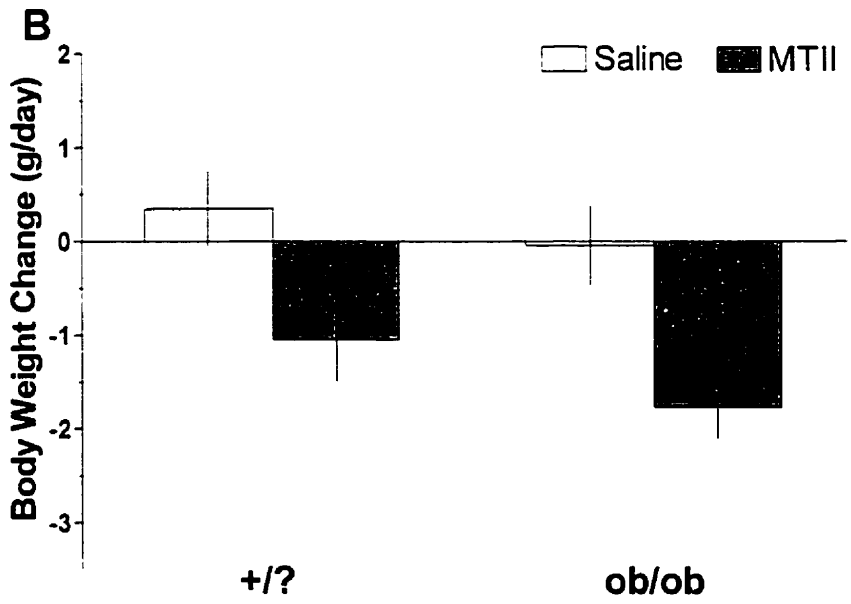

B
Saline
Leptin
Body Weight Change (g/day)
2
1
0
-1
-2
-3
a
b
a
c
+/?
ob/ob

Figure 10. Effect of intracerebroventricular infusion of 0.1 nmol MTII on daily food intake and daily body weight change in *ob/ob* mice and their lean controls (+/?). MTII was equally ineffective in altering food intake. although comparable decreases in body weight were detected ($P > 0.05$). Values are expressed as the mean ± sem.


A
Saline
MTII
Daily Food Intake (g)
7
6
5
4
3
2
1
0
+/?
ob/ob


B
Saline
MTII
Body Weight Change (g/day)
2
1
0
-1
-2
-3
+/?
ob/ob

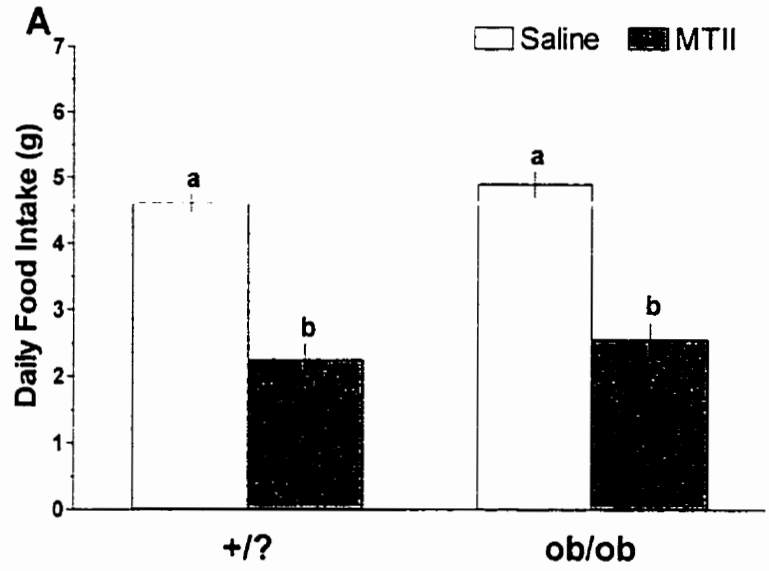
intake and body weight in lean and *ob/ob* mice (Figure 11A and 11B). Specifically, lean controls treated with 0.3 nmol MTII consumed 51% less food per day than after Sal treatment (2.25 ± 0.26 g/day vs. 4.61 ± 0.26 g/day). In addition, whereas Sal treatment resulted in weight gain (1.10 ± 0.50 g/day) in +/? mice, MTII infusion produced marked weight loss (-1.69 ● 0.57 g/day). Obese (*ob/ob*) mice treated with MTII (3 nmol) consumed 48% less food per day than when infused with Sal (2.56 ± 0.27 g/day vs. 4.90 ± 0.21 g/day). Whereas Sal treatment resulted in modest weight gain (33 ± 0.39 g/day) in *ob/ob* mice, 0.3 nmol of MTII produced marked weight loss (-2.22 ± 0.39 g/day). There was no statistically significant difference in food intake or body weight loss between *ob/ob* and +/? mice treated with 0.3 nmol MTII.

C) An MC4 antagonist prevents leptin induced decreases in food intake and body weight in ob/ob mice.

When mice were infused with 3nmol of the melanocortinergic antagonist SHU9119 alone, statistically significant effects on food intake and body weight were not detected in either lean or obese mice although, as expected, there was a tendency for increased food intake and body weight gain in lean mice after SHU9119 treatment (Figure 12 A and 12B). Interestingly, this tendency was not observed in *ob/ob* mice, but rather an opposite trend was noted (Figure 12A and 12B). Pre-treatment with SHU 9119 (3 nmol) before leptin infusion prevented leptin-induced decreases in food intake in +/? mice and *ob/ob* mice (Figure 13A and 13B). Specifically, +/? mice infused only with leptin (1μg) consumed 48% less food than when pre-treated with SHU 9119 (1.99 ● 0.18 g/day vs 3.55 ± 1.05 g/day). This percentage difference is similar to that observed following leptin treatment (Figure 9). Moreover, the 58% lower food intake in Sal pre-

treated *ob/ob* mice relative to infusion with SHU9119 (.2.16 ± 0.25 g/day vs 5.06 ± 0.06 g/day) is almost identical to the relative change produced by leptin alone as shown in Figure 9 (i.e., a 59% decrease). SHU 9119 also blocked leptin-induced decreases in body weight in +/? (-0.45 ±0.71 g/day vs. -2.06 ±.18 g/day) and *ob/ob* mice (-0.04 ± 0.38 g/day vs. -2.11 ± 0.35 g/day) as shown in Figure 13A and 13B.

Figure 11. Effect of intracerebroventricular infusion of 0.3 nmol MTII on daily food intake and body weight change in *ob/ob* mice and their lean controls. MTII infusion did produce significant (and comparable) decreases in food intake and body weight *ob/ob* mice and their lean controls. Bars with different letters are significantly different ($P < 0.05$). Values are expressed as the mean ± sem.


A
Saline
MTII
Daily Food Intake (g)
0
1
2
3
4
5
6
7
a
b
a
b
+/?
ob/ob

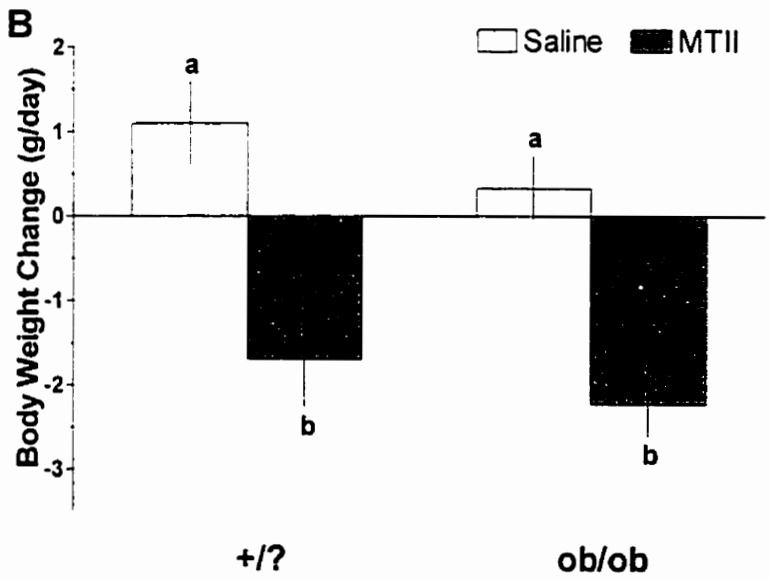

B
Saline
MTII
Body Weight Change (g/day)
-3
-2
-1
0
1
2
a
b
a
b
+/?
ob/ob

Figure 12. Effect of intracerebroventricular infusion of SHU 9119 (3.0 nmol) on daily food intake and body weight change in *ob/ob* mice and their lean controls. Infusion of SHU9119 did not produce statistically significant effects on food intake and body weight in either group although there was a tendency for increased food intake and body weight gain in lean mice after treatment. This tendency was not observed in *ob/ob* mice, but rather an opposite effect was noted. Bars with different letters are significantly different ($P< 0.05$). Values are expressed as the mean ± sem.

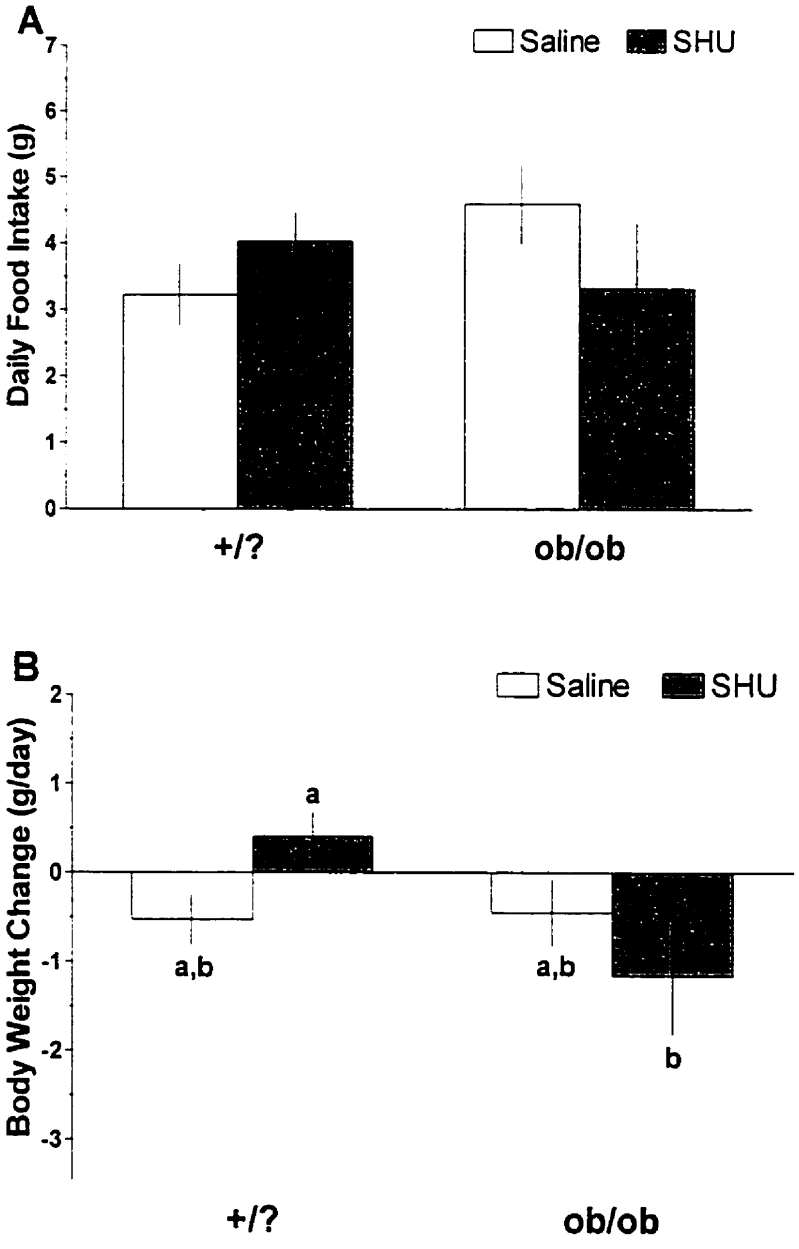
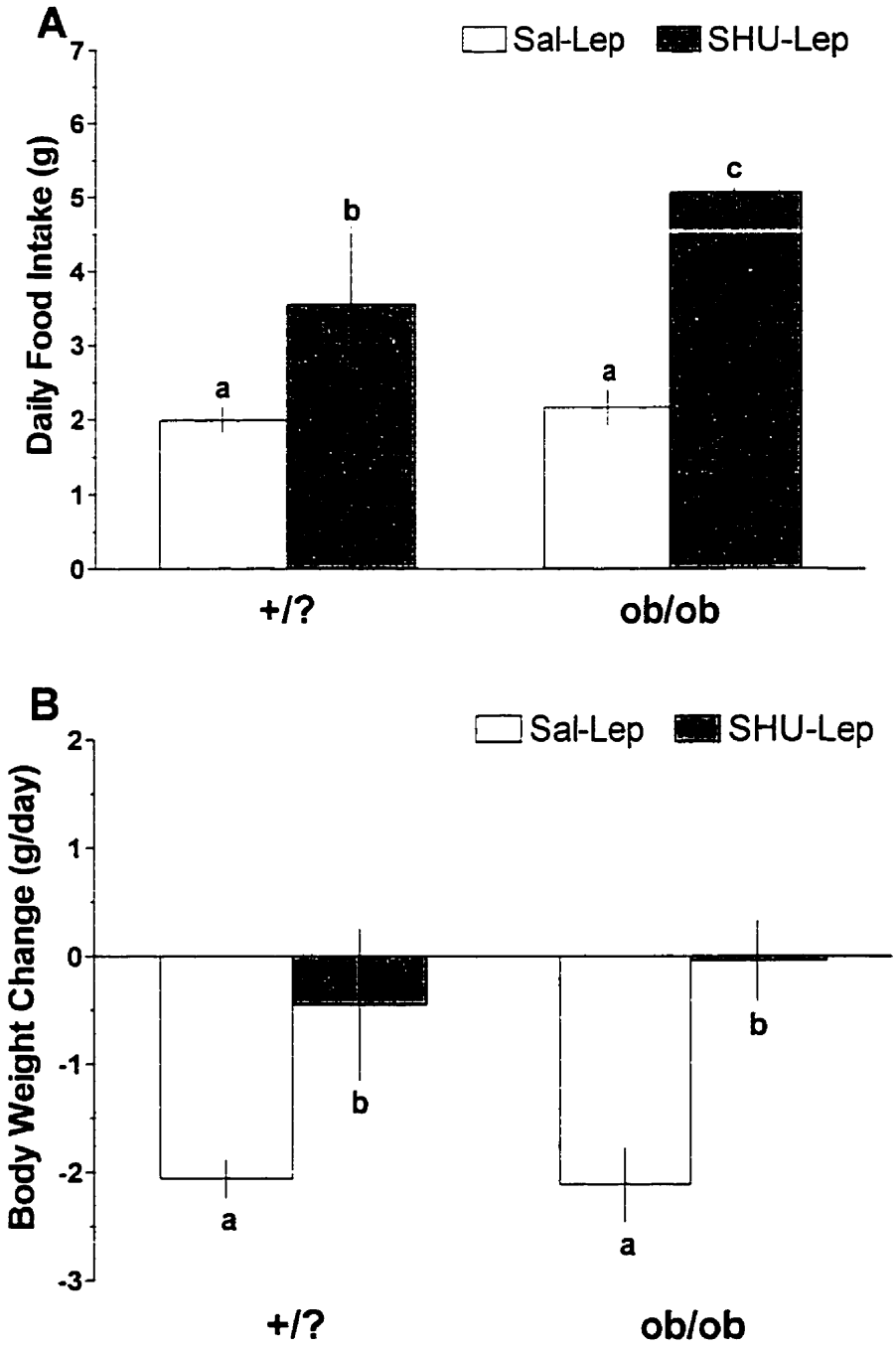

A
Saline
SHU
Daily Food Intake (g)
0
1
2
3
4
5
6
7
+/?
ob/ob
B
Saline
SHU
Body Weight Change (g/day)
2
1
0
-1
-2
-3
a
a,b
a,b
b
+/?
ob/ob

Figure 13. Effect of intracerebroventricular infusion of SHU9119 (3 nmol) on daily food intake and body weight change in *ob/ob* mice and their lean controls pretreated with leptin. Note that the *ob/ob* mice received 0.2 μg of leptin while their lean controls received 1.0 μg. Pretreatment with SHU9119 before leptin infusion prevented leptin-induced decreases in food intake and body weight in *ob/ob* mice and their lean controls. Bars with different letters are significantly different ($P < 0.05$). Values are expressed as the mean ± sem.


A
Sal-Lep
SHU-Lep
Daily Food Intake (g)
7
6
5
4
3
2
1
0
a
b
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ob/ob
B
Sal-Lep
SHU-Lep
Body Weight Change (g/day)
2
1
0
-1
-2
-3
a
b
a
b
+/?
ob/ob

DISCUSSION

Neuropeptide Y is synthesized in neurons of the arcuate nucleus and it has been proposed that these neurons play a critical role in promoting the development of obesity [77, 85]. In support of this proposal it has been demonstrated that in genetic obesity (e.g., *ob/ob* and *db/db* mice), hypothalamic NPY mRNA is elevated and in *ob/ob* mice lacking NPY, obesity is attenuated [81, 82, 84]. Furthermore, it is well established that NPY is extremely potent in promoting food intake when it is infused centrally (particularly intrahypothalamically) and that chronic infusion of NPY into the hypothalamus produces obesity [78, 111]. Similar to genetic obesity and hypothalamic obesity, the hormonal changes and increased weight gain produced by chronic NPY infusion is not dependent on hyperphagia [111]. Further support for a role of hypothalamic NPY neurons in promoting obesity was the demonstration that these neurons are inhibited by leptin (which has potent anti-obesity effects) [83].

In Experiment 1 the hypothesis that obesity due to hypothalamic lesions is associated with increased NPY mRNA was assessed. As reported previously, it was demonstrated that GTG treatment produces obesity and hyperphagia associated with a VMN lesion in mice [22]. Also in agreement with others it was demonstrated that MSG treatment is associated with an ARC lesion and, unlike GTG-induced lesions, MSG-induced lesions are not associated with a robust increase in body weight or food intake [112]. In addition, it was demonstrated that the GTG-induced lesion is associated with decreased hypothalamic NPY mRNA, while the MSG-induced lesion is associated with virtual elimination of hypothalamic NPY mRNA. The decrease in hypothalamic NPY mRNA observed in GTG-treated mice was contrary to our hypothesis as was the

demonstration that MSG treatment has no discernible effect on the hyperphagia and weight gain produced by GTG. Since MSG treatment results in virtual elimination of NPY mRNA in the ARC, the results of Experiment 1 strongly suggest that NPY does not play a role in mediating the hypothalamic obesity produced by GTG.

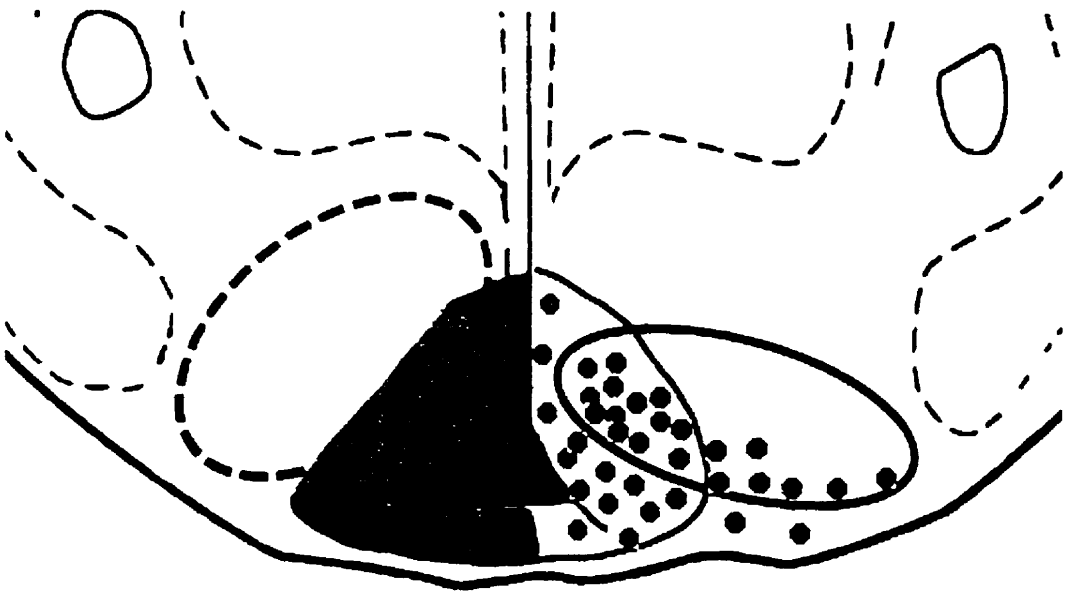
It is possible that NPY outside the ARC may mediate some effects of the GTG lesion. For example, obese agouti mice exhibit elevated NPY mRNA in the DMN following the development of obesity [113]. However, the present study did not show increased NPY mRNA in the DMN or any other part of the brain outside of the ARC. Although genetically obese *ob/ob* mice that are also NPY-deficient exhibit attenuated obesity and hyperphagia, it should be noted that these "double-knockouts" do remain obese and sensitive to leptin [84]. More recently, in support of the results of Experiment 1, it was reported that GTG is equally effective in producing obesity in both control mice and NPY knockout mice [114]. Taken together it appears that in contrast to the genetic obesity of leptin-deficient mice, the hypothalamic obesity of GTG-injected mice is not dependent on increased hypothalamic NPY activity.

Impairments in the synthesis, processing, or sensitivity to hypothalamic POMC have also been proposed to play an important role in several forms of obesity [94, 95, 96, 97, 104, 105, 107, 115, 116, 117]. In a separate study, our lab used tissues harvested from mice in Experiment 1 to assess the hypothesis that obesity due to hypothalamic lesions is associated with decreased hypothalamic POMC mRNA [118]. The results demonstrated that both MSG and GTG reduce hypothalamic POMC mRNA. However, it is important to note that MSG and GTG produce anatomically distinct lesions, and therefore probably do not destroy the same POMC-producing neurons. The MSG lesion

is centered in the ARC around the third ventricle, whereas the GTG lesion is centered lateral to this region. However, the lesions produced by MSG and GTG appear to overlap in the lateral portion of the ARC. The POMC-producing neurons of the hypothalamus extend from the ARC into an area well lateral to it, a distribution similar to (but somewhat medial to) that of the GTG lesion. Therefore, MSG appears to destroy the medial extent of the POMC field, GTG appears to destroy the lateral extent, and the two lesions may overlap to destroy the same neurons in the center of the POMC field (Figure 14). This hypothesis is supported by the observation that POMC mRNA is maximally reduced only in the presence of both lesions [118]. Therefore, as GTG produces a profound obesity that is characterized by hyperphagia, these results suggest that the lateral POMC field which is largely spared by MSG may be more important in the control of food intake and body weight than the medial POMC field. In support of this hypothesis, the lateral POMC field appears to be more sensitive to fasting and leptin [104]. Taken together, these results suggest that GTG-induced hypothalamic obesity may be due to impairments associated more with destruction of the POMC neurons than an increase in the activity of hypothalamic NPY neurons.

The hypothalamus is thought to be an important target of leptin since low doses of leptin administered ICV produce similar decreases in food intake and body weight as much higher doses administered peripherally [40]. In addition, hypothalamic nuclei such as the VMN and ARC express functional leptin receptors [65, 119]. Since these nuclei may be important targets of leptin, Experiment 2 assessed the hypothesis that leptin acts in the VMN to inhibit food intake and body weight and that GTG-induced obesity may be a result of destruction of leptin sensitive neurons in the VMN.

Figure 14. Monosodium glutamate and goldthioglucose produce anatomically distinct lesions in the hypothalamus, and probably do not destroy the same POMC producing neurons. The MSG lesion (blue) is centered around the third ventricle, whereas the GTG lesion (yellow) is centered lateral to this region. However, the lesions produced by MSG and GTG appear to overlap in the lateral portion of the arcuate nucleus (green). MSG appears to destroy the medial extent of the POMC field (red dots), GTG appears to destroy the lateral extent (green dots), and the two lesions may overlap to destroy the same neurons in the center of the POMC field.



Consistent with the hypothesis that leptin acts in the VMN to exert its effects, it was demonstrated that a dose 1/50 th of that typically used in ICV infusions to *ob/ob* mice (i.e., mice that are extremely sensitive to leptin) was capable of producing comparable decreases in food intake and body weight when infused directly into the VMN. In essence, doses of leptin that are ineffective when given ICV do significantly decrease food intake and body weight when infused directly into the VMN. These results suggest that the VMN may be a particularly important mediator of the effects of leptin on food intake and body weight. In addition, it was demonstrated that these leptin-induced decreases in food intake and body weight are blocked by GTG treatment. Although there is a trend toward decreased food intake and body weight following leptin infusion over the course of the second experiment after GTG treatment, it should be noted that in previous studies as well as the present study, the degree of hyperphagia and the rate of body weight gain peaks approximately 14 days after GTG injection. This transition from a dynamic to static phase of obesity coincided with the time period of leptin infusion during the latter part of Experiment 2. Therefore, this apparent trend toward decreased food intake probably reflects a transition from the dynamic phase to the static phase of obesity observed with GTG treatment. In any case, the results of Experiment 2 suggest that the VMN is particularly sensitive to leptin and the hypothalamic obesity that results from GTG treatment may be due, in part, to destruction of leptin-sensitive neurons in this region.

While the initial infusion protocol of Experiment 2 suggests that the VMN may be very sensitive to leptin, other reports have suggested that the ARC is the primary site of leptin action [120]. In support of this proposal it has been demonstrated that neurons in

the ARC do express the mRNA for the leptin receptor and NPY, a potent stimulator of food intake and body weight gain. Furthermore, NPY mRNA levels are decreased by leptin treatment [64, 83]. In view of this evidence, it is possible that the ARC, which is adjacent to the VMN, may have been exposed to leptin during the initial infusions of Experiment 2. The experiments performed do not exclude the possibility that leptin acts via inhibition of NPY neurons in the arcuate nucleus. This hypothesis is not supported by experiments demonstrating that NPY knockout mice respond normally to leptin, exhibit normal hormonal responses to a fast, and feed normally after a fast [114, 90]. In addition, the results of Experiment 1 demonstrated that NPY neurons do not mediate GTG-induced obesity and therefore are presumably not the site of leptin resistance in GTG-induced obesity. Taken together, the results demonstrated that doses of leptin that have a significant effect when infused into VMN-intact mice do not significantly affect food intake or body weight when infused into VMN-lesioned mice suggesting that GTG-induced hypothalamic obesity may be due to destruction of leptin-sensitive neurons in the VMN that normally regulate food intake and body weight.

Recent studies support the suggestion that the VMN is an important target of leptin action on food intake and body weight regulation. For example, very low-dose leptin infusions into the VMN of rats also decrease food intake and body weight [121]. These findings are consistent with the observation that VMN-lesioned rats are not sensitive to peripheral or central leptin administration [74, 122]. Studies also support the suggestion that dysregulation of the VMN leads to leptin insensitivity and hypothalamic obesity. For example, leptin insensitivity and increased plasma leptin is associated with GTG-induced obesity [118]. Since GTG injection produces impaired responsiveness to

glucose and glucoprivation, it is possible that the VMN neurons which are responsive to changes in blood glucose are also sensitive to leptin [24, 123]. Taken together with the present experiment, these observations suggest that GTG-induced obesity is associated with leptin resistance at the level of the VMN and may involve dysregulation of glucose-responsive neurons that are also sensitive to leptin. In addition, POMC neurons in the VMN play an important role in the regulation of food intake and body weight since their destruction by GTG is associated with profound obesity [118].

Recent studies suggest that one derivative of POMC, α-MSH, acts on melanocortinergic receptors to inhibit food intake and body weight gain [95]. Furthermore, the effects of leptin on food intake and body weight appear to be mediated by POMC neurons since the effects of leptin are blocked by the melanocortin receptor antagonist SHU9119 in rats [108, 109]. Experiment 3 extended this finding to mice demonstrating that SHU9119 effectively blocks the effects of leptin on food intake and body weight. Taken together with Experiment 2, these results suggest that both GTG and a melanocortin receptor antagonist can produce leptin insensitivity. These findings are consistent with the hypothesis that POMC neurons are important in mediating the effects of leptin and that GTG-induced obesity is a result of leptin insensitivity due to destruction of hypothalamic POMC neurons [118].

In genetically obese *ob/ob* mice, leptin deficiency is associated with increased leptin sensitivity [40]. In addition, the hypothalamic melanocortinergic system is important in mediating the effects of leptin [108, 109]. These findings suggest that three possibilities could account for increased leptin sensitivity in *ob/ob* mice: 1) increased sensitivity of melanocortinergic receptors to their agonists, 2) increased leptin sensitivity

mediated by non-melanocortinergic pathways, or 3) increased sensitivity of POMC neurons to leptin. Experiment 4 was designed to test these predictions. The results of Experiment 4 demonstrated that 1) *ob/ob* mice do, as reported by others, exhibit increased sensitivity to leptin, 2) *ob/ob* mice do not exhibit increased sensitivity of melanocortinergic receptors to an agonist such as α-MSH, and 3) the enhanced leptin sensitivity in *ob/ob* mice is not mediated via non-melanocortinergic receptors since the melanocortinergic receptor antagonist SHU9119 is equally effective in blocking the effects of leptin in both lean and *ob/ob* mice. Therefore the increased leptin sensitivity in *ob/ob* mice appears to be mediated largely by increased sensitivity of POMC neurons to leptin.

Recently, hypothalamic agouti-related protein (AGRP) has been suggested to play an important role in the regulation of food intake and body weight gain. AGRP appears to increase food intake and body weight gain through antagonism of hypothalamic MC-4 receptors [124, 125]. In addition, AGRP has been implicated in mediating the effects of leptin since hypothalamic AGRP neurons contain leptin receptors, and AGRP levels are decreased by leptin and increased in leptin-deficient *ob/ob* mice [125, 126]. Increased antagonism of MC-4 receptors by AGRP would appear to be associated with decreased sensitivity of these receptors to the effects of a melanocortin receptor agonist such as α-MSH. Thus, obesity in *ob/ob* mice may be mediated in part through increased antagonism of melanocortin receptors by the elevated AGRP levels in *ob/ob* mice. If this hypothesis is accurate, a melanocortin receptor agonist should be more effective in suppressing food intake in lean than in *ob/ob* mice. However, the results of Experiment 4

do not support this hypothesis since α-MSH was equally effective in reducing food intake in lean and *ob/ob* mice.

It has also been suggested that increased AGRP (in response to fasting or leptin deficiency) may play a greater role in promoting obesity than decreased α-MSH activity [127]. Thus, the effects of leptin on food intake and body weight gain may be due more to inhibition of AGRP than stimulation of POMC. If this hypothesis is accurate, obesity that occurs in leptin-deficient mice is a result of stimulation of AGRP neurons and alpha-MSH should be less effective in *ob/ob* mice than lean littermates. However, the present results do not support this hypothesis. It should be noted that the lack of increased sensitivity to the melanocortin receptor agonist (in spite of decreased levels of endogenous agonist may involve increased activity of AGRP neurons in the hypothalamus, as has been demonstrated in *ob/ob* mice.

Determining the factors that play a role in enhancing sensitivity to leptin is important since decreased leptin sensitivity is considered to play an important role in promoting obesity. It appears unlikely that enhanced sensitivity to leptin is a result of up-regulation of melanocortinergic receptors or increased antagonism of melanocortinergic receptors since *ob/ob* mice did not demonstrate increased sensitivity to α-MSH relative to their lean littermates. Furthermore, it appears unlikely that non-melanocortinergic pathways play an important role mediating leptin hypersensitivity since a melanocortinergic receptor antagonist was equally effective in blocking the effects of leptin in both lean and obese mice. Taken together these results suggest that the upregulation of leptin receptors on POMC neurons is an important factor in promoting

increased sensitivity of *ob/ob* mice to leptin and that the POMC neurons are important in mediating the effects of leptin in both lean and obese mice.

CONCLUSIONS

GTG treatment produces obesity and hyperphagia associated with a VMN lesion in mice as previously reported [22]. In agreement with others, MSG treatment is associated with an ARC nucleus lesion and, MSG-induced lesions are not associated with a robust increase in body weight or food intake [112]. The GTG-induced lesion is associated with decreased hypothalamic NPY mRNA, in the dorsolateral aspect of the ARC while the MSG-induced lesion is associated with virtual elimination of hypothalamic NPY mRNA. Taken together, these results strongly suggest that NPY does not play a role in mediating the hypothalamic obesity produced by GTG.

Infusion of leptin directly into the VMN of C57BL/6J mice (at a dose $1/50^{th}$ of that typically used in intracerebroventricular infusions to leptin-sensitive *ob/ob* mice) produces significant decreases in food intake and body weight gain compared to Sal-treated controls. GTG treatment (which produces a VMN lesion) blocks leptin-induced decreases in food intake and body weight gain. Taken together, these results suggest that the VMN is particularly sensitive to leptin and the hypothalamic obesity that results from GTG treatment may be due in part to destruction of leptin-sensitive neurons in this region.

The melanocortin receptor antagonist SHU9119 blocks the effects of leptin on food intake and body weight gain in CBA mice, as observed in rats [108, 109]. This finding suggests that the effects of leptin on food intake and body weight are mediated by POMC neurons. Taken together with Experiment 2, these results suggest that both GTG and a melanocortin receptor antagonist can produce leptin insensitivity. Thus GTG-

induced obesity may be a result of leptin-insensitivity due to destruction of hypothalamic POMC neurons.

ob/ob mice that lack a functional leptin gene, do, as reported by others, exhibit increased sensitivity to the effects of leptin on food intake and body weight gain [40]. Melanocortinergic receptors in *ob/ob* mice do not exhibit increased sensitivity to an agonist such as MTII since it is equally effective in reducing food intake in lean and ob/ob mice. Enhanced sensitivity in *ob/ob* mice is not mediated via non-melanocortinergic receptors since the melanocortinergic receptor antagonist SHU9119 is equally effective in blocking the effects of leptin in both lean and *ob/ob* mice. Taken together, these results suggest that upregulation of leptin receptors on POMC neurons is an important factor in promoting increased sensitivity of *ob/ob* mice to leptin and that the POMC neurons are important in mediating the effects of leptin in both lean and obese mice.

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