Alpha-Noradrenergic Regulation of Nutrient Intake in Genetically Obese (ob/ob) and Lean (+/?) Mice

A Thesis Manuscript Presented to

The University of Manitoba

In Partial Fulfillment of the Requirements for

the Degree of Master of Arts

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Faculty of Graduate Studies

July 1988

ISBN 0-315-48021-1

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BY

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF ARTS

Acknowledgements

I would like to express my sincere gratitude and appreciation to my thesis advisor, Dr. Linda M. Wilson, for her encouragement, guidance, and enthusiasm.

I would also like to thank the other members of my thesis committee including Dr. Dennis Fitzpatrick and Dr. J. Roger Wilson for their invaluable contribution.

Finally, I thank my parents, family, and friends for their continued support and encouragement.

This research was supported by a Natural Sciences and Engineering Research Council of Canada operating grant to L. M. Wilson (No. A7937).

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Abstract

Noradrenergic mechanisms in the hypothalamic paraventricular nucleus, principally of the alpha-2 subtype, are implicated in the regulation of food intake (Leibowitz et al., 1985). Given the increased levels of norepinephrine in the paraventricular nucleus of the obese (ob/ob) mouse, a genetically determined abnormality in the alpha-2 noradrenergic system may contribute to the hyperphagia of the ob/ob. Therefore, alpha-noradrenergic regulation of nutrient intake was examined in genetically obese and lean (+/?) mice. Experiment 1 determined the time required for ob/ob and +/? mice to adapt to a 6-h restricted feeding schedule. In Experiment 2 ob/ob and +/? mice were adapted to 6-h access to carbohydrate, fat, and protein before receiving vehicle injections ip 30 min before diet access, for 2 days, with intakes recorded at 1, 3, and 6 h. Then, separate groups (n=14) of ob/ob and +/? mice received 0.1 mg/kg or 0.5 mg/kg clonidine, an alpha-2 agonist, or vehicle 30 min prior to diet access. Clonidine decreased total caloric intake and intake from carbohydrate, fat, and protein in ob/ob and +/? mice (ps<.005). However, at 1 h, 0.5 mg/kg clonidine increased the proportion of energy from carbohydrate. Experiment 3 examined the specificity of the alpha-2 receptor mechanism in feeding. Yohimbine, an alpha-2 antagonist, was administered at doses of 3 mg/kg and 5 mg/kg ip, alone or prior to clonidine (0.5 mg/kg) or

vehicle. Mice administered either vehicle, 3 mg/kg, or 5 mg/kg ip yohimbine 30 min prior to clonidine and mice administered either dose of yohimbine prior to vehicle (n=7), decreased total food intake (p<.001), although the proportion of carbohydrate ingested was greater when 3 mg/kg or 5 mg/kg yohimbine preceded clonidine, or 5 mg/kg yohimbine preceded vehicle, than when either 3 mg/kg yohimbine or vehicle preceded vehicle, or vehicle preceded clonidine (p<.001). Although clonidine and yohimbine alone had similar effects, possibly implicating more than one receptor mechanism, joint administration tended to reverse the effect either drug had on its own. Given that previous research has suggested that clonidine can increase food intake in the rat (Leibowitz et al., 1985), the apparent difference in the effect of clonidine may be dose-dependent, or attributed to interspecies differences with respect to clonidine's mechanism of action. Despite suggestions to the contrary in the literature, there were no Phenotype x Drug interactions.

Alpha-Noradrenergic Regulation of Nutrient Intake in Genetically Obese (ob/ob) and Lean (+/?) Mice

The genetically obese mouse (ob/ob) is characterised by abnormal behavioural, physiological, and hormonal states, including hyperphagia, increased adiposity, and hyperinsulinemia (Mrosovsky & Melnyk, 1982; Sclafani, 1984; Storlien, 1984). Although the primary defect responsible for the syndrome has not been determined, an abnormality in central nervous system (CNS) function may contribute to the hyperphagia of the ob/ob mouse (Callahan, Beales, & Oltmans, 1984; Oltmans, Lorden, Callahan, Beales, & Fields, 1981). Evidence of CNS abnormalities in the ob/ob include decreased neuronal size in several brain regions (Bereiter & Jeanrenaud, 1979), altered dendritic orientation in lateral and ventromedial hypothalamic nuclei (Bereiter & Jeanrenaud, 1980), decreased levels of cholecystokinin in the cerebral cortex (Straus & Yalow, 1979), and increased hypothalamic norepinephrine (NE) levels (Feldman, Blalock, & Zern, 1979). This latter finding is of particular interest in terms of the hyperphagia exhibited by the ob/ob mutant, as hypothalamic NE has been proposed to play an important role in the regulation of feeding (Kuprys & Oltmans, 1982; Leibowitz, Brown, Tretter, & Kirschgessner, 1985).

Although hypothalamic catecholamines are known to modulate total energy intake (Hoebel, 1984; Morley & Levine, 1985) and have been implicated in specific macronutrient selection (Mauron, Wurtman, & Wurtman, 1980; Sclafani & Aravich, 1983; Shor-Posner, Azar, Insinga, & Leibowitz, 1985), research has yet to examine neurochemical regulation of macronutrient intake in the obese mouse. The current study, therefore, examined the impact of pharmacological manipulations of the noradrenergic system on macronutrient selection in the ob/ob mouse.

The Genetically Obese Mouse

The genetic aberration in the ob/ob is an autosomal recessive trait, a single point mutation in the genetic code leading to the production of a defective peptide (Connolly & Carnie 1984; Storlien, 1984). The homozygous recessive ob/ob displays marked obesity, involving increases in both size and number of adipocytes (Faust, Johnson, Stern, & Hirsch, 1978; Johnson & Hirsch, 1972). The rate of lipogenesis is increased in the liver and fat (Assimacopoulos-Jeannet, Singh, Le Marchand, Loten, & Jeanrenaud, 1974). In contrast, the homozygous dominant (+/+) wild type and the heterozygous +/ob are phenotypically lean. Procreation is confined to the mating of +/ob heterozygous mice because the ob/ob is infertile.

The ob/ob is hyperphagic, which is manifested in an increased caloric intake (Bray & York, 1971; Sclafani, 1984; Stricker, 1978). Obese mice maintain an elevated energy intake regardless of their pattern of self-selected nutrient intake (Romsos & Ferguson, 1982), and adult obese mice prefer to self-select a higher proportion of energy from fat than from protein or carbohydrate (Castonguay, Rowland, & Stern, 1985; Romsos, Chee, & Bergen, 1982). Obese mice allowed to self-select from diets varying in protein and carbohydrate consume as much protein as lean mice (Chee, Romsos, & Bergen, 1981; Chee, Romsos, Bergen, & Leveille, 1981; Romsos, Chee, & Bergen, 1982). When given access to diets varying in protein and fat, both obese and lean mice reduce their intake of protein (Romsos, Chee, & Bergen, 1982). The reduction in protein intake appears to be secondary to a greater preference for a high-fat diet rather than a high-carbohydrate diet and suggests that a nonprotein energy source can affect self-selected protein intake.

Romsos & Ferguson (1982) have found that energy derived from carbohydrate, fat, and protein averaged 12%, 68%, and 20%, respectively, for obese mice and 25%, 53%, and 22%, respectively, for lean mice. Therefore, both obese and lean mice self-select more energy from fat, although the ob/ob appears to maximise fat intake resulting in a greater increase in body weight gain. However, increased adiposity

has not been attributed to overeating alone because the ob/ob remains obese even when pair-fed with lean (+/?) littermates (Stock & Rothwell, 1982).

The ob/ob is also hyperinsulinemic (Dubuc, 1977; Storlien, 1984). Elevated plasma insulin concentrations are associated with an increase in the number and size of insulin-secreting beta cells of the pancreas (Storlien, 1984). The ob/ob exhibits glucose intolerance. Marked insulin resistance is associated with the progressive loss of insulin receptors (Herberg & Coleman, 1977). As a result, the hypoglycemic effect, normally associated with elevated levels of circulating insulin, does not characterise the ob/ob mutant. Also, enzymes involved in gluconeogenesis, found to be decreased in the hyperinsulinemic state, remain elevated in the ob/ob, an abnormailty which occurs early in development and may be associated with the loss of insulin receptor sites in the liver (Coleman, 1978).

Another major component of the ob/ob syndrome is the thermogenic defect (Seydoux, Rochner-Jeanrenaud, Assimacopoulos-Jeannet, & Jeanrenaud-Girardier, 1981). A chronically lower colonic temperature and resting metabolic rate at ambient temperatures below thermoneutrality suggest that the ob/ob is hypothermic and hypometabolic, as

indicated by a lowered rate of oxygen consumption (Boisonneault, Hornshuh, Simons, Romsos, & Leveille, 1978; Carlisle & Dubuc, 1984; Thurlby & Trayhurn, 1978).

Preweanling obese mice exhibit defective thermogenesis as well as differences in energy metabolism, which are evident as early as 5 days postpartum (Boisonneault et al., 1978), suggesting that abnormalities in both thermoregulation and metabolism are not secondary to obesity. That is, defective physiological thermoregulation precedes the divergence in body mass of lean and obese mice. Hypothermia, therefore, may be a primary contributor in the development of obesity, as a greater percentage of the diet is diverted to fat rather than to energy for thermogenesis. By expending less energy on thermoregulatory thermogenesis, obese mice store intake as white fat, resulting in gross adiposity (Thurlby & Trayhurn, 1979; Trayhurn & James, 1980). The lowered expenditure of energy on thermoregulatory heat production leads to an increased metabolic efficiency which appears to underlie the development and maintenance of obesity.

Hogan & Himms-Hagen (1980) have identified a mitochondrial defect in brown adipose tissue of reduced binding of purine nucleotides and a failure of the ob/ob to respond to cold stress with an increase in purine binding. Reduced NE turnover in brown adipose tissue is also

characteristic of the ob/ob (Knelans & Romsos, 1982). Therefore, an important mechanism in heat production, nonshivering thermogenesis, involving extra-muscular sources or areas of brown adipose tissue, is defective in the ob/ob mouse (Trayhurn, Goodbody, & James, 1982). As a result, the ob/ob displays an impairment in increasing heat production. The reduction in nonshivering thermogenesis, in turn, is manifested in a decreased metabolic rate and lowered colonic temperature (Batt & Hambi, 1982; Trayhurn & James 1980).

In addition to these symptoms, biochemical and neuroanatomical abnormalities have been cited as evidence of a CNS defect in the ob/ob mouse. Altered neuroanatomical organisation in the CNS includes reduced brain mass and cortical brain volume and significantly reduced soma cross-sectional areas in the ventromedial hypothalamus (VMH) of ob/obs when compared to lean controls (Bereiter & Jeanrenaud, 1979).

Elevated levels of NE (Feldman et al., 1979; Oltmans, 1983), pituitary dopamine (DA) (Lorden & Oltmans, 1977; Oltmans, 1983), 5-hydroxytryptamine (5-HT) (Garthwaite, Martinson, Tseng, Hagan, & Menahan, 1980), and beta-endorphin (Govoni & Yang, 1981) have also been detected in the ob/ob mouse. Endogenous hypothalamic NE levels are increased in the paraventricular nucleus (PVN) and the VMH

(Oltmans, 1983), areas which have been implicated in the regulation of feeding (Hoebel, 1984; Leibowitz et al., 1985). Following reserpine treatment, which acts to deplete catecholamines by affecting the ability of the adrenergic vesicle to store transmitter, hypothalamic NE levels of the ob/ob are not depleted to the same levels as in similarly treated lean mice (Oltmans, Olsauskas, & Comaty, 1980). Thus, increased NE levels and altered sensitivity to pharmacological manipulation in the ob/ob could involve a number of neurochemical mechanisms, including abnormal release mechanisms of the presynaptic neuron, modified storage properties, or altered reuptake mechanisms.

An increase in alpha-1 adrenergic receptor density is found in the ob/ob, although no differences in alpha-2 receptor density and affinity are known to exist between obese and lean mice (Callahan et al., 1984; Oltmans et al., 1981). No significant changes between lean and obese mice are found in either receptor number or affinity for alpha-adrenergic receptors in the cortex, or for dopaminergic or muscarinic receptors in the cortex or corpus striatum (Oltmans et al., 1981). Receptor abnormalities in the ob/ob, therefore, do not extend to all receptors, as the increase in alpha-adrenergic receptors in the hypothalamus appears relatively unique. However, a decrease in cholecystokinin (Straus & Yalow, 1979) and an increase in

cholecystokinin receptors (Hays & Paul, 1981) have been found in the cerebral cortex of obese mice, suggesting that multiple abnormalities in neural systems involved in the control of feeding exist in the ob/ob mutant.

Hypothalamic Control of Food Intake

Noradrenergic mechanisms located in the hypothalamic PVN have a physiological role in the control of feeding. Norepinephrine injected directly into this nucleus stimulates food intake in the satiated animal and potentiates food ingestion in the hungry rodent, and thus reflects the function of a physiological control mechanism (Hoebel, 1984; Lichtenstein, Marinescu, & Leibowitz, 1984; Morley & Levine, 1985). That is, the paraventricular and medial hypothalamic regions have been implicated in satiety control involving alpha-adrenergic and serotonergic (5-HT) systems (Blundell, 1979; Hoebel, 1984; Leibowitz, 1982). Norepinephrine acts to inhibit satiety; whereas 5-HT facilitates satiety. The PVN, therefore, is characterised by alpha-adrenergic inhibition and serotonergic excitation of a proposed satiety system.

In contrast, beta-adrenergic and dopaminergic systems in the perifornical lateral hypothalamic (LH) region inhibit feeding (Leibowitz, 1980; Leibowitz & Rossakis, 1978; Morley & Levine, 1985). Norepinephrine, therefore, injected into

the LH inhibits feeding through beta-adrenergic stimulation. Amphetamine treatment has been found to produce a dose-dependent decrease in food intake in both lean and obese mice, suggesting that amphetamine-induced release of NE contributes to the inhibition of feeding (Kuprys & Oltmans, 1982). However, when alpha-methyl-para-tyrosine is administered to ob/ob mice, the NE content, and presumably the synaptic availability of NE, is reduced and hyperphagia is potentiated (Batt, Wilson, & Topping, 1978).

Acute injection of NE is most effective in initiating a feeding response in rats when it is injected directly into the PVN region (Leibowitz, 1978). At this site, injection of NE produces the strongest feeding response at the lowest threshold dose. The ingestive response is similar to normal feeding in terms of magnitude and duration. In addition, chronic injection of NE into the PVN results in hyperphagia and increased body weight gain (Leibowitz et al., 1984).

Similar results are found following electrolytic lesions to the PVN. Lesions to the caudal aspect of the PVN are most effective in eliciting hyperphagia and body weight gain (Leibowitz, Hammer, & Chang, 1981). However, feeding behaviour induced by central NE injection is attenuated following discrete lesions to the PVN (Leibowitz, Hammer, & Chang, 1983). It appears that the integrity of the PVN is

essential for a normal feeding response to be elicited by central NE administration. Given that destruction of PVN noradrenergic innervation following local administration of 6-hydroxydopamine (6-OHDA) results in hypophagia, this would suggest that hyperphagia resulting from PVN electrolytic lesions cannot be attributed to the destruction of noradrenergic afferents to the nucleus, but reflects damage to PVN efferent projections through which NE may act to control satiety (Leibowitz & Brown, 1980; Shor-Posner, Azar, Jhanwar-Uniyal, Filart, & Leibowitz, 1986).

Norepinephrine injected into the PVN at near physiological doses stimulates food intake in the satiated animal, an effect which is blocked by selective alpha-2 adrenergic receptor antagonists, including yohimbine and rauwolscine, but not by beta-adrenergic receptor blockers, nor by blockers of serotonergic, cholinergic, or dopaminergic receptors (Callahan et al., 1984; Goldman, Marino, & Leibowitz, 1985; Leibowitz, 1980). Clonidine, an alpha-2 adrenergic agonist, increases total food intake in hungry and sated mice and rats (Callahan et al., 1984; Leibowitz et al., 1985, Marino, De Bellis, & Leibowitz, 1983). These effects are seen when these drugs are administered either centrally or peripherally (McCabe, De Bellis, & Leibowitz, 1984). Callahan et al. (1984) have found that peripherally administered yohimbine and

rauwolscine significantly reduced 3- and 6-h food intake in both ob/ob and lean mice; however, ob/ob mice were more sensitive to the anorectic effect than were lean mice. Anorectic doses of yohimbine did not affect water intake in water-deprived mice, suggesting a specific effect of the drug on food intake. Furthermore, clonidine (0.1 mg/kg) increased 1-h food intake in obese mice at doses which did not affect food intake in lean mice, suggesting that obese mice were also more sensitive to the hyperphagic effect of clonidine. However, higher doses of clonidine resulted in an apparent suppression of intake in both obese and lean mice. Given that the ob/ob has increased levels of NE in the PVN, the hyperphagia exhibited by this mutant may be attributed, at least in part, to an impaired satiety control mechanism.

Tricyclic antidepressants have also been found to enhance total food intake, specifically following injection into the PVN (Leibowitz et al., 1985). The response to such drugs as desimpramine, protryptyline, and amitriptyline is selectively blocked by alpha-noradrenergic antagonists and is also antagonised by drugs which inhibit the synthesis of endogenous NE (Leibowitz, Arcomano, & Hammer, 1978; Leibowitz et al., 1985). Antidepressant agents, therefore, known to block the neuronal reuptake of NE, elicit feeding by enhancing synaptic availability of endogenous NE specifically in the PVN.

The feeding response resulting from administration of tricyclic antidepressants is prevented by local administration of CA synthesis inhibitors, suggesting that antidepressant-induced feeding is mediated by presynaptic NE stores (Leibowitz, 1978). In contrast, feeding elicited by exogenous NE is unaffected by the same synthesis inhibitors, and is consistent with a postsynaptic mechanism for NE (Goldman et al., 1985). Electrolytic and neurotoxic lesions of the specific ascending noradrenergic fibres that innervate the PVN abolish the antidepressant feeding response, but leave intact or potentiate the postsynaptic NE feeding response (Leibowitz & Brown, 1980).

Given that NE may act via alpha-2 receptors located on the postsynaptic membrane (Langer, 1981), that is, alpha-2 receptors may exist post- as well as presynaptically in the brain, it is possible that clonidine, like NE, may affect food intake via a postsynaptic alpha-2 receptor mechanism. Clonidine-induced feeding is suppressed by injections of yohimbine but not prazosin or corynanthine, alpha-1 adrenergic antagonists (Goldman et al., 1985; Schlemmer, Elder, Casper, & Davis, 1981), implicating alpha-2 receptors rather than postsynaptic alpha-1 receptors in the regulation of feeding.

Postsynaptic alpha-2 receptors are known to mediate a number of physiological effects resulting from clonidine administration, including cardiovascular and sedative effects (Hamilton & Longman, 1982; Nasif, Kempf, Cardo, & Velley, 1983; Spyraki & Fibiger, 1982), which suggests that alpha-2 postsynaptic involvement in feeding is not improbable. Feeding induced by central injection of clonidine, like NE, is unaffected by local administration of alpha-methyl-para-tyrosine (Goldman et al., 1985). Injection of 6-OHDA into the PVN, in contrast to electrolytic lesions, leaves the feeding response evoked by peripheral injection of clonidine intact (Shor-Posner, Azar, & Leibowitz, 1984). As well, biochemical analyses examining the effect of clonidine on NE turnover have indicated that noradrenergic terminals in the PVN respond uniquely to peripheral clonidine administration, in a manner inconsistent with a presynaptic site of action (Jhanwar-Uniyal, Levin, & Leibowitz, 1985). Norepinephrine, clonidine, and tricyclic antidepressants, therefore, may act to stimulate food intake through the same noradrenergic system but at different parts of the synapse.

Statement of the Problem

Until recently, most studies examining the neuropharmacology of feeding have utilised a single, nutritionally complete diet with little concern for the

nutritional composition of the diet (Blundell & Hill, 1986). However, central and peripheral pharmacological manipulations may alter macronutrient selection, suggesting that specific brain neurotransmitters may have a function in balancing the proportion of carbohydrate, protein, and fat consumed by a rat (Kanarek, Marks-Kaufman, Ruthazer, & Gualtieri, 1983; Leibowitz, Roosin, & Rosenn, 1984). For example, Leibowitz et al. (1985) have shown that endogenous NE, acting on alpha-adrenoceptors in the PVN, regulates carbohydrate ingestion in the rat.

Similarly, clonidine and tricyclic antidepressants selectively stimulate carbohydrate ingestion in the rat. Although clonidine has also been found to affect the ingestion of protein and fat, this effect is considerably less than the potentiation of carbohydrate intake, resulting in a significant decline in the proportion of fat and protein consumed (Leibowitz, 1985). That is, both fat and protein intake are increased following clonidine administration; however, when proportion of total energy intake is considered, a reduction in percentage concentration of protein and fat is found. Thus, while NE, clonidine, and tricyclic antidepressants all increase total food intake, these drugs also selectively potentiate carbohydrate ingestion. Therefore, noradrenergic neurons innervating the PVN in the rat have been implicated in the

regulation of carbohydrate selection, and this neurochemical system mediates the stimulating action of clonidine and antidepressants on carbohydrate ingestion.

However, although hypothalamic catecholamines are known to modulate total energy intake and have been implicated in specific macronutrient selection, research has yet to examine neurochemical regulation of macronutrient intake in an obese pathological model, such as the ob/ob mutant, which exhibits hyperphagia. Given the elevated NE levels in the ob/ob, it is possible that a genetically determined abnormality in the PVN-noradrenergic system may lead to the hyperphagia exhibited by the ob/ob mutant. Altered neurochemical mechanisms in this region may allow an increase in the amount of neurotransmitter reaching the postsynaptic membrane. It follows that increased PVN-NE would result in increased postsynaptic alpha-2 receptor stimulation which, in turn, may lead to the hyperphagia exhibited by this mutant.

The present research, therefore, examined the impact of pharmacological manipulations on macronutrient selection in the ob/ob, using a self-selection feeding paradigm, in which mice were given free access to separate sources of each of the three macronutrients, carbohydrate, protein, and fat. The alpha-2 adrenergic agonist clonidine, known to alter

total food intake, was administered to assess its effects on specific macronutrient selection in both ob/ob and +/? mice. Although clonidine was known to alter total food intake and especially carbohydrate ingestion in the rat, its effects on macronutrient intake in the mouse, and in particular the ob/ob, were unknown. The impact of yohimbine, an alpha-2 adrenergic receptor blocker, was also examined to assess its effects on total food intake and nutrient selection in lean and obese mice treated with clonidine. It was hypothesised that total food intake in ob/ob and +/? mice would be altered following clonidine administration. Clonidine was also expected to increase the proportion of carbohydrate ingested by mice. In contrast it was expected that yohimbine would antagonise the effect of clonidine on food intake in obese and lean mice.

Experiment 1

Nutrient selection can be altered by a number of external environmental factors including a restricted feeding regimen (Blundell, 1983; Li & Anderson, 1984), suggesting that drug effects may differ depending on the extent of food deprivation. However, Leibowitz et al. (1985) have found that a 6-h restricted feeding schedule yields a relatively stable baseline nutrient selection pattern and total diet intake scores similar to 24-h food intake scores under ad-lib food conditions in the rat. Therefore, 24-h food

intake measures may also be accurately measured within a 6-h feeding paradigm in the rat. However, previous research monitoring feeding behaviour has shown that obese mice adapt less readily to restricted feeding schedules or meal-feeding paradigms (Callahan et al., 1984; Jagot, Dickerson, & Webb, 1982; Kuprys & Oltmans, 1982), although diet adaptation has only been assessed in research examining macronutrient intake, in lean and obese mice, where nutrient intake was not restricted (Romsos, Chee, & Bergen, 1982; Romsos & Ferguson, 1982). The purpose of Experiment 1 was to determine the time required for ob/ob and +/? mice to adapt to a 6-h feeding regime, implemented in Experiments 2 and 3 where the effects of clonidine and yohimbine were examined, and to assess the comparability of macronutrient self-selection in free-feeding and meal-feeding mice.

Method

Subjects

Obese (C57Bl/6J, ob/ob, n=16) and lean (C57Bl/6J, +/?, n=16) adult male mice, 10 weeks of age at the start of the experiment, were obtained from Jackson Laboratories, Bar Harbor, ME, USA.

Heterozygote crosses between mice carrying the obese (ob) gene lead to animals of three genotypes: the homozygous, dominant (+/+) wild type, the heterozygote (+/ob), and the

homozygous recessive type (ob/ob). Both the +/+ and +/ob are phenotypically lean, while the ob/ob is obese. However, the +/ob mouse is not identifiable from its +/+ littermate, and for this reason, lean littermate controls were identified as +/?.

Mice were individually housed in hanging wire cages (24.5 cm x 18 cm x 18 cm), suspended over wood-chip bedding, with a nest of bedding in each cage. All mice were maintained at 23°C on a 12-h light-dark cycle (lights on 0730 h). Colony room humidity ranged from 30-40%.

Apparatus

Diets were presented individually in circular aluminum containers with a stainless steel cover with four 1-cm holes, to allow access to the macronutrient but minimise food spillage. Water was available in calibrated 100-ml Wahmann drinking tubes. Body weights (g) and food intake (g) were measured using a Mettler Digital Balance (Model No. PB 300) to the nearest .01 g.

Diets

Three single-energy-source diets (cf. Leibowitz et al., 1985) were presented simultaneously to each animal. The carbohydrate ration was composed of 43.9% dextrin (R. Wine Baril), 43.9% corn starch (St. Lawrence Starch Ltd.), 4% minerals (United States Biochemicals), 3% vitamins (United