The Role of Leptin During Neonatal Rat Development: Impact on the Hypothalamic-Pituitary-Adrenal Axis and Energy Balance

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TABLE OF CONTENTS

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ABSTRACT

This work examined the consequences of elevated leptin levels in neonatal rat pups and the possible relationship between leptin and dietary fat levels during development with respect to neuroendocrine and energetic parameters. While acute leptin injection elevated circulating leptin levels, it failed to have an effect on the HPA axis. In contrast, chronic injection of leptin produced a decrease in stress responsiveness as well as a decrease in body weight gain and fat deposition in 10 day old rat pups. Tonic exogenous administration of leptin also resulted in a decrease in stimulated CRF expression, a decrease in NPY expression and downregulation of the leptin receptor in these pups. Also, dams nursing leptin injected litters spent more time in ano-genital grooming of their pups. We also conducted experiments feeding dams a high-fat diet throughout gestation and lactation and found that a high fat diet produced increased plasma leptin in the offspring and is associated with decreased stress responsiveness, increased fat deposition and decreased NPY expression, but not with any changes in maternal behaviour.

RESUME

Le but de notre étude était de déterminer le rôle de la leptine dans la régulation de l 'axe corticotrope au cours du développement chez le rat et de comprendre les relations existant entre des taux élevés de leptine suivant une augmentation de l'apport alimentaire en graisses et le contrôle du stress et de la balance énergétique chez les ratons. Nous avons tout d'abord testé l'effet d'injections aigues de leptine sur la réponse hormonale au stress du jeune rat au jour 10 et 21 de la vie, puis nous avons évalué cette même réponse suivant des injections répétées de leptine entre le jour 2 et 9-10 de la vie. Bien que les deux régimes d'administration de la leptine augmentent fortement les taux de leptine circulants dans le plasma, seul le régime d'injections répétées diminue la réponse au stress des jeunes rats au jour 10 de la vie et produit une diminution de leur poids corporel ainsi que du poids du tissu adipeux. L'administration chronique de leptine chez les jeunes rats diminue également l'expression du CRF hypothalamique sous stimulation de stress ainsi que celle d'un peptide fortement impliqué dans la prise alimentaire, le NPY se trouvant dans le noyau arqué. De plus, l'administration chronique de leptine réduit fortement la concentration des récepteurs pour la leptine dans le noyau arqué, bien qu'elle produise une augmentation des récepteurs aux glucocorticoides dans l'hippocampe. Chez l'adulte, l'administration de leptine augmente la thermogenèse et diminue la prise alimentaire, ce qui pourrait se traduire au cours du développement par des changements du comportement maternel envers les rats injectés avec la leptine. A part une augmentation significative et importante du toilettage maternel reçu par les ratons injectés avec la leptine, il ne semble pas que le traitement chronique avec ce peptide modifie le comportement maternel de la mère face à ses petits. Enfin, lorsque des ratons sont exposés à un lait riche en graisses, les taux circulants de leptine sont augmentés, la réponse au stress et l'expression de NPY hypothalamique est diminuée comme chez les ratons injectés avec la leptine mais au contraire de ces derniers, le dépôt de graisses est augmenté. Nous cherchons actuellement à déterminer l'origine de ces différences d'action physiologique de la leptine au cours du développement.

I. INTRODUCTION

A. STRESS AND THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

Stress is a ubiquitous, yet poorly defined concept in human society and indeed in all of the animal kingdom. Based on the pioneering work of Selye, stress can generally be defined as any physical or psychological factor that alters the homeostasis of an organism. This being so, it is obvious that stress has its place in that it allows the organism to recognize and potentially adapt to environmental changes. However, left unchecked, the responses of the body to stress can have serious deleterious consequences. For this reason, a huge number of disorders and diseases ranging from the metabolic; such as diabetes; to the psychiatric; such as depression; have been linked to stress. Several lines of evidence indicate that the physiological response of an organism to stress can be programmed by various factors, particularly during development. One such factor, examined here, is the nutritional status and/or nutritional composition during the neonatal period. An understanding of possible mechanisms relating to the effect of dietary changes during development on the physiological response to stress could lead to insight on how some individuals could be predisposed to a particular stress response and particularly susceptible to stress-related disorders both during infancy and as adults.

A.1. The HPA Axis

The central nervous system (CNS) coordinates all psychological and physical stress inputs and outputs the effects of stress via two primary pathways. The first is the hypothalamic-pituitary adrenal (HPA) axis and the second is the sympathetic/ adrenomedullary (SA) system (for review see Chrousos, 1995 and 1992). Activation of the

SA system, whereby autonomic innervation of the adrenal medulla leads to the release of epinephrine and norepinephrine, ultimately affects a number of target organs such as the heart and vasculature, producing an increase in heart rate and blood pressure. At the origin of the HPA axis, neurons expressing corticotropin-releasing-factor (CRF) and arginine-vaspressin (AVP) located in the parvocellular portion of the hypothalamic paraventricular nucleus (PVN) receive integrated input from higher centers or sensory pathways activated by stress. These neurons innervate the median eminence, and their products reach the anterior pituitary via the hypophyseal portal circulation. CRF secreted into this system interacts with specific receptors on the cells of the anterior pituitary, stimulating the release of adrenocorticotropic hormone (ACTH) which itself is produced by the tissue-specific cleavage of a large multi-precursor molecule, POMC. AVP is synergistic to the actions of CRF. ACTH secreted into the general circulation acts on the adrenal cortex to stimulate the synthesis and release of glucocorticoids (GC's) into the circulation. The primary GC in humans is cortisol (F), and in rodents, corticosterone (B). These GC's act metabolically to increase glucose availability via lipolysis and glycogenolysis as well as protein catabolism. There are also suppressive to immune responsivesss under conditions of stress.

The mineralocorticoid (type I, MR) and glucocorticoid (type II, GR) receptors mediate GC effects by modulating cellular transcription. Once the cortiocosteroid enters the cell (GC's are highly lipophilic), and binds to the cytoplasmic receptor, it translocates to the nucleus where it becomes capable of binding to a particular sequence of DNA known as a glucocorticoid response element (GRE) and can induce alterations in the rate of transcription. Therefore, the actions of the GC's depend not only on their own presence and density intracellularly, but also on the presence of the intracellular receptor, and on the GRE sequence which is located on the DNA. The MR has a very high affinity for GC (as

well as endogenous aldosterone, a mineralocorticoid) while the GR has a lower affinity for GC's. Although most tissues contain corticosteroid receptors, their distribution is specific. Thus, particular distribution coupled with the differing affinity of the GR and MR can lead to further control of the range and extent of the effects of corticosteroids in general, and GC's in particular. For example, the PVN contains primarily the low-affinity, GR and the hippocampus is rich in the MR (see Meaney et al,1993 for review).

Glucocorticoids travel through the blood stream primarily bound to a high affinity carrier molecule known as corticosteroid-binding -globulin (CBG). In adults, less than 1% of GC's travel freely through the circulation (Hammond et. al., 1991). When bound to CBG, B and F are unable to penetrate the cell membrane to interact with their receptors which reside inside the cell. Therefore, fluctuations in the plasma levels of CBG determine the amount of free-corticosteroid available to exert actions in cells. CBG itself is down-regulated by GC's (Hammond et. al., 1991).

Glucocorticoids have profound metabolic effects which are necessary for survival under conditions of threatened homeostasis (reviewed in Meaney et al, 1993). GC attempt to maintain the necessary energy available for response to the given stressful condition by stimulating the catabolic processes of gluconeogenesis, glycogenolysis, and lipolysis (Taskinen, et. al., 1983). In order to conserve energy which may be necessary to fight or flee, various neuroendocrine systems are suppressed by these effector hormones. For instance, GC's inhibit the reproductive axis at multiple levels including the suppression of GnRH, LH and FSH, as well as testosterone and oestrogen production directly (Rabin,1988). They are also responsible for the inhibition of TSH secretion and the suppression of thyroid hormone production . The activation of the HPA axis over long periods of time can also impinge on growth by suppressing the secretion of growth hormone (GH) and the action of

IGF-1 (Diegez, et. al., 1988). Gastric motility is also impaired in the stress response by the actions of CRF (Tache et. al., 1994) and/or the SA system, mediated by the vagus nerve (Chrousos, 1995). In addition, the HPA axis interacts with the immune system. GC's suppress the inflammatory reaction of the immune system as well as the effects of various cytokines, but GC's also have some stimulatory actions on this system (Stratakis, 1995).

As mentioned, dysregulation of the HPA axis can have drastic negative effects. Over the long-term, for example, the effects of chronic HPA axis activation on the immune system would increase susceptibility to infection. One important result of long-term exposure to GC's is the resulting syndrome known as metabolic syndrome-X which results in characteristics such as insulin resistance, obesity, elevated triglyceride levels and hypertension. The particular relevance of this condition is discussed in detail below.

Because of the hazardous nature of an unchecked HPA axis, tight regulation is a necessity. This is accomplished by multiple feedback loops. GC's feedback negatively at the level of the pituitary, the hypothalamus, and other extrahypothalamic sites to turn-off the release of ACTH and CRF (Chrousos, 1995). Prevention of endogenous GC production by removal of the adrenals (i.e. adrenalectomy, ADX) results in a marked increase in the synthesis of CRF and POMC mRNA as well as increased secretion of ACTH; effects which are reversed by the administration of exogenous corticosteroids (Dallman et. al., 1987). Due to the high density of MR and GR, the hippocampus is also an important site for feedback actions of GC's on the PVN. The hippocampus connects indirectly to the PVN via the BNST and its influence is thought to be inhibitory on PVN secretion (Meaney et al, 1993).

Finally, it should be noted that the HPA axis exhibits a circadian rhythm with low circulating levels of GC's at the beginning of the sleep phase and higher concentrations of GC's marking the onset of the activity period (Dallman, 1987). For rodents, the sleep/wake

cycle would be approximately opposite to humans, with the activity period corresponding to the night. This cyclicity is controlled by neurons in the suprachiasmatic nucleus of the hypothalamus (SCN) which send afferents to innervate CRF neurons in the PVN (Dallman, 1987). Other important behaviors such as feeding are also entrained by the sleep/wake cycle.

A.2. The Ontogeny of The HPA Axis

The status of the HPA axis is of particular interest during the time of development. The developing brain is particularly sensitive to damage from chronic exposure to GC's, therefore it is essential that regulation of stress response be tight and efficient in this period. Various organizational effects of GC's on the development of the rat have been examined, and it is generally accepted that exposure to chronic GC can interfere with neuronal development and cellular proliferation (Munck, et. al., 1984).

In rats the early postnatal period is characterized by a phase of blunted adrenocortical responsiveness during the first 2 weeks of life (Walker, 1986). Although there appears to be a suppression of glucocorticoid secretion in response to endogenous or exogenous ACTH stimulation, the functional components of the HPA axis have the capacity to react at this young age (Walker, 1994). In fact, several of the central mechanisms controlling the activity of the HPA axis are functional as early as the first days of life although the system does undergo maturation over the course of the neonatal period (Walker et al, 1991). Thus, the primary difference between the adult and the neonate appears to be in adrenal sensitivity to ACTH, however, several reports have also pointed to the sharp decline in CBG levels around the time of birth which would lead to a relative increase in the availability of B for negative feedback (Challis et. al., 1995).

Various environmental factors have been shown to influence aspects of the HPA

response to stress in pups. Of particular interest is the interaction between mother and the pups. In landmark studies by Levine and others, it was shown that handling of pups over the first 10 days of life resulted in a decreased response to stress in adulthood (Levine, 1957). This effect was later shown by Meaney et al (1993) to be associated with an increase in licking and grooming behavior by the mothers of pups who were handled. It was further shown that individual differences in the amount of grooming given by mothers (and also arched back nursing posture) could correlate with the activity of the HPA axis in offspring as adults (Liu, et al, 1998). Thus, contact with the mother may translate into predisposition to a particular magnitude of stress response in adult life.

A number of mechanisms have been proposed to maintain low adrenal sensitivity, including those mediated by maternal contact (Hofer, 1994). However, it is becoming increasingly clear that the stress response is part of a larger, integrated system of energy balance. For this reason, we now turn to an orientation on metabolism and energy flow, and how this may relate to the development and functioning of the HPA axis.

B. METABOLISM AND ENERGY BALANCE

Metabolism is essential to control the production and storage of energy for utilization by physiological systems. Energy balance may be represented as the equilibrium between energy availability in the form of food intake or catabolic processes leading to substrate availability; and energy expenditure via physical activity, raised metabolic rate, or increased thermogenesis (Coppack, 1994).

Teleologically, it can easily be seen how information on the state of energy balance of the body would be required by the CNS which controls processes such as feeding or

thermogenesis. The mobilization of energy stores or the laying down of fat depots depend on the energetic requirements at any given time. It can be regulated on a diurnal basis in coordination with other diurnal rhythms like feeding, GC secretion and body temperature; or it can be solicited in particular circumstances that require energy availability like environmental threats or exercise. As any kind of disruption from homeostasis, stress by definition places new demands on the energy utilization system. It is therefore critical that systems regulating the energy balance and those involved in the response to a stressor be fundamentally intertwined.

B.1. Lipostatic Theory of Metabolic Regulation

Over the past 50 years, it has become increasingly clear that energy homeostasis is not maintained simply by stimulating food intake when blood glucose falls below a certain threshold. Such a "glucostatic theory" (Mayer, 1967) does not correlate well with energy expenditure and cannot, over the long term, explain the influence of other variables (time of day, habit) on feeding behavior (Woods, 1998). On the other hand, the "lipostatic theory" (Kennedy, 1953) suggests that stored energy is one factor integrated by the CNS which ultimately determines whether intake of food or energy expenditure is required in a particular state. Thus, a negative energy balance would be characterized by an anabolic requirement and would lead to increased food intake and body weight gain, while a state of positive energy balance would be a condition where catabolism is favored and food intake is ultimately decreased (Woods, 1998).

One major energy source is body fat. The mobilization of lipid for useable fuel is achieved by the process of lipolysis, where triacylglycerides are hydrolyzed into fatty acids and glycerol (Coppack, 1994). The primary site of lipolysis is the adipocyte, and free fatty

acids (FFA's) are released into circulation where they form a complex with albumin (Spector, 1975 / Coppack, 1994). Consequently, the adipocyte is the major regulator of the lipid energy source in the body (Coppack, 1994). Because there is metabolic competition between lipids and carbohydrates as energy substrates (Coppack, 1994), an increase in lipid oxidation and FFA availability leads to a corresponding decrease in glucose uptake and utilization (Coppack, 1994). Several hormones participate in the regulation of this balance (Woods, 1998).

One major hormonal regulator of energy balance is insulin, which increases glucose uptake and utilization while providing the primary hormonal antilypolytic effect (Brindley, 1995). Insulin has been shown to strongly inhibit output of FFA at concentrations below even those needed to elicit glucose uptake (Coppack, 1994). Insulin also increases food intake when injected acutely due to immediate glucose uptake by cells (Bray, 1985), but chronic insulin administration (without insulin resistance) tends to decrease food intake by inhibition of hypothalamic centers controlling feeding (Bray, 1985).

In contrast to insulin, catecholamines - epinephrine and norepinephrine - have predominantly a stimulatory action on lipolysis via beta-adrenergic receptors on adipocytes. However this effect is thought to be most relevant during conditions of physical stress (exercise) (Coppack, 1994). During rest, the activation of alpha-adrenergic receptors on adipocytes is inhibitory to lipolysis (Coppack, 1994). Growth hormone also promotes lipolysis (MacGorman, 1987), but the magnitude of this contribution in relation to the overall regulation of FFA availability is not clearly known.

There is also a very close association between the HPA axis and the nutritional state of the organism (see Dallman et al, 1993 for review). Dallman points out that GC's are unique among hormones in that they are increased in both fed and fasted state and can either

synergize or antagonize the effects of insulin. In both humans and rats, an increase in corticosteroids has been shown with energy intake (onset of feeding), but fasting has also been observed to result in an increased activity of the HPA axis in both species (Dallman, 1993). One important difference is that under conditions of feeding, there would be a rise in insulin levels, while in the fasted state, insulin remains low. These findings not only underscore the importance of the HPA axis in the control of caloric flow, but also highlight the interplay between insulin and cortisol.

Administration of low doses of GC's stimulates energy intake, but high doses, inhibit it (Dallman, 1993). This phenomenon is likely due to different actions mediated by occupancy of MR and GR receptors which bind ligands with different affinity. Thus, low amounts of GC would activate primarily the high affinity type I (MR) receptors resulting in anabolic actions, while higher doses would activate the lower affinity type II (GR) receptors and produce catabolic results (Bray, 1985). These important actions of HPA axis in relation to energy balance have led to extensive study in pathological conditions associated with elevated GC's such as obesity and Cushing's Syndrome. The use of genetic models of obesity have further unraveled the interplay between hormones of the stress axis and metabolic systems under both normal and abnormal physiological conditions.

B.2. The Use of Obesity as a Model and the Discovery of Leptin

Obesity is a disorder characterized by a severe disruption of energy balance, in which energy intake/deposition far outweighs expenditure. It is defined as a body mass index of 30 kg/m^2 or more (Seidell, 1999). On its own, obesity represents a severe health problem in Western society with almost 25% of Americans classified as clinically obese (Wickelgren, 1998). A variety of factors can lead to the development of obesity. These include

nutritional imbalance, physical inactivity, genetic mutations, drugs, endocrine dysfunction, and hypothalamic injury (Bray, 1979). The obese phenotype is associate with other features of the metabolic syndrome-X such as type II diabetes, hypertension, hyperlipidemia, and, most prominently, hypercortisolemia and insulin resistance.

It is of great significance that the full obese phenotype, regardless of its etiology, requires the presence of the adrenocortical system for maximal expression (Dallman, 1984). This supports a strong link between the HPA axis and energy homeostasis. Much of the early experimental work done to understand energy balance in obesity was done using models of hypothalamic lesion (Bray, 1979). In particular, it is well documented that bilateral lesions of the ventromedial hypothalamic nucleus (VMH) leads to the development of obesity (Bray, 1979). The VMH regulates the rhythms of food intake and insulin secretion that are disrupted in the syndrome (Dallman, 1987).

More recently, focus has shifted from lesion studies to animal models which carry genetic mutations resulting in the obese phenotype. Several monogenic mutant strains have been described in rodents, in particular the *ob/ob* mouse, the *db/db* mouse, and the *fa/fa* rat (for an extensive review see Bray, 1979). These strains demonstrate characteristics almost indistinguishable from animals with VMH lesions (Misra, 1996) including hyperphagia, hyperinsulinemia, increased adiposity, decreased oxygen consumption and hypothermia as well as type II diabetes with severe insulin resistance. These mutants also have stunted growth, decreased lean body mass, and cold intolerance (Bray, 1979). Normal rodents who are pair fed to these mutants do not deposit fat at the same rat as the mutants, confirming the fact that this obesity is the result of increased energy intake coupled with decreased energy expenditure (Trayhurn, 1996).

Since it is now generally accepted that the principal indicator of energy balance is the

amount of stored fat (Trayburn, 1996), it is reasonable to suppose that the magnitude of the fat depot might be reflected to the brain by a circulating fat metabolite or related protein that also has access to the CNS. As early as the 1970's, such an obesity factor was suspected from parabiosis experiments (Coleman et al, 1973). These experiments create an exchange of blood and blood-borne factors between two mice by surgically fusing the pelvic bones. The parabiosis of a genetically obese *db/db* mouse to a normal mouse led to severe weight loss and ultimately death of the normal mouse, with no effect on the *db/db*-partner. Conversely, when *ob/ob* mice were parabiosed to normal or *db/db* mice, it was the *ob/ob* partner who lost weight. Coleman suggested that the *ob/ob* mouse must be defective in some circulating factor that was supplied by the fusion with the normal partner or the *db/db* mouse. It was also suggested that the *db/db* mouse would be defective in the receptor for this factor and might have, as a consequence, unregulated levels of the factor itself. In 1994 Freidman's group used positional cloning to isolate the product of the ob gene and identified it as a secreted protein (Zhang et al, 1994). It was given the name "leptin" from the Greek "leptos" meaning thin. They suggested that leptin might signal the size of the adipose depot to the brain and also inhibit food intake.

C. LEPTIN AND ENERGY BALANCE

C.1. Characteristics of Leptin and Its Receptor

The murine ob gene on chromosome 6 (Zhang et al, 1994) expresses a 4.5 kb mRNA containing a 167 amino acid open reading frame (Hamann, 1996) that is translated into an 18 kDa protein. The secreted protein is 146 amino acids long with a size of 14-16 kDa after post-translational modification (Hamann, 1996; Trayburn, 1996). Crystal structure analysis

portrays leptin as a four helix bundle, related to the family of cytokines including IL-6, IL-1, and IL-12 (Prolo, 1998). The human gene for leptin is located on chromosome 7 and contains three exons and two introns. The promoter of this gene has multiple binding sites, including response elements for GC's and cAMP (another important intracellular signaling molecule). This indicates that leptin is likely subject to regulation by GC's and cAMP.

Leptin was originally thought to be expressed exclusively by adipocytes (Misra, 1996); and not in undifferentiated cells (Hamann, 1996). However, it is now known that the protein is produced by cells of the placenta (Hassink, 1997; Hoggard, 1997) which suggests a role for leptin in development (see below). Leptin also appears to be synthesized in the gastrointestinal tract (Prolo, 1998).

Leptin can be detected in the plasma of most mammals including humans, with the corresponding human protein bearing 83-84% homology to the mouse or rat form. The half lives of human and rat leptin have been measured to be 24.9 ± 4.4 min. (Klein, 1996) and 9.4 ± 3.0 min., respectively (Zeng, 1997). These relatively short half-lives are thought to be determined primarily by the rate of renal clearance and might indicate the important of rapid fluctuations in circulating plasma leptin levels.

At present, two strains of mice with ob mutations have been identified, one containing a point mutation and the other a deletion mutation in the promoter region. As a result, no leptin is translated in these animals and they develop obesity. It is likely that additional mutations will be found which will more or less strongly associate with the obese phenotype. In obese humans, as in some other animal models, leptin levels are elevated up to 20-fold (Hamann, 1996). This is indicative of leptin resistance and is reminiscent of the situation in the *db/db* mouse which was originally posited to have a receptor defect.

Tartaglia et al identified binding sites for leptin in mouse choroid plexus and

subsequently isolated the cDNA for Ob-R, the leptin receptor, by expression cloning in 1995. Ob-R is a member of the class-I cytokine receptor family that transduces a signal via the JAK/STAT pathway (Rosenblum, 1996). The receptor maps to the db locus on chromosome 4 and its mRNA is subject to alternative splicing which generates at least 5 distinct isoforms, Ob-Ra-e (Lee, 1997). Of these, only Ob-Rb, the long form of the receptor, contains an intracellular domain (302 amino acids) which is functionally coupled to intracellular signaling systems (Misra, 1996). The *db/db* phenotype contains a point mutation resulting in the premature termination during translation and thus, a receptor without the intracellular portion.

Leptin receptor gene expression has been found in a variety of tissues including heart, kidney, brain, liver, and muscle. *In situ* hybridization studies for the expression of Ob-Rb in the brain have found it expressed principally in the hypothalamus, particularly in the DMH, VMH, and LH nuclei (Fei, 1997). Ob-Ra, a short form without any intracellular domain is expressed also in a wide range of tissues but in the brain is heavily localized in the choroid plexus (Hoggard, 1997). This has led several investigators to postulate that Ob-Ra might be the transporter carrying leptin across the blood-brain barrier. Leptin has been shown to cross the blood-brain-barrier in a saturable, unidirectional manner 20 times faster than albumin, indicating a transport system similar to other peptides of the same size like IL-1a (Banks, 1996). However, it has recently been shown that this particular form of the receptor may actually have a signaling role. Murakami et al (1997) demonstrated that leptin treatment of CHO cells expressing only the Ob-Ra isoform express c-fos, c-jun and jun-B like those expressing Ob-Rb. Since Ob-Ra and Ob-Rb exhibit different distribution in discrete brain regions, they might serve different physiological roles *in vivo*.

Of the remaining isoforms, little is known about Ob-Rc and Ob-Rd, which are both

short forms of the receptor (Fei, 1997). It has been suggested that they may represent bi-products of the splicing process. Ob-Re, however, contains no transmembrane domain, indicating that it is a secreted protein which might participate in the transport of leptin in plasma (Hoggard, 1997). Indeed, Houseknecht et al (1996) have shown that most of the leptin in the plasma is bound to three different binding proteins in rodents and two binding proteins in humans. One of these proteins has a molecular weight of 85kDa which corresponds to that of the Ob-Re. The identity and exact function of other binding proteins remains to be characterized.

C.2. Regulation of Leptin

Several hormones implicated in metabolic regulation have been shown to influence leptin secretion. For example, insulin stimulates leptin mRNA production and secretion directly from adipocytes (Wabitsch, 1996; Hardie, 1996, Hamann, 1996). Glucocorticoids have also been shown to stimulate leptin secretion (Sleiker, 1996) but high doses are necessary (Hamann, 1996). In addition, TNFα and IL-1 have both been shown to increase levels of leptin *in vivo*, consistent with the known anorexic properties of these cytokines (Grunfeld, 1996). In addition, leptin, TNFα and IL-6 are all secreted by the adipocyte and TNFa knockout mice have been observed to have reduced plasma leptin concentration (Mohamed-Ali, 1996).

In contrast, catecholamines produced by activation of the sympathetic nervous system diminish leptin production through beta3-adrenergic receptors (Gettys, 1996). Since the production of cAMP is a downstream event in the beta3-adrenergic signaling pathway, this finding is consistent with the report that leptin production is also inhibited by administration of intracellular cAMP itself (Sleiker, 1996). It has been suggested that the sympathetic

nervous system may experience negative feedback in this way. Sympathetic outflow would lead to a reduction in circulating leptin which, in turn, would decrease the signal for thermogenesis and sympathetic activation (Mohamed-Ali, 1998).

Leptin injection causes down-regulation of *ob* gene expression, but this effect is not observed in cultured adipocytes, suggesting that there are other hormones implicated in this short-loop feedback system of leptin (Hamann, 1996). Thyroid hormones also suppress leptin production possibly via increasing the sensitivity of the beta-adrenergic influence on leptin mRNA synthesis (Mohamed-Ali, 1998) or by their effects on thermogenesis.

C.3. Metabolic Actions of Leptin

As already mentioned, metabolic processes can be viewed as either anabolic, favoring energy intake and weight gain; or catabolic favoring weight loss and reduction in energy intake. There is a strong positive correlation between circulating leptin levels and the fat depot (Hamann, 1996). This is also true of insulin, but only insulin is acutely upregulated by food intake (i.e. within minutes). Over a period of hours, leptin is also increased by food intake, an effect which is probably mediated by insulin (Woods, 1998). Injection of leptin results in a strong catabolic influence on the organism achieved in several ways. First, leptin inhibits food intake. Although leptin receptors are expressed widely, it is now understood that this action of leptin takes place at the level of the hypothalamus (Misra, 1996). Using induction of Fos as a marker of neuronal activation, it as recently been shown that leptin can activate multiple hypothalamic nuclei including the ventromedial, dorsomedial, ventral premammillary and paraventricular nuclei, as well as distinct neuronal populations in the brain stem (Elmquist, 1997). Furthermore, the hypothalamus contains multiple nuclei and neuropeptidergic neurons involved in integrating

peripheral information and affecting energy homeostasis. For instance, Neuropeptide Y (NPY) is an important peptide in this system because it is one of the most potent stimulators of food intake (Woods, 1998). NPY expressing cells in the hypothalamic arcuate nucleus (ARC) project to the PVN, the primary energy balance integration site. NPY also inhibits sympathetic outflow thereby reducing energy expenditure and augmenting a state of positive energy balance. *Ob/ob* and *db/db* mice (as well as *fa/fa* rats) greatly overexpress NPY. Injection of recombinant leptin to *ob/ob* mice causes decreased food intake and body weight, a dose dependent effect that is more potent with central than peripheral administration (Hamann, 1996). Leptin reduces the expression of NPY in these animals as well as normal mice (Woods, 1998). The role of leptin appears to be direct on arcuate neurons expressing the leptin receptor since similar results are not observed with receptor-mutants such as *db/db* (Woods, 1998). Via its receptor, leptin has been shown to modulate synaptic transmission by affecting calcium currents in the arcuate nucleus (Glaum, 1996). A second hypothalamic system worthy of consideration here is the melanocortin system. Alpha-melanocyte-stimulating-hormone (αMSH) is produced as one produce of the POMC multihormone precursor in the cells of the ARC (Kalra, et. al., 1999). This hormone also acts on the PVN cells but, in contrast to NPY, it is anorectic in nature (Woods, 1998). Leptin receptors are also expressed on POMC neurons (Cheung, 1997), and POMC mRNA is reduced in *ob/ob* mice (Woods, 1998).

The second catabolic outcome of leptin is to increase energy expenditure. Leptin increases the activity of the sympathetic nervous system (Traynhurn, 1997). This results in an increased metabolic rate (Woods, 1998) and thermogenesis (Tuominen, 1997). For this reason, animals injected with leptin lose weight to a greater extent than would be predicted by the reduction of energy intake (food) alone. Leptin injection in *ob/ob* mice (which lack the

production of leptin) also normalizes glucose, insulin and corticosterone levels which tend to become increased in the metabolic syndrome (Hamann, 1996).

C.4. Leptin and Stress

An interplay between elements of the HPA axis and metabolic systems has already been emphasized. Several lines of evidence indicate that leptin reduces corticosteroid secretion. It was demonstrated that leptin directly inhibits cortisol and corticosterone secretion in cultured cells from the adrenal glands of humans and rat, respectively (Bornstein, 1997; Pralong, 1998). Coupled with the fact that GC's regulate the expression of leptin, these data provide evidence for at least one level at which an interaction between leptin and the HPA axis occurs. However, a major step towards considering leptin as a stress-related peptide came when Lincinio et al. (1997) reported that the pulsatility of leptin secretion was inversely related to cortisol pulses in humans. This finding was later confirmed by Korbonits (1997).

Leptin receptor mRNA has been identified not only in the hypothalamus, but also in the pituitary (Deitrich, et. al., 1998) and adrenal gland (Hoggard, et. al., 1997). It is becoming increasingly clear that an inverse relationship exists between leptin and the HPA axis. It has now been demonstrated that leptin reduces the rise in plasma ACTH and corticosterone seen in fasted rats (Ahima, 1996). Leptin has also been shown to block the response of the HPA axis to restraint stress in normal mice (Heinman, 1997).

The effects of leptin on hypothalamic CRF are more controversial. *In vitro* application of leptin to hypothalamic explants stimulates the release of CRF (Costa, 1997). However, *in vivo* data supports an inhibitory role for leptin on the stress axis since administration of leptin to *ob/ob* mice completely prevents the rise in c-fos expression of

PVN CRF neurons that is normally seen following food deprivation (Huang, 1998). At this point, methodological differences may account for these discrepancies. The potential of a system *in vitro* is not always indicative of its function *in vivo*. However, it should also be noted that CRF is, itself, an anorectic peptide. Central administration of CRF reduces food intake and body weight (Wood, 1998). Thus, if CRF represents one mediator of leptin's action to decrease food intake and increase energy expenditure, then leptin may be both stimulatory and inhibitory to CRF neurons in the PVN, depending on the conditions.

D. LEPTIN AND DEVELOPMENT

Development is a time of dynamic fluctuations in energy balance. The energetic requirements for growth an maturation are part of intricate system which are subject to various hormonal influences. It has become increasingly clear that leptin, in addition to being an important central signaling molecule, may be one hormone involved in the developmental process. In addition to metabolic defects, *ob/ob* mice have abnormal myleination in the brain (Sena, 1985), as well as lower brain weight and cortical volume (Steppan, et.al., 1999). Consequently, there has been a recent mounting interest in the role of leptin during the neonatal period, since very little is currently known about the function of leptin during development.

Leptin is detectable in the blood of normal rats as early as day 1 of life (Rayner, 1997) and Devaskar et. al. (1997) have measured leptin levels in plasma from 2-day old mice. Various studies implicate leptin as possibly having a role in neonatal rodents. For example, Ahima et al (1998) report a discrete leptin surge in mice between d7 and d10. This finding is in agreement with the observation that leptin mRNA is elevated over the first 2 weeks of life

in mice (Devaskar, 1997). Rayner (1997) has shown that an increase in leptin also occurs in both normal and *fa/fa* rats, although absolute levels are significantly higher in the genetically obese animals.

There is also data from human studies supporting a role for leptin in infancy. Leptin has been detected in human fetal cord blood (Ertl, et. al., 1999) and is present as early as 18 weeks of gestation (Jaquet, et. al., 1998). It has also been shown that leptin levels are correlated positively with birth weight (Sivan, et. al., 1997, Gomez, et. al., 1999). It has also recently been discovered that leptin is expressed in the placenta (Hassink, 1997; Hoggard, 1997). Leptin has been isolated from milk (Houseknecht, 1997) and has been shown to be transferred to the suckling neonate from the mother via nursing (Casabiell, 1997), but since leptin is also produced endogenously in the infant, it is unclear whether this source of leptin is biologically significant. However, increases in leptin mRNA and concentrations during development can be augmented by feeding a diet high in fat (Trottier et al, 1998, Rosseau, 1997; Ahren, 1997). When taken together with the neurostructural and endocrine abnormalities seen in *ob* mutants (Ahima, 1998), and the restorative effects on reproductive function, these findings data provide a powerful case for leptin as an important protein during development.

Garcia-Mayor (1997) observed an increase in leptin levels in both male and female children prior to puberty, with females having larger overall leptin values. However, after puberty, males showed a decrease in plasma leptin coincident with increasing testosterone (Blum, 1997). This is interesting since Devaskar saw no differences in plasma leptin between male and female mouse pups. The increase in plasma leptin with developmental stage reported by Hassink (1996) occurred in both control and obese children. But while Hassink reported no sex differences in adults with respect to leptin levels, other studies have shown an

inverse correlation between leptin and age through adulthood (Ostlund, 1996). These data are supported by animal models since the infertility of *ob/ob* mice is restored by administration of exogenous leptin (Barash, 1996; Chehab, 1996). Further, administration of leptin can actually accelerate sexual maturation in rats, indicating that the protein is at least permissive to the onset of puberty (Cheung, 1997). However, leptin is clearly not the only factor involved in triggering sexual maturity. It is possible that leptin's relation to energy balance is simply a signal to the organism that the body is energetically capable of meeting the metabolic demands of puberty.

The apparent relationship between leptin and the reproductive axis notwithstanding, there is currently very little data on how leptin might act with respect to other systems that it is known to modulate in adults. Flier has recently addressed the issue of brain development (Ahima, et.al., 1999) by administering leptin to neonatal *ob/ob* mice. This study reported that leptin normalized levels of some neuronal and synaptic proteins such as growth associated-protein and syntaxin-1, while not affecting others. Thus, it appears that leptin may have specific roles in the development of neuronal connections and CNS development in mice, but there are currently no data on this role for leptin in rats or humans.

The precise mode of action of leptin during the neonatal period is not clearly specified. The ontogenic profile of the leptin receptor remains to be elucidated. Similarly, very little is known about leptin's impact on the HPA axis during the neonatal period. Since leptin administration is associated with weight loss in adults, the fact that infants continue to grow despite high levels of leptin has led some to suggest that infants may experience a resistance to leptin during their development. However, exogenous administration of leptin to rat pups has been shown to decrease body weight and fat depot (Stehling, 1996). This effect was accompanied by an increase in oxygen consumption and core temperature but no

decrease in food intake. Also, by observing the suckling of *fa/fa* rats, Rayner (1997) determined that the inability of leptin to signal in these animals does not affect the milk consumption. Therefore, while leptin signaling is undoubtedly of significance during the neonatal period, there are important differences with respect to the role leptin plays in modulating energy balance as compared to the adult.

The studies described in this thesis examine the role of leptin in rat pups during development with respect to energy balance and the activity of the HPA axis. Because these two systems are related, we hypothesize that leptin might represent one important link between them. Our laboratory has previously shown that pups nursing on milk that is high in fat have increased plasma leptin levels on day 10, and also display a blunted ACTH response to stress. We hypothesized that leptin might be one mediator between these two effects. If so, leptin could be protective as well as permissive in the developing animal, limiting the exposure of the CNS to glucocorticoids while promoting growth and maturation.

II. SPECIFIC AIMS

The possibility of a neuroendocrine link between energy balance and stress responsiveness has particular relevance during development where both systems are tightly regulated and essential in function. This makes the role of leptin not only important, but essential to understanding the processes by which animals grow and develop. In examination of the role of leptin during development, this Master's thesis was designed to accomplish several specific objectives. First, we intended to discover whether high levels of circulating leptin could affect the stress responsiveness of neonatal rats. We addressed this issue by looking at hormonal stress responses following acute increases in leptin by exogenous administration. Second, since we previously showed decreased stress responsiveness with elevated leptin levels in a high fat milk paradigm, we sought to elucidate some of the consequences of tonically elevated circulating leptin levels over the first 10 days of life on the HPA axis, and whether prolonged exposure to leptin had any energetic effect on neonatal rats. Further, we questioned whether chronic elevation of leptin had any regulatory effects on expression of the leptin receptor and other related proteins such as NPY. In addition, we raised the question as to whether high leptin in pups is translated into any changes in the behaviour of the dam which could be mediating some of the neuroendocrine or metabolic effects observed. Finally, in an attempt to link our leptin studies back to the model of a high fat diet, we reexamined stress responsiveness in rat pups nursing milk with elevated fat content. We also compared these pups in terms of their leptin and NPY levels, as well as the maternal behaviour, building on our previous findings. It is our hope that these studies have shed new light on the complex systems integrating stress and energy balance, particularly during development; and that this insight might ultimately contribute to our knowledge of these systems in humans and aid in our study of human endocrine disorders.

III. MATERIALS AND METHODS

a) Rationale and Experimental Design

Experiment 1. Our laboratory has previously demonstrated that dietary fat content influences HPA function in neonatal rats. We have also observed that a high fat diet is associated with increased leptin levels in these rats. Therefore, because of leptin's putative role as an indicator of fat depot, we sought to determine the extent to which leptin might be involved in mediating the HPA changes observed. We hypothesized that leptin might act to suppress the peak ACTH response to stress as we had seen with pups feeding on high fat milk and has also been demonstrated with adult rodents acutely challenged with leptin. Experiment 1 tested the effect of an acute administration of leptin to pups at various ages during development. Pregnant female rats were received and housed in our animal facility as described below. Litters were designated as either to receive leptin (LEPT) or vehicle injection (VEH). The pups were allowed to mature undisturbed until either day 10 or day 21, when leptin was injected intraperitoneally at a dose of 3mg/kg body weight. The acute challenge used here consisted of each animal receiving an injection 18 and 3 hours prior to stress testing (see below). These pups were then examined for differences in hormonal response to stress (see Table 1).

Experiment 2. We further hypothesized that because a high fat diet is a chronic condition, that the effects of leptin might require repeated administration over a period of days in order to be observed. Using the same experimental groups, a chronic paradigm was used for leptin administration. In this case, we followed 3 different regimes, all of which were, effectively, chronic exposure of the pups to leptin. First, IP injection of leptin was

given from D2 through D10, at a dose of 1 mg/kg and 3 mg/kg, with stress testing conducted on D10. This regime was conducted using either homogeneous litters (i.e. each pup receiving the same treatment) or litters that were mixed with one half receiving leptin and the other vehicle. Second, pups were injected from D2 through D9 with no injection on the day of the stress test, D10. Finally, leptin was administered at the high dose, 3 mg/kg beginning on D6 and continuing through the day of stress, D10. End points measured in this series are outlined in Table 1.

Experiment 3. The original high-fat feeding paradigm was conducted by feeding various diets from the beginning of lactation through the dates of testing. In order to confirm the previous findings regarding the impact of high fat diet on HPA function, we repeated this paradigm as our third series of experiments. However, in this instance we wanted to test the effect of dietary changes during pregnancy and continuing through lactation rather than during lactation only. In addition, after having measured some energetic and behavioural parameters in the leptin-injection experiments, we wished to compare these results by measuring similar end points in the high-fat regiment. Female rats mated in our facility were then fed a control, high fat, or low carbohydrate diets as described below. The composition of the diets is listed in Table 2. After the pups were born, the mothers were maintained on the specific diets. Stress testing of the litters was conducted on D10. Table 1 lists the end points compared for this third series.

b) Animals.

For experiments 1-2, pregnant female Sprague-Dawley rats were received in our animal facility on day 15-16 of gestation (Charles River, St. Constant, Canada). The mothers

were singly housed in clear plastic cages with unlimited access to food (Purina chow diet, Ralston-Purina, St. Louis, MO) and water. The standard rat chow diet consisted of 4.5% fat, 22.5% protein, and 52.1% carbohydrates, with an energy density of 3.34 kcal/g. Constant conditions within the facility of 22-25°C and humidity of 70-80% were maintained, with a 12:12 hour light:dark cycle in effect (lights on at 08h00). The day of parturition was designated at Day 0 (D0) and litters were culled to 10-12 pups per litter on D2 without manipulating the sex ratio of the litters. Litters were assigned to receive either leptin or vehicle, with dose and frequency depending on the experimental conditions described above. All pups of a given litter received the same treatment (i.e. homogeneous litters), except for one series of heterogeneous litters in which half of the litter received leptin and the other half vehicle. For this experiment, pups were identified by a magic marker band around the base of the tail. All protocols were approved by the Animal Care Committee at McGill University and followed ethical guidelines from the CCAC.

For experiment 3, female Sprague-Dawley rats were received from Charles River at a weight of 130-150g. Mating was conducted by placing three females in a large clear plastic cage with one male rat. Body weight gain of the females was recorded daily during the procedure. When weight gain was sufficient to indicate pregnancy, the female was transferred into a clear plastic cage and singly housed thereafter. Pregnant females were fed either a control, high fat, of low carbohydrate diet as described earlier from the time they were introduced to the male, to ensure the dietary consistency over the entire duration of the pregnancy. These diets (Harlan Teklad, Madison, WI) were in powdered form, given in clear, glass, anti-spill jars with screw on metal tops, and were available *ad libitum*. Except for cage cleaning (once weekly), litters were undisturbed until the day of the stress testing which was D10, post partum.

c) Leptin Administration.

Murine leptin was obtained lyophilized from Preprotech Inc. (Rocky Hill, NJ) and reconstituted in 10mM Tris buffer at a pH of 9.5. After dissolution, the pH was readjusted to 7.4 by addition of HCl. Leptin or vehicle (10mM Tris-HCl pH 7.4) was injected intraperitoneally to the pups in a volume of 50ul, with all injections given in the morning between 08h00 and 10h00. Doses were calculated daily, based on the weight of the litter the previous day, and adjusted for 24 hours of pup growth.

d) Maternal Behaviour.

Maternal behaviour was recorded on D8-9 of lactation and each mother was recorded only once. Mothers and their litters were kept in a quiet room during the entire duration of the experiment and access to the room was prevented during recording sessions. Videotape recording started one hour after administration of the treatment to the pups and continued for a period of 8 hours during the light portion of the light:dark cycle. Tapes were analyzed to determine the total time each dam spent with her pups (total nesting time / 8 hour, min/hr), the frequency of nesting bouts (total number of nesting bouts / 8 hours, bouts/hr), the average duration of each bout (total nesting time / number of bouts, min/bout) and the average time spent in ano-genital grooming of the pups prior to the onset of a nesting bout (total grooming time / 8 hours, min/hr). In addition, milk ejection reflex episodes were identified by recording the stretching reflex of the pups and reorganization of the litter to the nipples that typically occur following a milk ejection reflex (MER). The average frequency of MER was determined per nursing bout for 2-3 bouts per mother within the first 3 hours of recording.

e) Ether Stress.

All experiments were conducted between 10h00 and 13h00 to minimize the effect of daily hormonal fluctuations on stress response (Dallman, et al, 1993). Pups from different treatment groups were separated from their mothers 20-30 min. prior to the onset of stress and placed in an opaque housing cage kept in a quiet room. This was done to allow randomization of the litters within one treatment group and also to reduce the disturbances typically associated with repeated intrusion into the mother-litter cage. All disturbances prior to and during the testing were avoided as much as possible. The stressor consisted of 3 minutes exposure to ether vapors and the time points for analysis were determined to be 0 (control), 5, 30, and 60 minutes following the onset of stress. Control (0 min.) animals were removed from the cage and immediately weighed and sacrificed, a process which took less than 5 sec. The exposure to ether for the experimental pups consisted of 1 min. in a glass jar saturated with ether vapors and 2 minutes under a nose cone containing cotton impregnated with ether. After completion of the 3 min. exposure, pups were returned to clean cages and sacrificed by decapitation at the predetermined time points (5, 30, or 60 min.) thereafter.

f) Blood and Tissue Collection.

Trunk blood was collected in Eppendorf tubes containing 10ul of EDTA (60mg/ml) and plasma was kept frozen at -20°C until used for hormone assays. For experiments 2 and 3, the left retroperitoneal fat pad was dissected from pups and weighed. Brains from the 0 min and 60 min time points were rapidly dissected and postfixed in a solution of 4% paraformadlehyde in phosphate buffer (0.5M, pH 7.4) at 4°C for 4 days, followed by immersion in a solution of 10% sucrose in phosphate buffer for 2 days at 4°C. Brains were

then frozen at -80°C before being processed for *in situ* hybridization. In experiment 3, the dams were also sacrificed by decapitation within 2 hours after removal of the pups, and trunk blood was collected for determination of leptin levels. Fat pads were also dissected and weighed.

g) Hormone Assays

Plasma ACTH levels were measured by a specific radioimmunoassay as described previously (Walker, et al., 1990). The limit of detection of the assay was 15.6 pg/ml and the inter and intraassay variability was 26% and 8%, respectively. Plasma corticosterone concentrations were determined by RIA using a kit from ICN Biomedicals (Costa Mesa, CA) with slight modifications. The limit of detection was 0.2 ug/dl, inter- and intraassay variability was 12% and 3%, respectively. Plasma leptin levels were measured in basal (0 min) samples by specific RIA using a kit from Linco Research (St. Charles, MO). The limit of detection was 0.5 ng/ml and interassay variability was 9%. Plasma corticosteroid-binding globulin (CBG) was measured by CBG binding according to a protocol described previously (Tannenbaum, et al., 1997).

h) In situ Hybridization

In situ hybridization was performed for CRF and NPY according to a protocol described earlier for similar neuropeptides (Laurent-Huck and Felix 1991). The NPY probe was a 48 base oligomer (Kozak et. al., 1998) and the CRF probe was a 45-base oligomer, complementary to bases 523 to 667 of the 2nd exon of the CRF gene (Sheldon Biotechnology Center, Montreal, PQ).

The probes were 3'-end labeled with ^{35}S and terminal deoxynucleotyltransferase using a kit from Boerhinger Manheim (Laval, Quebec) and purified on Nensorb columns (Dupont NEN, Boston, MA). Brain sections (25 um) of vehicle and leptin-treated rats were collected onto slides coated with poly-L lysine and stored at -80oC until hybridization. Sections were fixed with 4% paraformaldehyde in 0.1M phosphate buffer for 10min and dehydrated in graded ethanol prior to being submitted to a series of washes in saline sodium citrate (SSC) 4X containing 1% Denhart's solution (1x 1hr), 0.2M triethanolamine (TEA) and NaCl 18% (1x 5min), 0.2M TEA, NaCl 18% and acetic anhydride 0.25% (1 x 10 min), and SSC 2X (3 x 5min). The sections were then dehydrated in graded ethanol, rinsed in choloroform, followed by ethanol 100% and 95% and air dried. Sections were incubated with 75ul of hybridization solution containing 7.5-9.0 $x10^5$ cpm of the ^{35}S-labelled probe and coversliped before being incubated overnight at 42oC. The hybridization solution consisted of 0.6M NaCl, 0.01M Tris buffer, 500ul/ml formamide, Denharts solution (1X), 0.1M phosphate buffer, sarcosyl (1X), 1mM EDTA, 0.5mg/ml tRNA, and 0.25mg/ml salmon sperm DNA. The imperfect hybrids were disrupted by successive washes in SSC 4X, SSC 1X, SSC 0.5X and SSC 2X and the sections were dehydrated again in graded ethanol and air dried before exposure to beta-Max Hyperfilm (Amersham, Arlington Heights, IL) for 6 days at –80°C. Radioactive standards prepared from brain paste with high activity [^3H] and [^{14}C] were exposed simultaneously. Hybridization signal on the autoradiograms was quantified from sections placed in the medial portion of the PVN and using a computerized densitometry by means of an MCID image analyzer system (Imaging Research Inc, Ste Catherine, ON).

In situ hybridization for the leptin receptor was performed according to a protocol described earlier (Huang, et. al.). The probe was a 473 base oligomer (Mercer, et. al., 1996).

i) Statistical Analysis.

Body weight values were obtained by dividing daily litter weights by the number of pups per litter and calculating the average daily body weight gain as a function of treatment group. Fat pad weights were transformed into a ratio as a function of body weight and these data were analyzed by one-way ANOVA. For ACTH and B levels, two-way ANOVA was used with time and treatment as variables, and leptin levels were analyzed by one-way ANOVA. All significant interactions were determined by F tests for simple main effects, with pairwise comparisons performed using Tukey's honestly significant difference test. Maternal behaviour, CRF and NPY mRNA levels were analyzed using Students t-test, where appropriate. The level of significance for all analyses was set as $p < 0.05$. All values are expressed as mean ± SEM.

IV. RESULTS

A. ACUTE INJECTION OF LEPTIN

A.1. Effect of Leptin Injection on Plasma Leptin Concentrations.

Our first objective was to find a way of elevating circulating concentrations of leptin in rat pups. We achieved this by injecting leptin intraperitoneally and we selected a dose of 3mg/kg for our acute injections. We adopted a regime of injecting this dose of leptin 18 hours and then 3 hours before the onset of the ether stress. This acute injection of leptin at a dose of 3 mg/kg significantly elevated the circulating levels of leptin in rat pups, measured 3 hours after injection on D10 of life. As shown by Figure 1, leptin injected pups had plasma leptin concentrations of 55.4 ± 6.5 ng/ml compared to vehicle injected pups, at 3.38 ± 0.4 ng/ml ($p<0.001$).

A.2. Effect of Acute Leptin Injection on Hormonal Response to Stress.

Having already seen high leptin levels in pups consuming high fat milk, concomitant with a blunted ACTH response to stress, we hypothesized that high circulating levels of leptin produced exogenously might similarly inhibit the stress response in pups consuming a normal diet. Stress responsiveness was measured at 2 different ages (D10 and D21) by determining the plasma concentration of ACTH and B following 3 min. ether stress at various time points (Table 3). No differences were observed between leptin and vehicle treated-pups with respect to either ACTH or B levels in 10 or 21 day-old pups.

B. CHRONIC INJECTION OF LEPTIN

B.1. Effect of Leptin Injection on Plasma Leptin Levels.

Although we saw no effect of exogenous leptin administration on stress responsiveness with an acute injection, we recognized that the effect of diet is a chronic condition and that chronic treatment with leptin might be necessary to induce comparable effects. We used several chronic treatment regimens (see Materials and Methods) to create a background of high circulating leptin levels in pups over the first 10 days of life. Chronic injection of leptin in pups between D2 and D9 significantly elevated plasma leptin levels measured 24 hours after the last injection (i.e. on D10). Figure 2 shows a dose-related increase in leptin concentrations between the 1 mg/kg and the 3 mg/kg treatments. The importance of the background effect of chronic leptin treatment on plasma leptin concentrations can be seen in Figure 3. Pups who received an injection of leptin 3 hours prior to testing on D10 in addition to chronic injection beginning on D2 exhibited much higher plasma levels than those receiving only an acute injection at -18hr and -3hr prior to sacrifice (see also Part A.1 for comparison).

B.2. Effect of Leptin Injection on Body Weight and Fat Deposition.

Because of leptin's known effects on metabolic function in adults, we sought to test the biological activity of leptin in pups by measuring body weight gain and fat deposition following chronic treatment. As shown in Figure 4, leptin injection (1 mg/kg) significantly reduced body weight gain of the pups compared to vehicle injection ($p<0.05$). A similar effect was observed for the 3 mg/kg dose, however, because there were only two litters in this group, statistical calculations could not include this group. The average body weight gain of

this group was indeed less than that of the other two. The biological activity of leptin in pups was confirmed by the measurement of retroperitoneal fat deposition in these animals, which was significantly and dose dependently reduced by leptin treatment (Figure 5, normalized to body weight, $p<0.001$). When fat pad was expressed in absolute values, a similar effect was observed (VEH = 25.2 ± 2.5 mg; 1 mg/kg dose = 12.4 ± 1.4 mg; 3 mg/kg dose = 8.5 ± 0.9 mg, $p<0.001$).

B.3. Effect of Leptin Injection on Hormonal Stress Response.

Plasma ACTH responses to ether stress in 10-day old pups injected chronically with either leptin or vehicle are displayed in Figure 6 (D2-9, 1 mg/kg dose) and Figure 7 (D6-10, 3 mg/kg dose). Two-way ANOVA revealed a significant main effect of dose (1 mg/kg, $p<0.001$; 3 mg/kg $p<0.001$) and of time ($p<0.001$) as well as a significant interaction between dose and time ($p<0.001$). Pairwise analysis at both doses showed that while peak ACTH response was not altered, leptin-injected pups showed a faster return to baseline levels compared to vehicle-injected pups. The reduced magnitude of the total pituitary response to stress was obvious when expressed as a function of the total area under the curve (AUC, Figure 6 and 7, lower panels). Although leptin treatment significantly reduced the ACTH response to stress in these neonatal rats, it did not affect basal levels of ACTH or B under either treatment. Plasma B under resting conditions or following stress were not different between treatment groups with either leptin dose (Table 4). At the 0 min. time point, pups in the D6-10 injection group exhibited higher levels of ACTH and B compared to those injected from D2-9, likely reflecting a residual effect of the injection given 3 hours prior to the 0 min. point in the D6-10 regiment (compare Figures 6 and 7). We consistently observed no sex differences between pups under either basal or stimulated conditions (see Table 5).

Since inhibition of the duration of the HPA response to stress could be indicative of increased glucocorticoid feedback on the HPA axis, we tested circulating concentrations of corticosteroid-binding-globulin to determine whether bioavailable corticosterone levels might be altered. Although we detected no significant differences, there was a trend towards increased plasma CBG levels in pups injected with leptin (Figure 8). We also measured expression of the glucocorticoid receptor and mineralocorticoid receptor in brain regions where feedback occurs (see Table 6) and found significantly greater expression of the GR in the CA2 region of the hippocampus.

One possible site at which leptin could affect the HPA axis is at the level of the hypothalamus. By inhibiting the synthesis or release of CRF, leptin could potentially limit the ACTH response to stress. Therefore, we examined expression of CRF in rats that were chronically injected with leptin at the highest dose (3mg/kg). Basal expression of CRF in the hypothalamic PVN was not altered by leptin treatment as shown by *in situ* hybridization for CRF mRNA in Figure 9 (left). In contrast, 60 min. after the onset of stress, stimulated expression of CRF mRNA in the PVN was significantly reduced by leptin administration in the PVN of these rat pups (Figure 9, right). Autoradiograms are displayed in Figure 9-A. Because the *in situ* hybridization for the 0 and 60 min. time points were not performed within the same series, we could not directly compare time effects on CRF mRNA levels.

B.4. Effect of Leptin Injection on Maternal Behaviour.

Because leptin was affecting fat pad weight and body weight gain in the pups, we hypothesized that an increased energy expenditure induced by leptin treatment could modify some aspects of maternal behaviour. For example, increased thermogenesis in pups might

cause mothers to spend less time in the nest, since this variable is highly dependent on nest temperature. As seen in Figure 10 (top), the amount of time spent in ano-genital grooming was significantly greater for dams caring for leptin-injected pups compared to those nursing vehicle-injected pups ($p<0.05$). In contrast to our predictions, there was also a trend for mothers of leptin-injected pups to show longer nesting bouts with their litters (Figure 10, bottom, not significant). In spite of the fact that leptin treated pups gained less weight on a daily basis, there was no difference in the number of milk ejection reflexes per nesting bout, indicating that milk availability was not significantly altered by the administration of leptin to the pups. No significant differences were observed between the treatment groups for any of the other behavioural parameters measured, which included total nesting time, and frequency of nesting bouts (Table 7).

B.5. Effect of Leptin Injection on Ob-R Expression.

If leptin exerts its effects during development through one or more isoforms of its receptor, Ob-R, then chronically elevated levels of leptin in the plasma might change central leptin receptor density or sensitivity in areas related to the regulation of HPA activity. To examine the effect of leptin treatment on leptin receptors, we conducted *in situ* hybridization for all forms of Ob-R in the hypothalamus and in particular, the arcuate nucleus of the hypothalamus. Preliminary analysis of Ob-R mRNA (on 2 animals per treatment group) indicates a reduction in the expression of the leptin receptor with chronic leptin treatment (Figure 11, $p<0.05$).

B.6. Effect of Leptin Injection on NPY Expression.

Since we had observed leptin's ability to modulate expression of CRF, we questioned

whether leptin could affect other proteins as well. In particular, NPY which has effects on feeding behaviour opposite to leptin (i.e. NPY is a potent stimulator of food intake) is a protein that is highly involved in energy balance as well. Since leptin-deficient rodents have high levels of NPY, we hypothesized that injection of leptin might result in a downregulation of NPY in these rat pups. As shown in Figure 12, chronic injection of leptin resulted in a significant reduction in the expression of NPY mRNA in the hypothalamic arcuate nucleus ($p < 0.05$). Both the 1 mg/kg and the 3 mg/kg dose induced a 50% decline in NPY expression compared to vehicle treatment.

C. MODULATION OF DIETARY FAT CONTENT

C.1. Effect of Dietary Fat Content on Milk and Plasma Leptin Levels..

Experiment 3 was designed not only to confirm our previous findings on stress responsiveness with various diets, but also to compare some of the behavioural and energetic parameters which we observed to be affected by direct injection of leptin to pups. Thus we hoped to further clarify the extent to which leptin itself is influential in mediating the differences in hormonal metabolic state in pups as a function of diet. Both a HF and LC diets have a net effect of increasing the proportion of caloric intake from fat (Trottier, 1999). As can be seen from Figure 13 (right) there was no significant effect of diet on plasma leptin of the mothers, but there was a trend towards increased levels in the LC and HF groups. However, pups nursing on milk from HF and LC-fed dams did have significantly elevated plasma leptin levels over those on the control diet (Figure 13, left). While there was no significant difference in milk leptin concentrations between diet groups, there was a trend towards higher levels in HF and LC dams compared to mothers in the CD group (Figure 14).

This indicates that the increased leptin in pups is likely due to the fact that they consumed more fat and not due to differences in the amount of leptin passing from mother to pup through the milk.

C.2. Effect of Dietary Fat Content on Fat Deposition.

Retroperitoneal fat pad weight was recorded on D10 of life. For both pups from the HF and LC groups, fat deposition was significantly greater than vehicle as expressed in fat pad per body weight (Figure 15, left, $p<0.001$). Thus, although these pups had high circulating leptin levels, this protein did not appear to be efficient in inhibiting fat deposition in these animals. Likewise, dams maintained on a HF diet had significantly more fat than those consuming the control diet (Figure 17, right, $p<0.05$).

C.3. Effect of Dietary Fat Content on Hormonal Stress Response.

In pups from mothers fed a high fat diet during lactation only, we previously reported decreased peak ACTH secretion following stress for HF and LC pups. In these experiments, we wanted to test whether increased fat deposition or fat consumption throughout pregnancy and lactation had a larger effect on stress responsiveness in pups. Figure 16 displays the plasma ACTH profile over a 120 min. time course following 3 min. ether stress on D10 across the treatment groups. Two-way ANOVA revealed a significant main effect of time ($p<0.001$) and a significant interaction between time and diet ($p<0.05$). While there was no difference in peak ACTH secretion, subsequent pairwise comparisons showed that HF and LC pups had significantly lower levels of ACTH by the 30 min. time point ($p<0.05$) and HF remained lower at the 120 min. time point ($p<0.05$). As for B secretion, there were no differences between groups with respect to either basal or stimulated B levels (Table 8). We

consistently observed no sex differences between pups within any group (see Table 9).

C.4. Effect of Dietary Fat Content on Maternal Behaviour.

We expected that since the pups from high-fat fed mothers had higher circulating levels of leptin, they might induce nesting behaviour from their mothers similar to what we had observed when pups were given leptin injection. However, there were no significant differences in any of the maternal behaviours measured between HF fed mothers and those on the CD (Table 10).

C.5. Effect of Dietary Fat Content on NPY Expression.

Since NPY is a stimulator of food intake, we hypothesized that it would be decreased in pups from HF-fed mothers due to the increased energy density of their diet. Having already seen that leptin injection can down-regulate NPY expression in the ARC, and that HF and CD pups have increased plasma leptin, we expected that NPY in these animals would be affected. Preliminary analysis of NPY mRNA in the arcuate nucleus showed that NPY expression was indeed reduced in pups from LC-fed dams, but levels were no different from control for the HF group (Figure 17). Further elaboration on these parameters is necessary to clearly specify an effect, however since the energy density of the LC diet is greater than even that of the HF diet, it is reasonable that the strongest effect on NPY would be seen in the LC group.

V. FIGURES AND TABLES

Experimental End Points

Body Weight Gain		X	
Fat Deposition		X	X
Leptin Levels	X	X	X
ACTH Levels	X	X	X
B Levels	X	X	X
CFR mRNA		X	
NPY mRNA		X	X
Ob-R mRNA		X	
Maternal Behaviour		X	X

Table 1: List of the experimental end points measured over the course of each of the three experimental paradigms. X indicates that a parameter was measured in at least one series of a particular experiment.

Composition of Diets

Casein	171.5	172.0	172.5
L-Cystine	3.0	3.0	3.0
Corn Starch	328.5	253.8	-
Maltodextrin	150	150	80.4
Sucrose	150	150	150
Soybean Oil	24	99	149.0
Shortening (Primex)	24.1	99.1	149.3
Cellulose	97.34	10.0	234.71
Mineral mix (AIN-93G-MX)	35	42.0	39.9
Calcium Phosphate, dibasic	6.0	7.2	6.8
Magnesium Oxide	0.3	0.36	0.34
Ferric Citrate	0.25	0.3	0.29
Vitamin Mix (Teklad)	10.0	12.0	11.4
Choline Bitartrate	-	1.2	2.3
Ethoxyquin (antioxidant)	0.01	0.04	0.06

Table 2: Detailed composition of the diets given to the dams in experiment 3. Maternal diets were provided by Harlan Teklad (Madison, WI) and all values are proportionally as g/kg diet. Proportion of macronutrient composition expressed as % by weight.

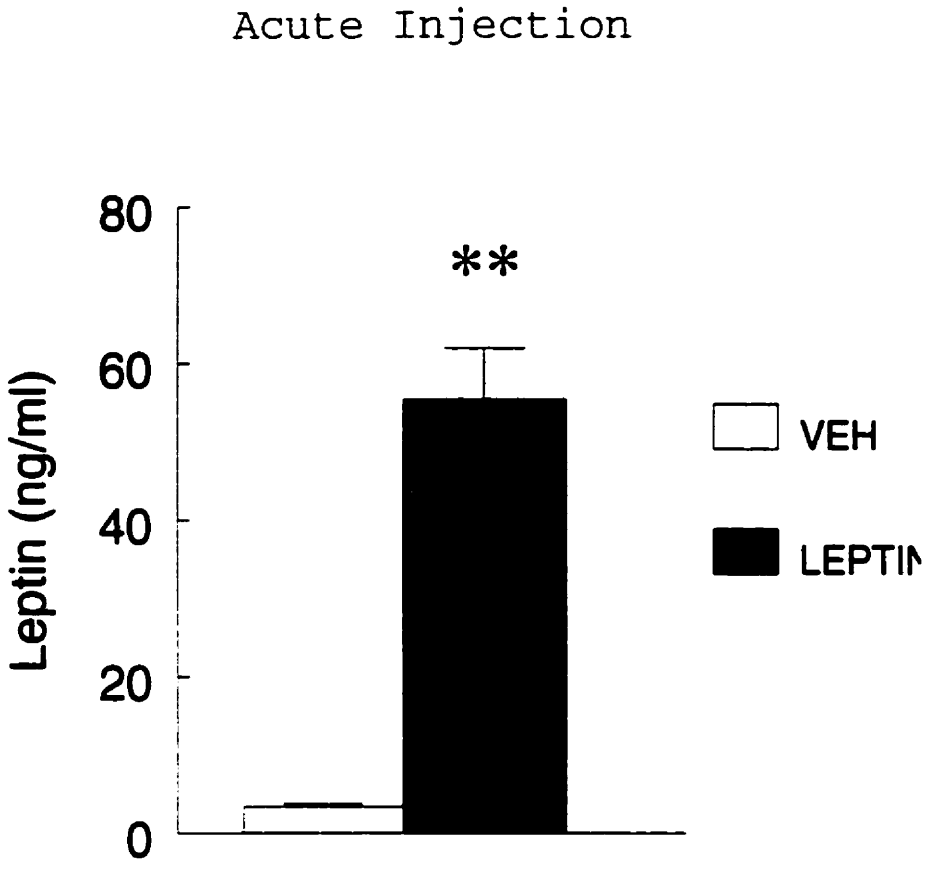


Figure 1: The acute leptin injection paradigm resulted in significantly increased plasma leptin levels compared to vehicle. The graph compared rats tested on D10 that received either leptin (3mg/kg) or vehicle at -18h and -3 hours prior to sacrifice. Values represent means ± SEM for of 13-14 animals per group. (**, $p < 0.01$).

Stress Response, Acute Leptin Injection

0 min.	VEH	109.9 ± 10.3	2.7 ± 0.6
	LEPT	119.4 ± 14.3	3.0 ± 0.3
5 min.	VEH	162.6 ± 28.2	
	LEPT	173.6 ± 12.5	
30 min.	VEH	125.3 ± 14.8	3.9 ± 0.7
	LEPT	183.9 ± 36.2	4.0 ± 0.6
60 min.	VEH	158.5 ± 42.6	3.7 ± 0.4
	LEPT	149.8 ± 9.0	4.3 ± 0.5
0 min.	VEH	56.8 ± 28.4	18.3 ± 2.5
	LEPT	36.7 ± 21.2	16.8 ± 2.0
5 min.	VEH	405.6 ± 15.9	25.3 ± 12.6
	LEPT	322.8 ± 41.1	13.4 ± 1.7
30 min.	VEH	285.7 ± 44.5	21.7 ± 1.6
	LEPT	443.9 ± 74.5	24.9 ± 1.7
60 min.	VEH	343.7 ± 57.7	23.8 ± 2.1
	LEPT	338.5 ± 77.7	24.8 ± 3.6

Table 3: ACTH and B responses over 60 min. following 3 min. ether stress in rats injected acutely with leptin (3mg/kg; -18h, -3h) or vehicle. Values expressed are means ± SEM. No significant differences were observed

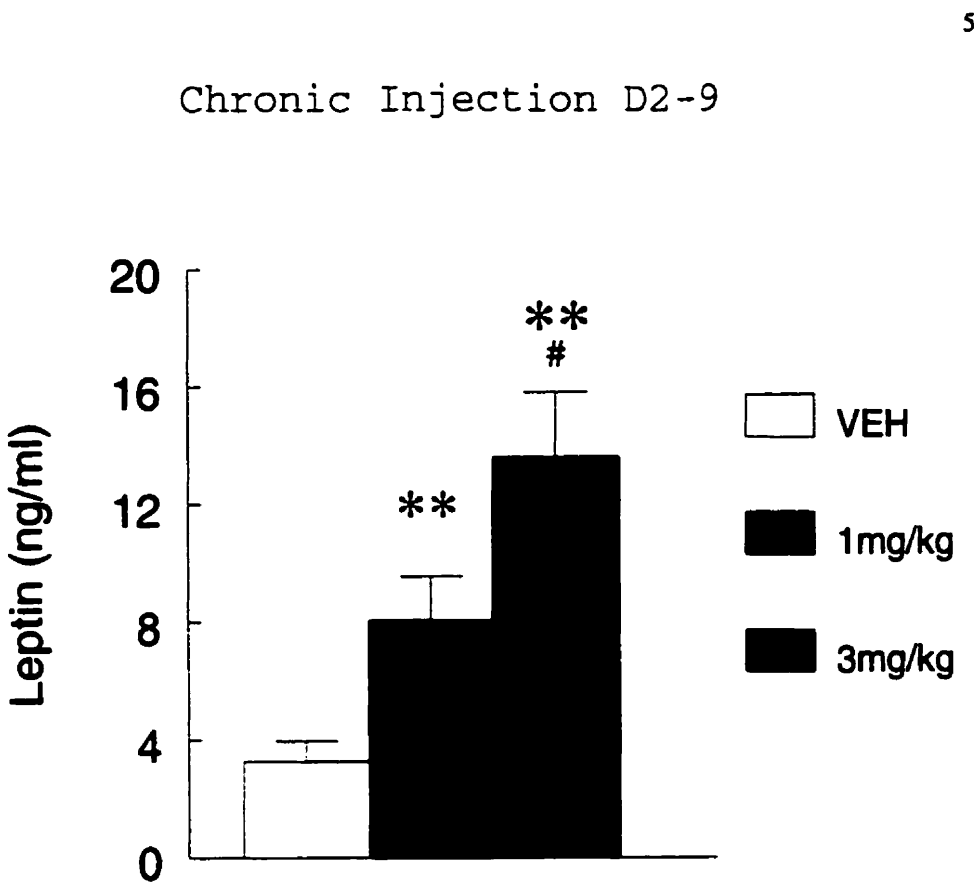


Figure 2: The chronic injection paradigm produced a dose dependent increase in circulating leptin levels in 10 day-old pups. Rats received either leptin at 1mg/kg, 3mg/kg, or vehicle from D2 to D9, and were sacrificed on D10. Values represent means ± SEM of 22-24 animals per group. One way ANOVA showed a significant effect of treatment group, $F(2,26) = 14.2$, $p < 0.001$. ** = different from vehicle, $p < 0.01$; # = different from 1mg/kg, $p < 0.05$.

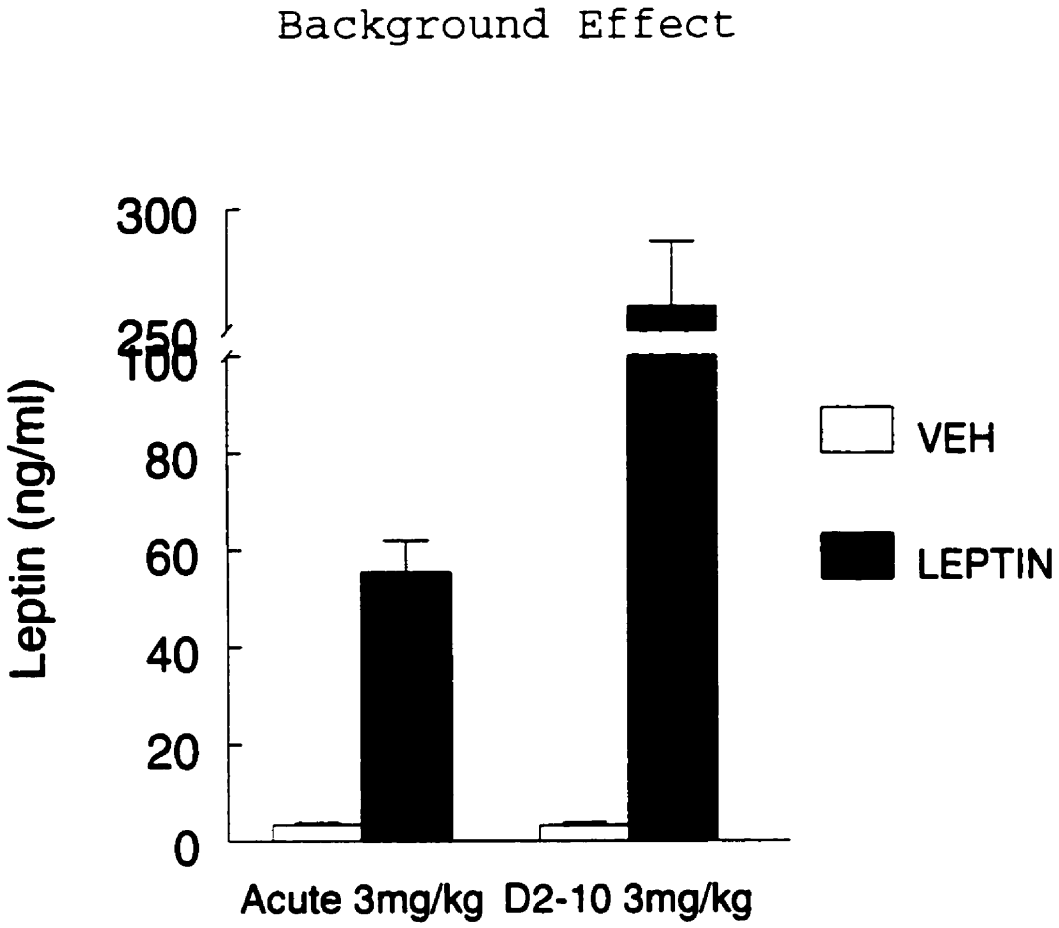


Figure 3: The effect of a chronic background on an acute injection of leptin. Plasma leptin levels following either acute injection only (-18h, -3h), or chronic injection with the last injection taking place 3h prior to testing (D10). In both cases leptin was administered at a dose of 3mg/kg and compared to vehicle injection over the same time period. Values represent means ± SEM of 6-7 animals per group.

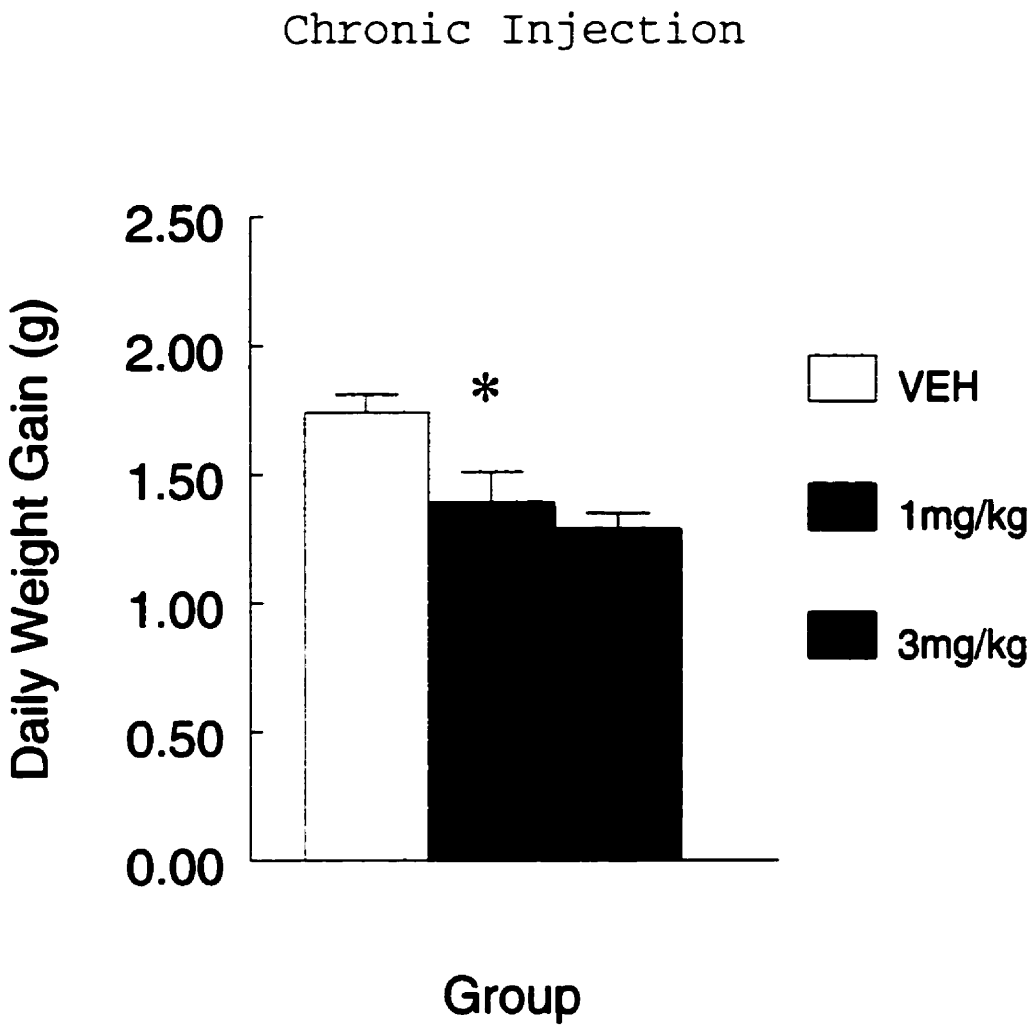


Figure 4: Average daily body weight gain of rat pups receiving chronic leptin injection from D2-D9 at a dose of either 1mg/kg (n=7 litters) or 3mg/kg (n=2 litters) versus vehicle (n=12 litters). Values represent means + SEM. One way ANOVA showed a significant effect of treatment, $F(2,18) = 5.30$, $p < 0.05$. * = different from vehicle, $p < 0.05$. 3mg/kg group was not significantly different due to the low sample size of 2.

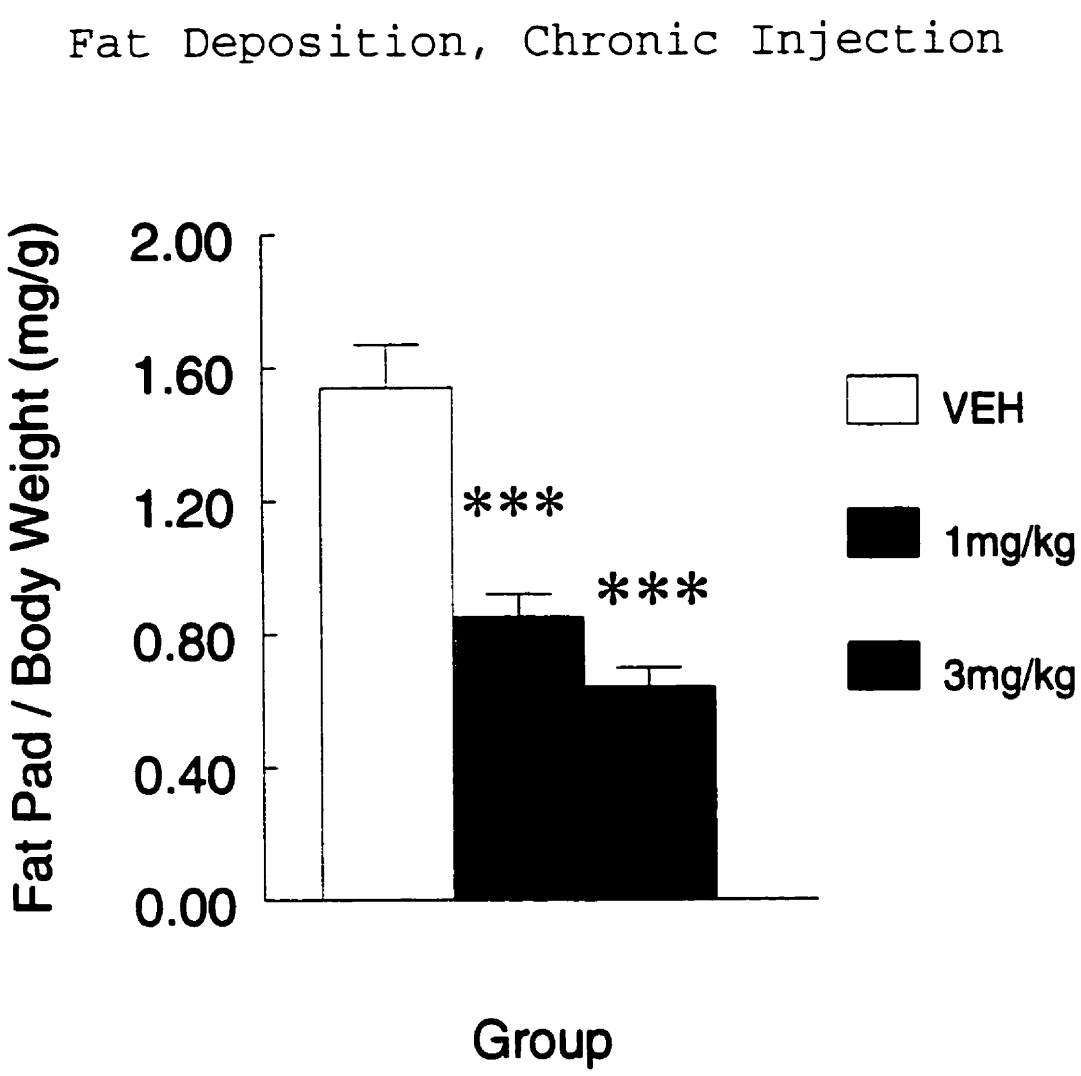
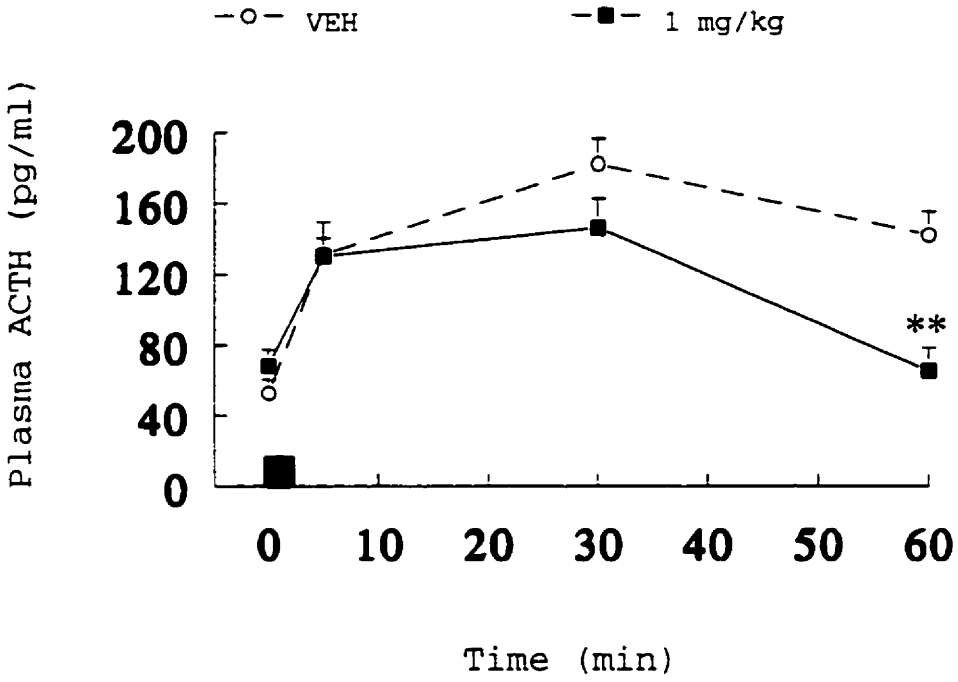


Figure 5: Retroperitoneal fat pad weight normalized to body weight in pups injected chronically with leptin (1mg/kg, n=38 or 3mg/kg, n=12) versus vehicle (n=26). Values represent means ± SEM. One way ANOVA showed a significant effect of treatment group, $F(2,73) = 18.1$, $p < 0.001$. *** = different from vehicle, $p < 0.001$.

Chronic Injection 1mg/kg



Total ACTH Secretion

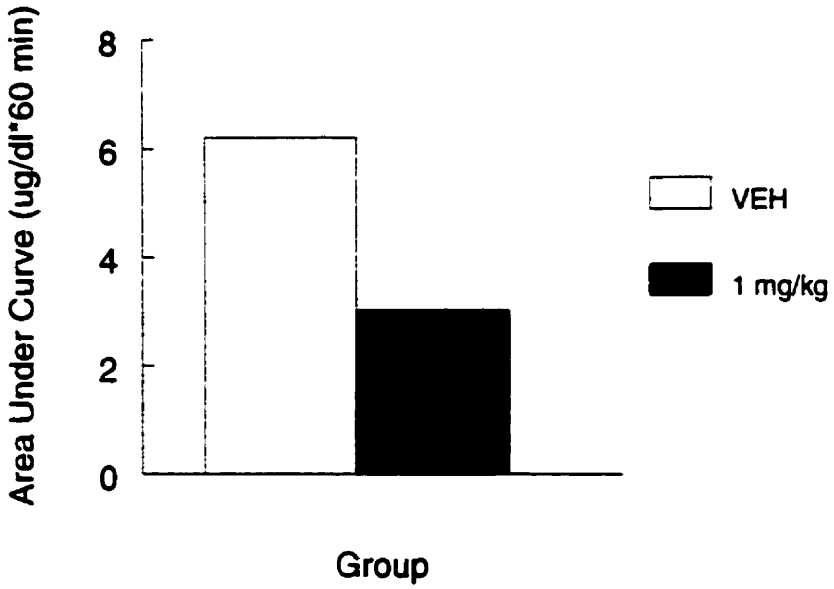
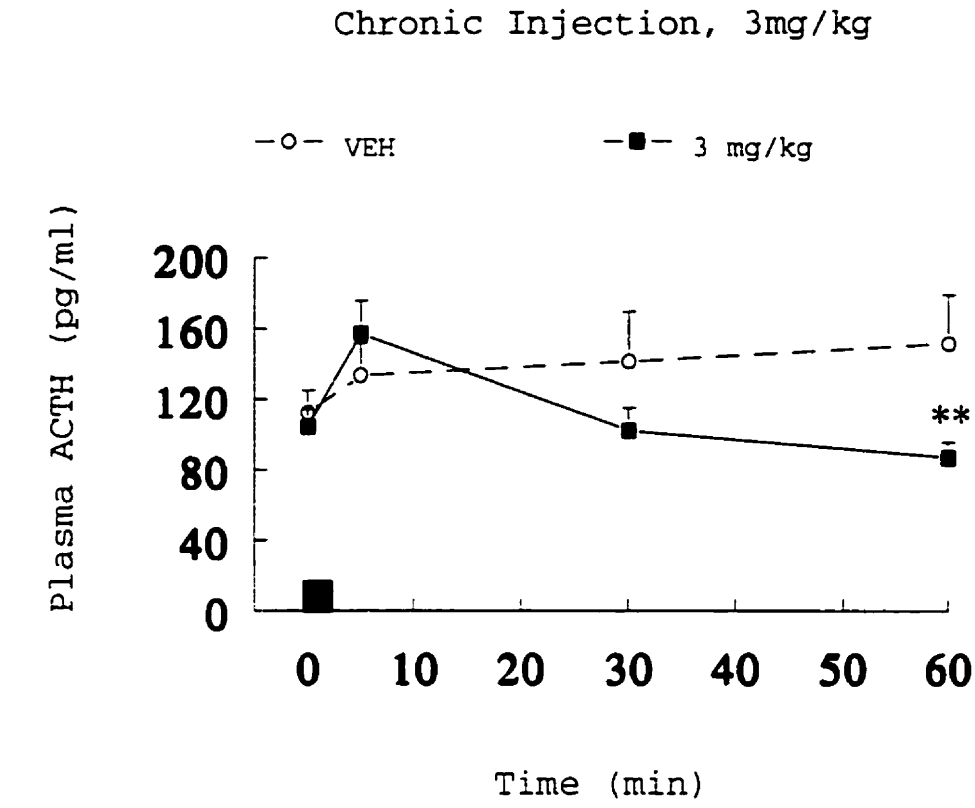


Figure 6: ***Top:*** ACTH response to a 3 min. ether stress in rat pups on D10, subjected to chronic injection of leptin at a 1mg/kg dose. Two way ANOVA showed that there was a significant main effect of dose ($F(1,127) = 6.37$, $p < 0.01$) and time ($F(3,127) = 24.92$, $p < 0.001$) as well as a significant interaction between dose an time ($F(3,127) = 3.58$, $p < 0.01$). Values represent means ± SEM(n = 17-18 per time point). ** = different from vehicle at same time point, $p < 0.01$.

Bottom: Total ACTH secretion over 60 min. time course of the same experiment, determined by calculating the area under the curve (AUC).


Chronic Injection, 3mg/kg
VEH
3 mg/kg
Plasma ACTH (pg/ml)
200
160
120
80
40
0
**
0
10
20
30
40
50
60
Time (min)

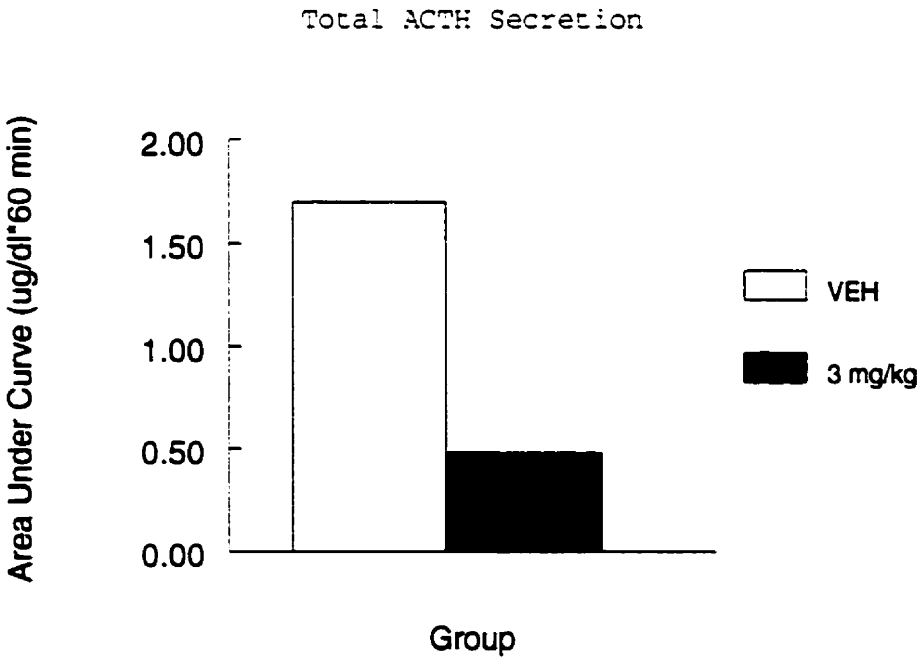

Total ACTH Secretion
Area Under Curve (ug/dl*60 min)
2.00
1.50
1.00
0.50
0.00
VEH
3 mg/kg
Group

Figure 7: ***Top:*** ACTH response to 3 min. ether stress in rat pups on D10, subjected to chronic injection of leptin at the high dose of 3mg/kg. Two way ANOVA showed significant main effect of dose ($F(1,80) = 3.11$, $p < 0.05$) and of time ($F(3,80) = 7.02$, $p < 0.001$) as well as a significant interaction between dose and time ($F(3,80) = 4.10$, $p < 0.01$). Values represent means ± SEM ($n = 6$-7 per time point). ** = different from vehicle at the same time point, $p < 0.01$.

Bottom: Total ACTH secretion over 60 min. time course of the same experiment, determined by calculating the area under the curve.

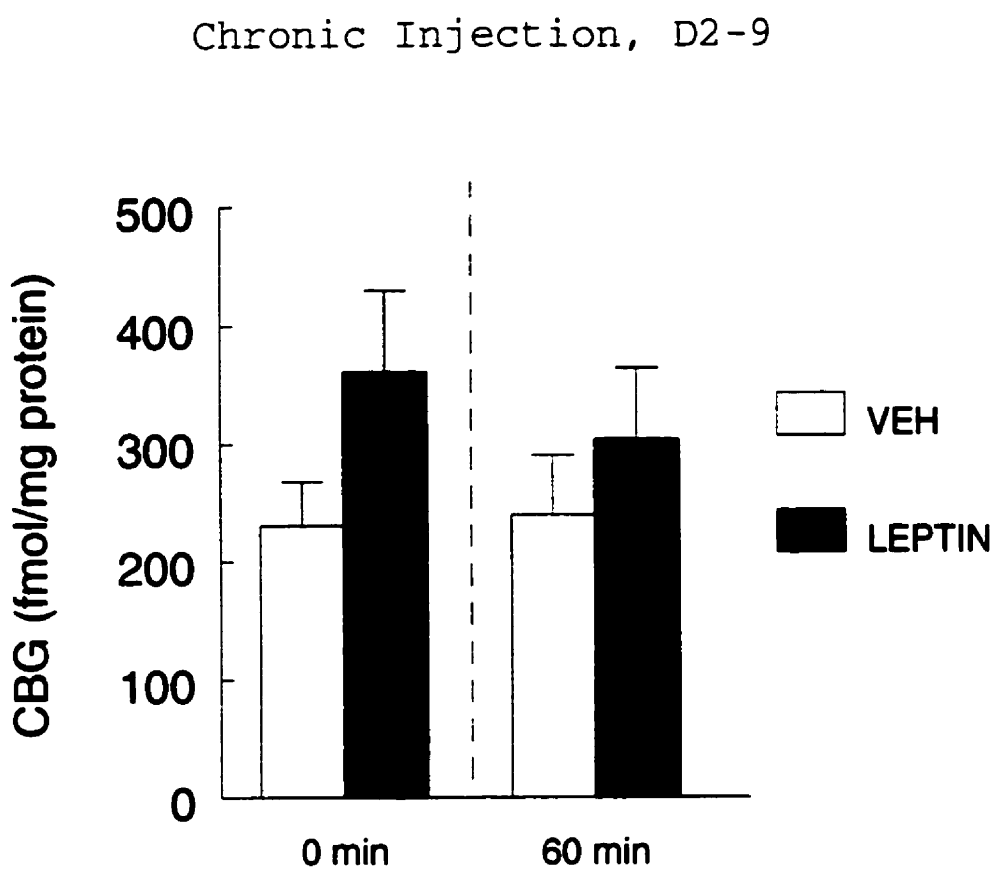


Figure 8: Basal (0 min.) and stimulated (60 min.) plasma levels of corticosteroid-binding-globulin measured in 10 day old pups injected with leptin (D2-9) or vehicle. Values represent means ± SEM for 11-12 animals per group. No significant differences were observed.

Chronic Leptin Injection

D2-9 VEH	1.24 ± 0.11	2.31 ± 0.22	5.52 ± 0.60	4.41 ± 0.63
D2-9 1mg/kg	1.12 ± 0.11	2.00 ± 0.25	4.94 ± 0.61	6.01 ± 1.33

D2-9 VEH	2.05 ± 0.24	2.02 ± 0.07	4.76 ± 0.33	6.19 ± 1.04
D2-9 3mg/kg	3.13 ± 0.45	3.78 ± 0.38	6.49 ± 0.96	4.02 ± 0.91

Table 4: Plasma corticosterone response over 60 min. following 3 min. ether stress in rats injected chronically with leptin (1mg/kg or 3mg/kg) or vehicle between D2-9. Values represent means ± SEM for 12-14 animals per group. No significant differences were observed between the treatment groups.

Chronic Leptin Injection

male	76.42 ± 12.49	159.62 ± 25.27	80.22 ± 12.24	130.32 ± 13.29
female	76.31 ± 12.52	153.64 + 30.20	75.42 ± 16.34	153.92 ± 38.99

Table 5: Plasma ACTH response compared by sex within treatment groups (leptin, 1mg/kg; D2-9 and VEH). Values represent means ± SEM for 7-11 animals per group. No significant differences were observed.

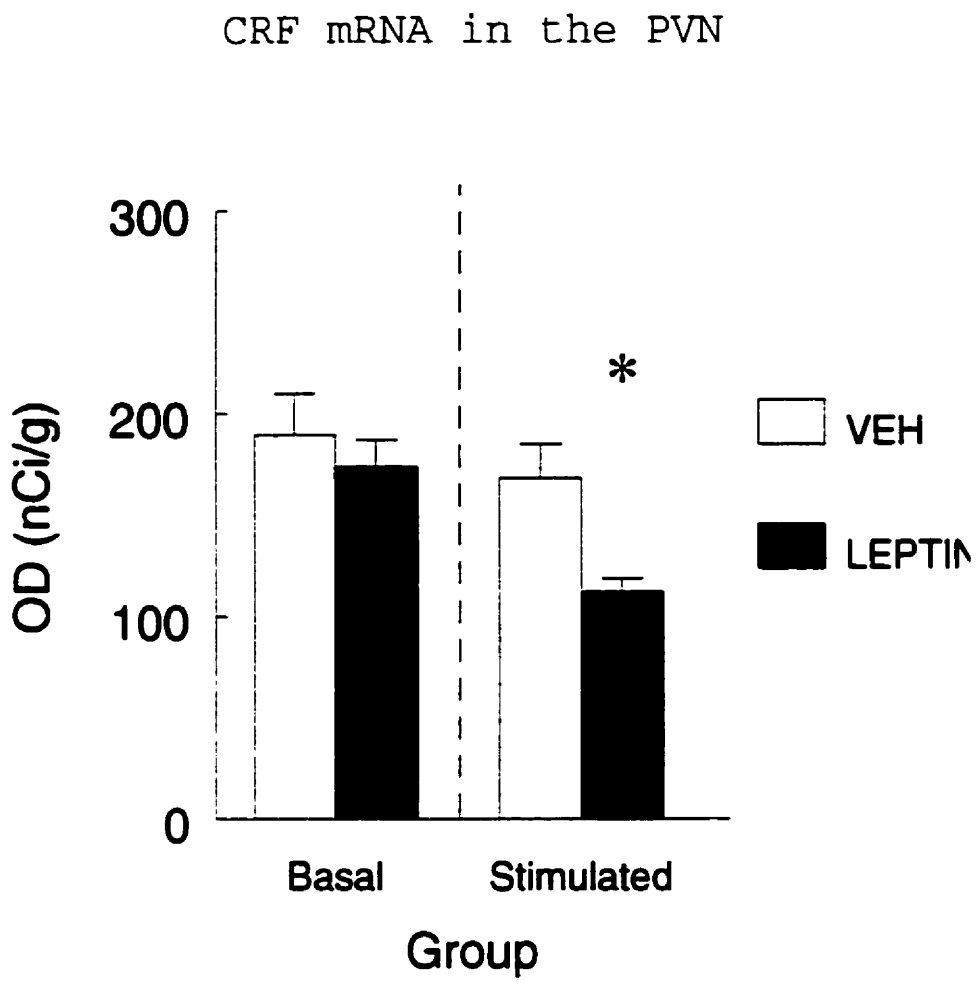


Figure 9: CRF mRNA expression in the paraventricular nucleus of the hypothalamus measured by *in situ* hybridization in rat pups injected chronically with leptin (3mg/kg) or vehicle. Both basal conditions (left panel), or stimulated (60 min. following 3 min. ether stress, right panel) are shown. Values represent means ± SEM (5-6 animals per group, with 2-6 sections per animal). * = different from vehicle, $t(9) = 2.95$, $p < 0.05$.

Vehicle

Leptin

Basal

Stimulated

Figure 9-A: Autoradiogram of sections of the PVN subjected to *in situ* hybridization for CRF from leptin or vehicle injected pups.

Glucocorticoid Receptor

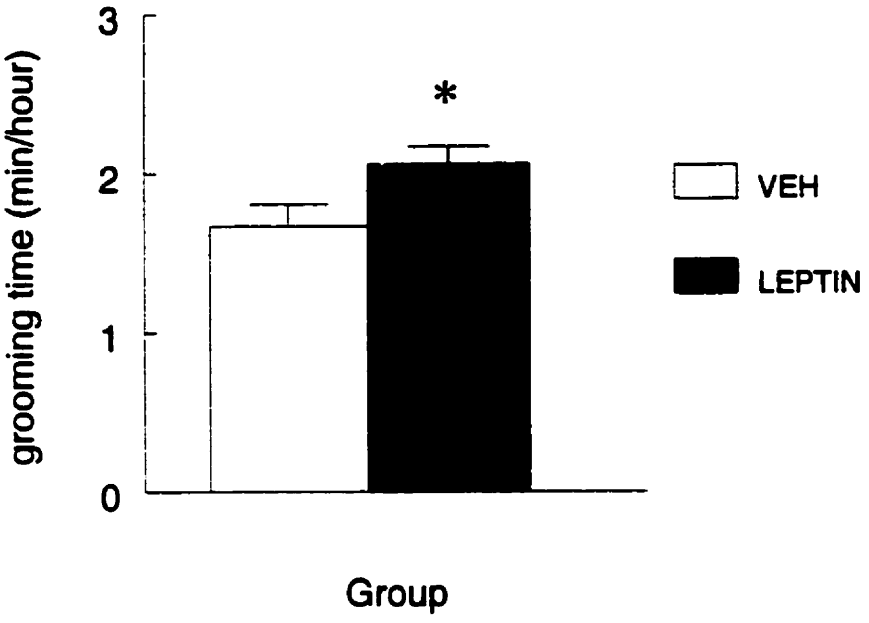
VEHICLE	12.61 ±0.27	11.52 ± 0.35	9.92 ± 1.16	7.84 ± 1.35
LEPTIN 3mg/kg	17.27 ± 3.56	15.29 ± 0.08	13.17 ± 0.23	5.99 ± 1.00
p		**		

Mineralocorticoid Receptor

VEHICLE	5.32± 0.13	9.53 ± 2.01	6.89 ± 0.39	5.77 ± 0.69
LEPTIN 3mg/kg	4.67 ± 1.01	8.71 ± 0.33	5.99 ± 0.41	6.05 ± 0.61
p				

Table 6: Image analysis of GR and MR expression in various brain regions of pups injected with leptin (3 mg/kg) or vehicle, expressed in OD (nCi/g). Values represent means ± SEM for 2-3 animals per group, 4-5 sections per animal. Students t-test showed a significant difference in the CA2 region for the GR, $t(3) = 8.273$; $p < 0.01$.

Ano-genital Grooming



Nesting Bout Duration

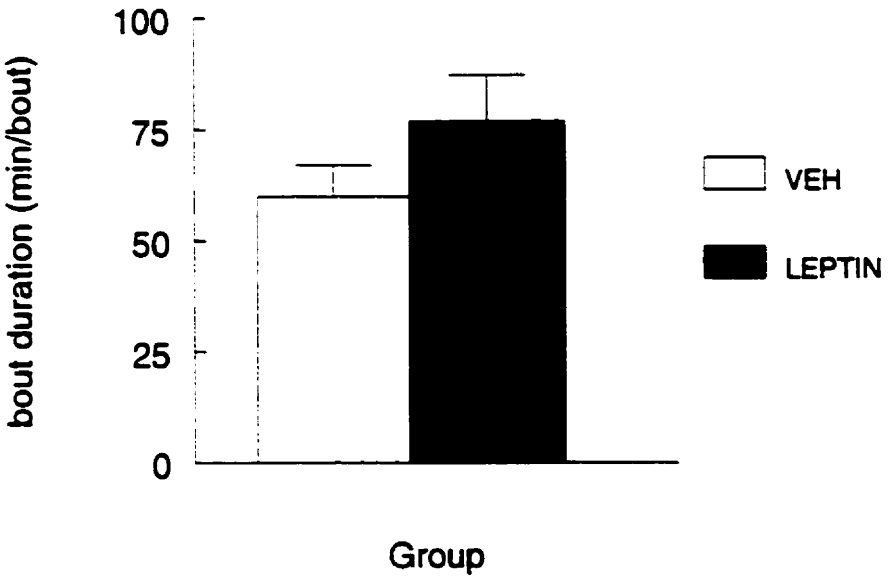


Figure 10: ***Top:*** Average time spent in ano-genital grooming of mothers nursing pups chronically treated with leptin (1mg/kg) compared to mothers nursing vehicle-treated pups. Behaviour was measured on D8 over an 8 hour period. Values represent means ± SEM for 7 litters per group. Student's t-test showed a significant difference between groups, $t(12) = 2.09$, $p < 0.05$.

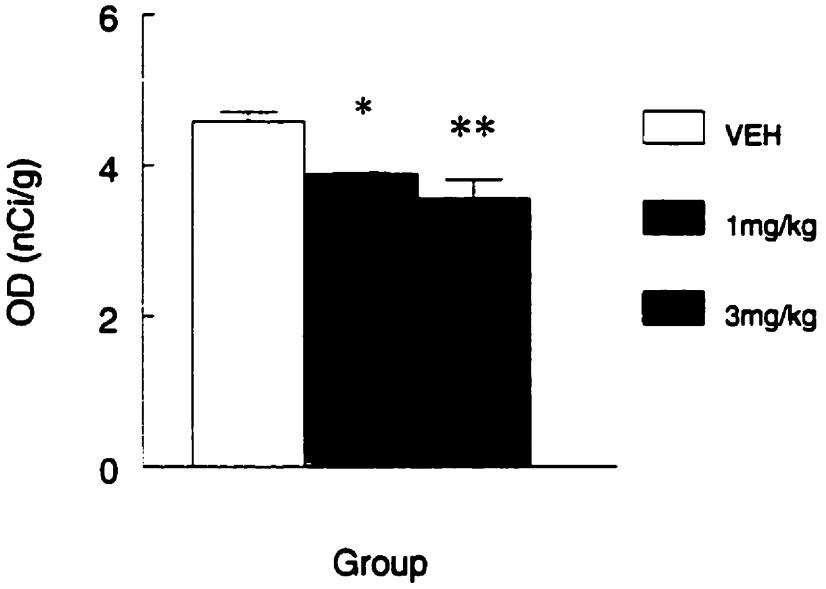
Bottom: Average nesting bout duration of nesting bouts observed over an 8 hour period in dams nursing leptin (1mg/kg) or vehicle-injected pups. Values represent means ± SEM for 7 litters per group. No significant differences between groups.

Chronic Leptin Injection

VEH	40.5 ± 4.4	60.0 ± 7.0	0.71 ± 0.04	2.9 ± 0.4	1.67 ± 0.14
LEPTIN	44.4 ± 3.3	76.8 ± 10.3	0.68 ± 0.05	2.8 ± 0.4	2.06 ± 0.11
p					*

Table 7: Overview of the maternal behavioral parameters measured in dams nursing pups injected chronically with leptin (1mg/kg) versus vehicle. Mothers were recorded for 8 hours on D8 of lactation. Values represent means ± SEM for 7 litters per group. * = $p < 0.05$.

Ob-R Image Analysis



Ob-R Grain Count

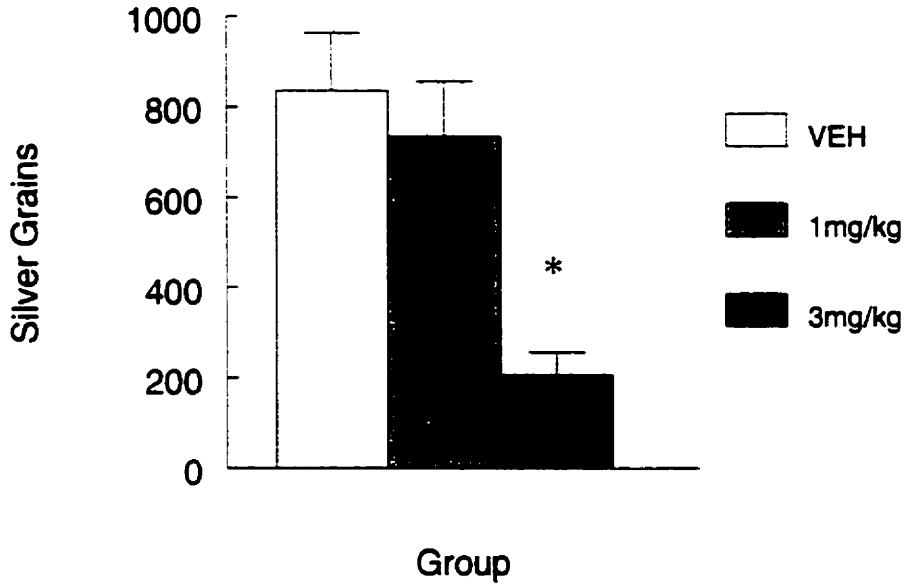


Figure 11: ***Top:*** Ob-R mRNA expression in the arcuate nucleus measured by *in situ* hybridization in rat pups chronically treated with leptin (1 mg/kg, D2-9 an 3mg/kg, D6-10) Values represent means ± SEM of 2-4 animals per group (2-6 sections per animal). One way ANOVA showed a significant effect of treatment group, $F(2,7) = 13.79$, $p < 0.01$. * = different from vehicle, $p < 0.05$; ** = different from vehicle, $p < 0.01$.

Bottom: Total silver grain count for Ob-R mRNA expressed in cells of the arcuate nucleus of the hypothalamus in 10 day-old rat pups. Values represent means ± SEM for 2-6 animals per group (3-5 sections per animal) One way ANOVA showed a significant effect of treatment group, $F(2,10) = 6.02$, $p < 0.05$. * = different from vehicle, $p < 0.05$.

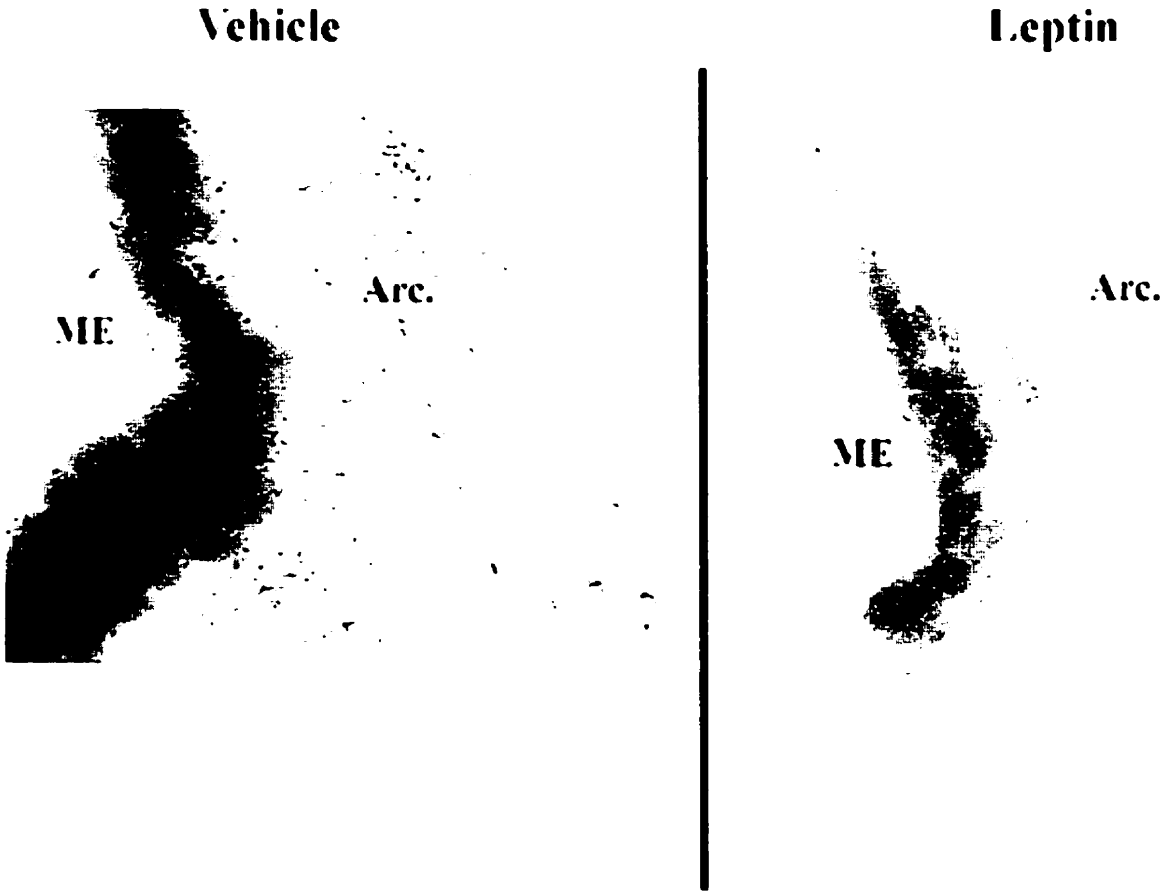

Vehicle
Leptin
ME
Arc.
ME
Arc.

Figure 11-A: Photographic representation of ARC sections expressing Ob-R.

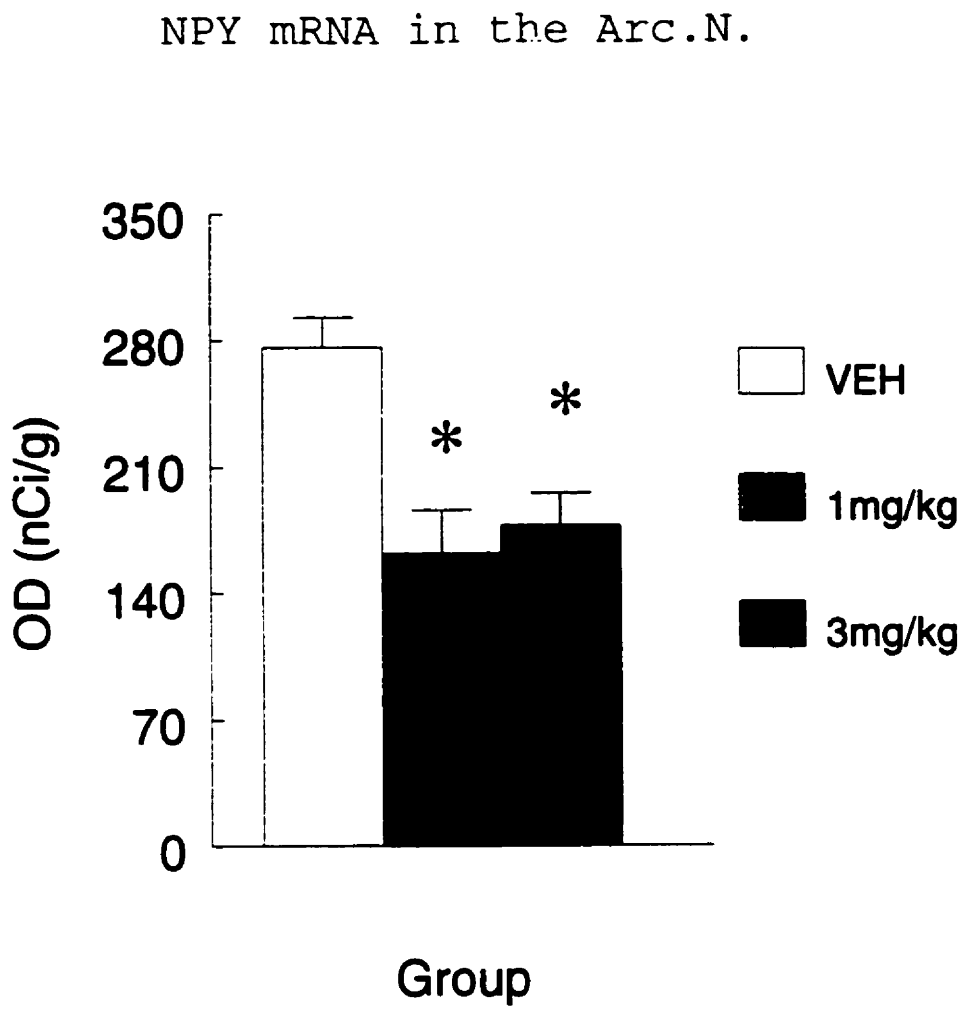


Figure 12: ***Top:*** NPY mRNA expression in the arcuate nucleus measured by *in situ* hybridization in rat pups injected chronically with leptin at either 1mg/kg or 3mg/kg from D2-D9 versus vehicle treated pups over the same period. Values represent means ± SEM of 3 animals per group (3-5 sections per animal). One way ANOVA showed a significant effect of treatment group, $F(2,8) = 9.57$, $p < 0.05$. * = different from vehicle, $p < 0.05$.
Bottom: Sections of the ARC subjected to *in situ* hybridization for NPY.

Vehicle

1 mg/kg

3 mg/kg

Figure 12-A: Autoradiogram of sections of the ARC nucleus subjected to *in situ* hybridization for NPY from leptin or vehicle injected pups.

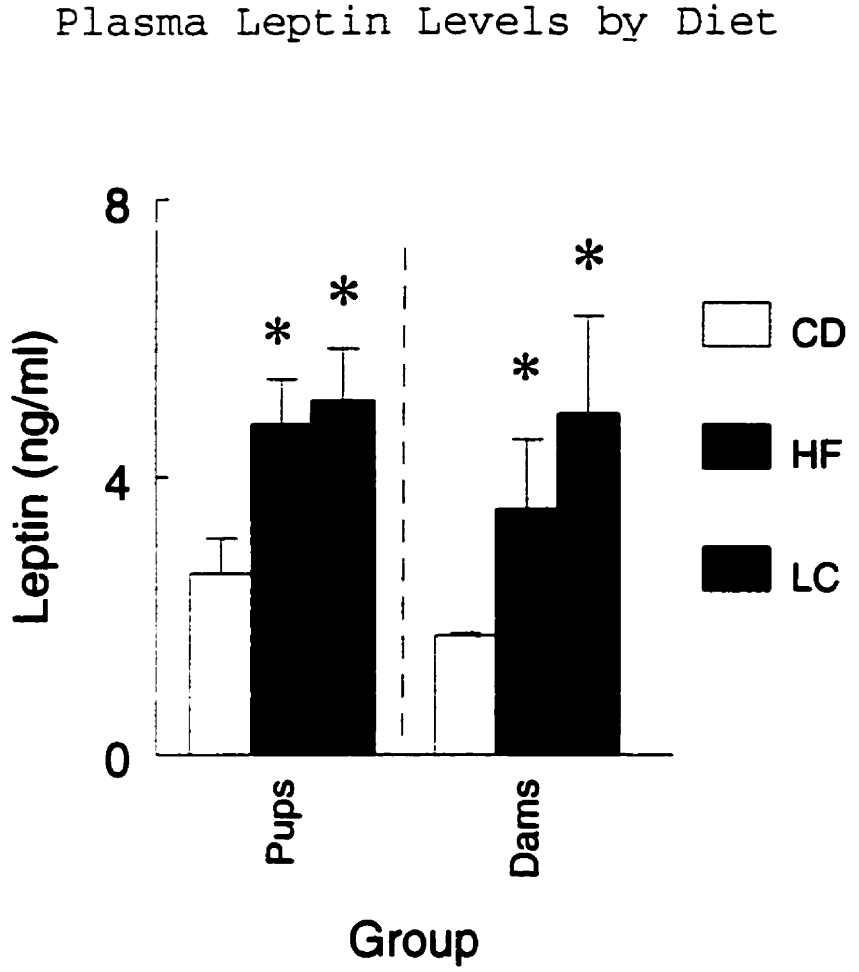


Figure 13: Plasma leptin levels in pups (left panel, n = 12-15) and dams (right panel, n = 5-7) compared by diet. Mothers were receiving either the control (CD), high fat (HF) or low carbohydrate (LC) diets from the beginning of gestation and leptin levels were measured on D10 of lactation. Values represent means ± SEM. One way ANOVA showed significant group differences in pups, $F(2,38) = 5.01$, $p < 0.05$ (* = different from CD, $p < 0.05$), but not in dams, $F(2,15) = 2.34$ ($p = 0.13$, not significant).

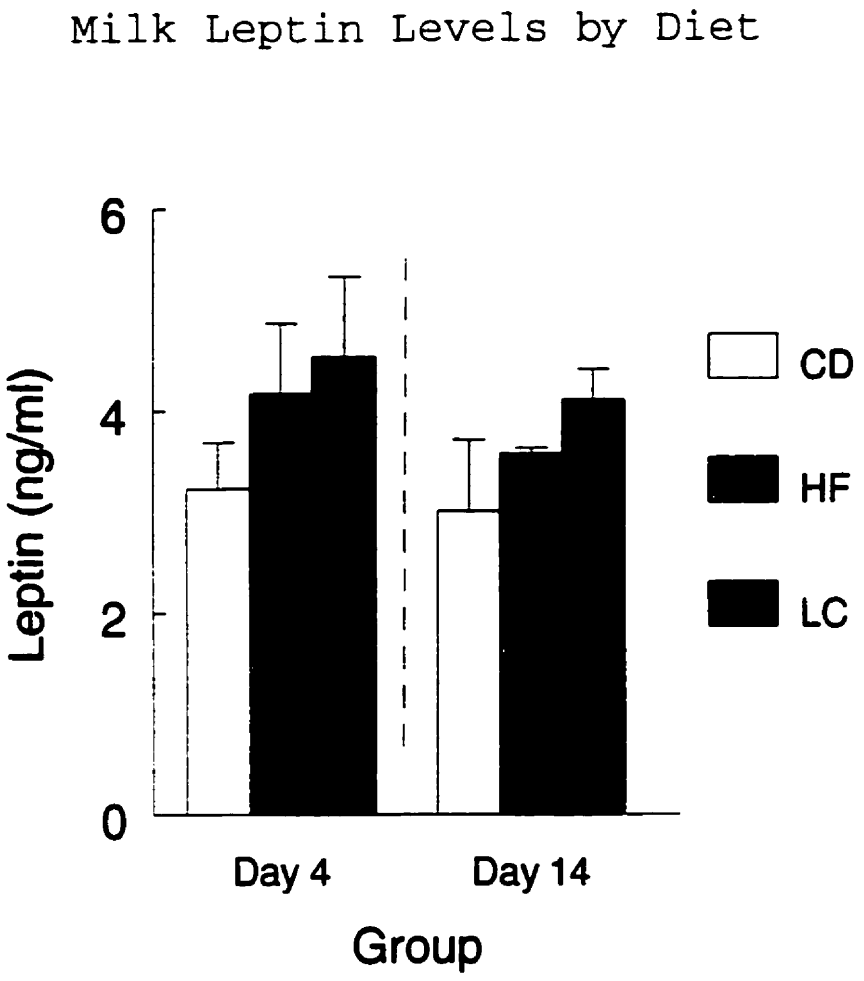


Figure 14: Milk leptin levels measured in dams fed either control (CD), high fat (HF) or low carbohydrate (LC) diets from the beginning of lactation. Values represent means ± SEM for 3-5 animals per group, no significant differences.

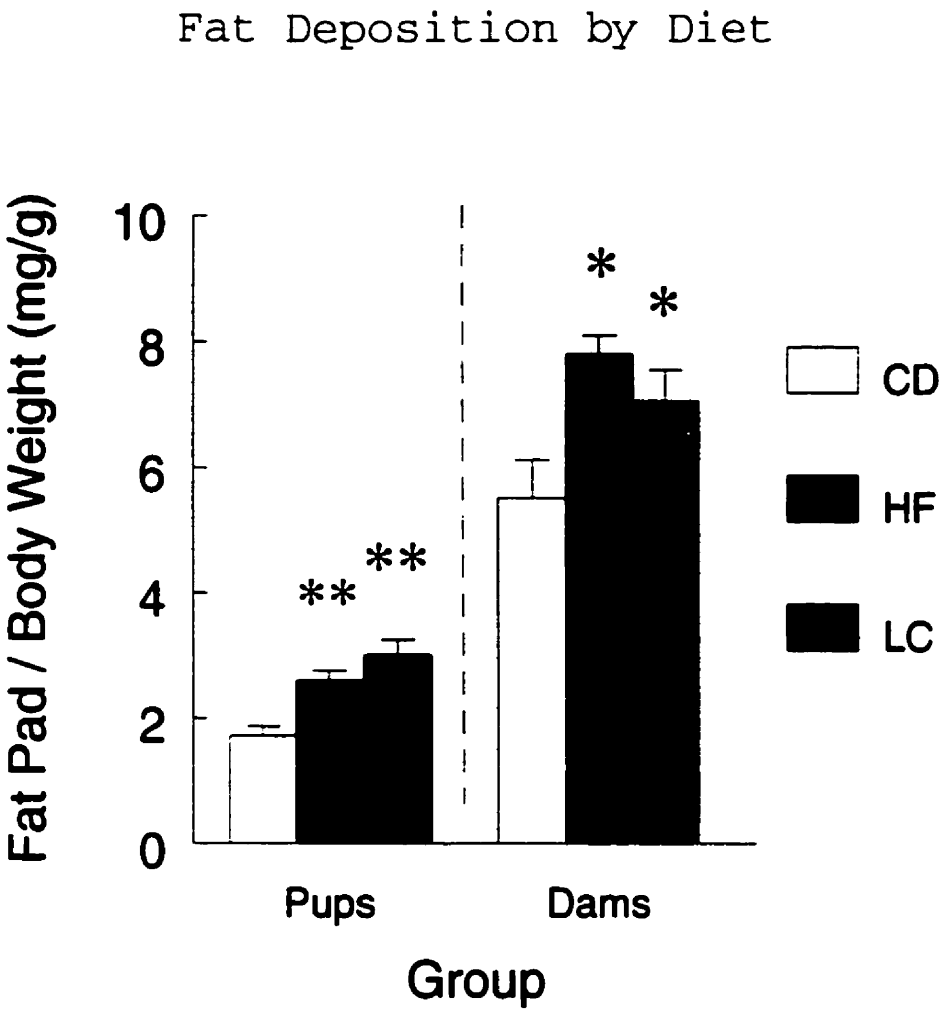


Figure 15: Retroperitoneal fat pad weight normalized to body weight in pups (left panel, n = 13-14) and their dams (right panel, n = 4-6) who were receiving either CD, HF, or LC diets from the beginning of gestation. Measurements were done on D10 of lactation. Values represent means ± SEM of 4-14 animals per group. One way ANOVA showed a significant effect of group in pups, $F(2,38) = 11.95$ ($p < 0.001$; ** = different from CD, $p < 0.01$) and in dams, $F(2,13) = 4.79$, $p < 0.05$ (* = different from CD, $p < 0.05$).

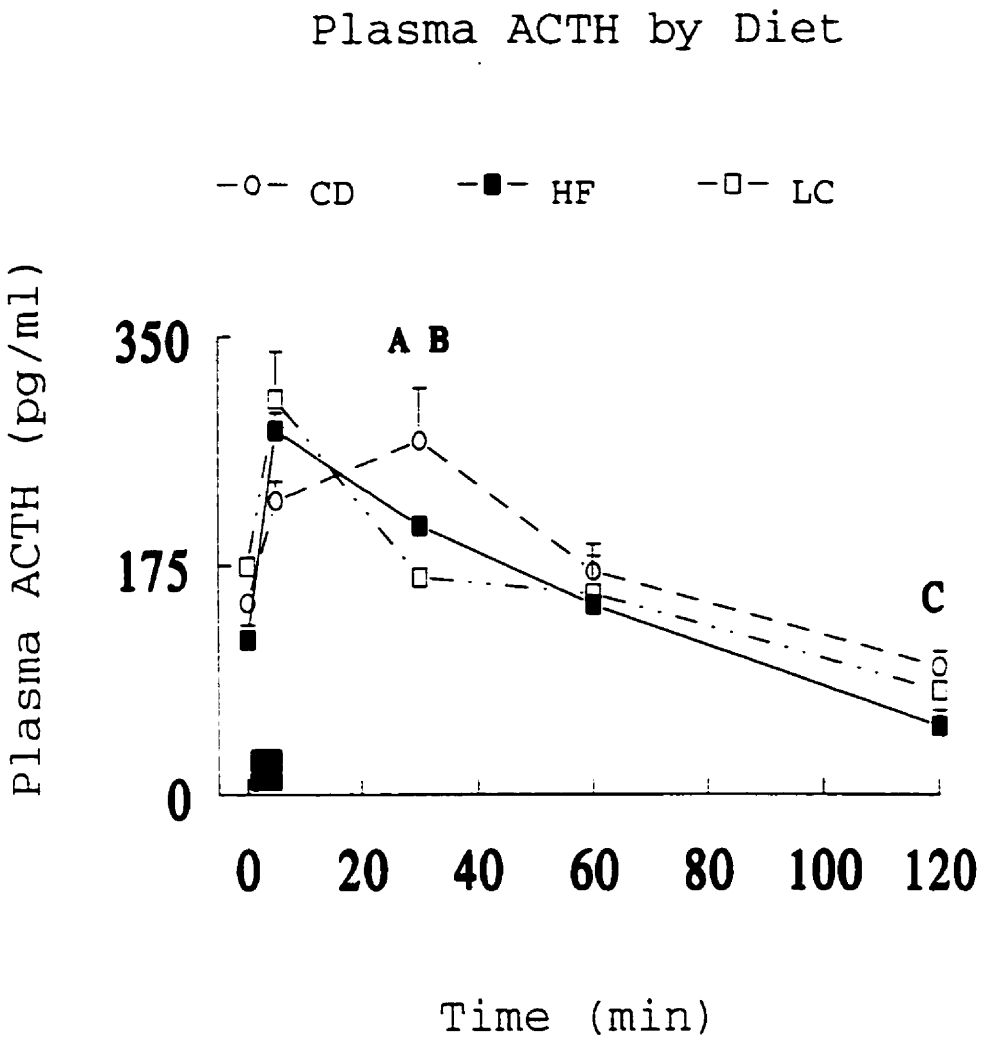


Figure 16: ACTH response over 120 min. to a 3 min. ether stress in 10-day old pups. Pups were nursing on milk from dams receiving either CD, HF, or LC diets from the beginning of gestation. Two way ANOVA showed a significant main effect of diet ($F(1,59) = 3.83$, $p < 0.05$) and time ($F(4,59) = 3.21$, $p < 0.05$) as well as a significant interaction between diet and time ($F(4,59) = 32.03$, $p < 0.01$). A: HF < CD, $p < 0.05$; B: LC < CD, $p < 0.05$; C HF < CD, $p < 0.05$. Values represent means ± SEM for 6-7 animals per group, per time point.

Plasma Corticosterone by Diet

CD	2.32 ± 0.30	3.39 ± 0.82	4.97 ± 1.78	5.35 ± 1.49	4.99 ± 1.01
HF	3.05 ± 0.72	2.29 ± 0.28	4.96 ± 1.78	2.45 ± 0.46	2.59 0.45
LC	2.51 ± 0.14	4.91 ± 0.38	5.53 ± 0.67	6.81 + 0.99	3.24 ± 0.80

Table 8: Corticosterone response over 120 min. following 3 min. ether stress in pups reared by dams consuming either CD, HF or LC diets from the beginning of gestation. Values represent means ± SEM for 6-7 animals per group at each time point. No significant differences.

Maternal Diets

male pups	137.30 ± 9.46	120.36 ±15.33	171.78 ± 9.32
female pups	154.45 ± 11.95	114.55 ± 15.25	181.40 ± 19.80

Table 9: Plasma ACTH levels compared by sex between diet groups. Values represent means ± SEM for 2-6 animals per group. No significant differences.

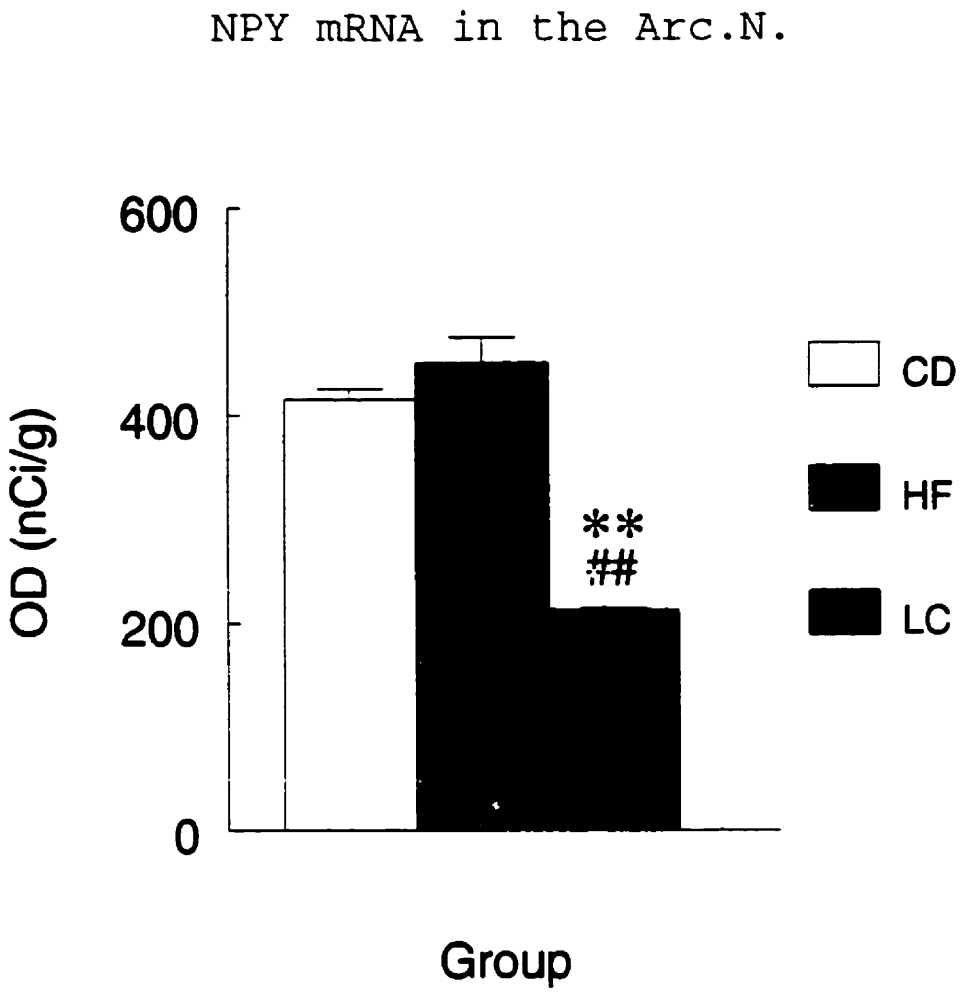


Figure 17: NPY mRNA expression in the arcuate nucleus of the hypothalmus in pups reared on either HF or CD milk. One way ANOVA showed a significant effect of diet, $F(2,8) = 29.56$, $p < 0.001$. Values represent means ± SEM for 2-4 animals per group, 5-6 sections per animal. ** = different from vehicle, $p < 0.01$; ## = different from HF, $p < 0.01$.

Control

High Fat

Low Carb

Figure 17-A: Autoradiogram depicting NPY expression in the ARC for HF or CD reared pups.

Maternal Behaviour by Diet

Control	39.6 ± 3.19	71.1 ± 3.99	0.56 ± 0.04	3.0 ± 0.47	2.42 ± 0.04
High Fat	44.2 ± 2.45	65.1 ± 5.11	0.675 ± 0.04	2.9 ± 0.32	2.59 ± 0.31

Table 10: Overview of maternal behavioural parameters measured in dams receiving either CD, HF, or LC diets. Mothers were recorded for 8 hours on D8 of lactation. Values represent means ± SEM for 6 litters per group. No significant differences.

VI. DISCUSSION

The state of energy balance represents a physiological integration point for a variety of systems, because all systems draw on the bodies pool of energy reserves in order to execute their particular functions. This equilibrium between food intake and energy expenditure is subject to complex metabolic and hormonal regulation, including regulation by the HPA axis. Leptin, the product of the ob gene which is secreted by adipose tissue, is now generally accepted as being a major indicator of peripheral fat depot to the CNS. In the studies comprising this Master's thesis, we demonstrate that leptin modulates both indices of energy balance and neuroendocrine functions in neonatal rats, possibly representing a link between these two systems. Our lab has previously emphasized the importance of developmental influences on the regulation of the stress axis, and we now extend our hypotheses to encompass the relationships between stress and the regulation of energy balance during the perinatal period.

A. LEPTIN LEVELS DURING DEVELOPMENT

Leptin levels in adults are thought to be determined primarily by the magnitude of peripheral adipose tissue. According to this principle, rat pups who have almost no fat tissue at birth would be expected to have very low leptin levels. In fact, our lab and others have reported that normal circulating leptin levels are comparatively higher in neonates than in adults. It has also been noted that leptin levels appear to surge in rodents during the course of the first 2 weeks of life, and that this increase is relatively independent from adiposity. This indicates that leptin concentration is not strictly dependent on fat stores and the origin of high levels of

leptin during a time of adipose tissue acquisition poses the question of biological activity. Leptin has been detected in the placenta and has been shown to surge during the late gestational phase. During development, adipose tissue might be synthesizing higher levels of leptin compared to adults, as suggested by the increase in leptin MRN seen in mice at this period (Hoggard, 1997). Alternatively, large amounts of leptin seen in pups may be due to the fact that their diet consists exclusively of milk, which is high in fat content. In addition, it is possible that a proportion of plasma leptin originates from milk and is transferred from dam to pup at this time. We have measured leptin in milk and shown that milk leptin levels vary as a function of maternal diet. Others have reported that radiolabelled leptin can pass from mother to pup via the milk (Casabiell et al, 1997). Although the source of leptin in neonates is still debated at this point, it is clear from our studies that leptin is physiologically active. In our studies which modified dietary fat intake of rat dams, we detected no significant differences in milk leptin levels (although we saw a trend towards higher leptin levels with a high fat diet), but consistently observed higher plasma leptin levels in pups who were nursing on the high fat milk. Whether these increased leptin concentration results from alterations in direct synthesis by the pup's adipose tissue, or are a consequence of altered leptin metabolism in pups receiving a high fat diet remains open to speculation.

B. IMPACT OF LEPTIN ADMINISTRATION ON BODY WEIGHT GAIN, FAT DEPOSITION AND MATERNAL BEHAVIOUR

In developing rats, the first 2-3 weeks of life represent a critical period for a number of maturational processes which are strongly favored by a positive energy balance (Vitiello, DiBenedetta, Cioffi, and Gombos, 1980). Intact pups steadily increase their body weight

(Walker and Aubert, 1988) and fat depots during the suckling period, despite high levels of circulating leptin (Trottier, 1998). Since leptin causes decreased food intake and weight loss in adults, this has led some researchers to suggest that the developmental period represents a time of leptin resistance. In the present studies we found that exogenous administration of leptin in pups during the first 9-10 days of life significantly reduced body weight gain in a dose-dependent fashion. This decrease in body weight gain in suckled pups could result either from a decrease in the amount of milk ingested or from an increased energy expenditure. With respect to changes in milk intake, we found no evidence for differences in milk ejection frequency in mothers who were nursing leptin injected litters compared to those nursing vehicle injected litters. Milk ejection is, to a certain extent, dictated by suckling intensity. Provided that pup treatment did not alter the amount of milk available per milk ejection episode (which we did not measure), this result indicates that milk availability was not modified by the treatment and also that the intensity of the suckling stimulus was probably comparable in both groups. These results are in agreement with earlier studies conducted in 9 and 14 day-old food deprived pups which failed to detect any differences in acute food intake between leptin and vehicle-treated rats (Stehling, Doring, Ertl, Preibisch, and Schmidt 1996). In this report, it was also demonstrated that increased energy expenditure induced by leptin administration in artificially reared pups was responsible for the reduction in body weight gain and fat deposition and also led to an increase in core body temperature (Stehling, 1996). In the nest, changes in litter temperature can have important consequences on maternal behavior, in particular on nesting bout duration (Woodside and Leon 1980). Indeed, it is well known that nesting bout duration is reduced by increasing nest temperature. We thus hypothesized that an increase in pup core temperature induced by leptin administration would lead to an increase in litter temperature and decrease nesting bout

duration as well as total nesting time. This was not the case, however, since dams nursing leptin treated pups showed a tendency to have longer nesting bouts than controls and were not different in their total nesting time in an 8 hr period. Although we have not tested nest and individual pup temperature in our experiments, our data suggest that the variation in nest temperature brought about by a leptin-induced increased thermogenesis is minimal and does not modify the nesting behaviour of the mother.

Our observation that exogenous leptin treatment causes a dramatic decrease in retroperitoneal fat pad weight is particularly interesting because we previously showed (Trottier, 1998), and confirmed here in Experiment 3, that pups nursing on a high fat milk have circulating levels of leptin similar to those obtained in our chronic paradigm, but continued to increase their body weight and deposit more fat than pups nursing on a control diet milk. The fact that exogenous leptin appears to have the opposite effect on fat deposition than endogenous increases due to a high fat diet lends itself to various interpretations. Firstly, the decreased fat pad in leptin-injected animals could be due to increased energy expenditure in these animals that is not compensated for by ingestion of a higher fat diet, as it is for pups nursing on a high fat milk. Thus, a negative energy balance is induced by exogenous leptin administration, but not in pups fed a high fat milk. Secondly, the pattern of leptin exposure might be important in determining the direction of changes in energy balance. In the case of exogenous leptin administration, we observed a large increase in circulating leptin concentrations 3 hrs after an injection and plasma levels that were still 2-3 fold higher than vehicle-injected pups 24 hrs after an injection. This pattern of changes in circulating leptin levels are likely very different from tonically elevated endogenous production and it is possible that the high levels of leptin present immediately after injection may be sufficient to induce specific receptor changes or affect other metabolic systems that

do not occur with the more consistent levels seen in a high fat milk paradigm. In fact, we did see clear changes in leptin receptor expression in pups receiving repeated injections of leptin. Finally, it is possible that factors other than leptin such as elevated free fatty acids or milk triglycerides which are present in high fat milk may counteract the effect of leptin on fat deposition in pups.

C. HYPOTHALAMIC-PITUITARY-ADRENAL RESPONSIVENESS

In addition to its effects on weight gain and fat deposition, leptin has been documented to modify the activity of the HPA axis in adult rodents. Fluctuations in leptin levels are inversely related to those of corticosterone (B) levels in adult rats and humans, and acute injections of leptin have been shown to blunt the stress induced rise in ACTH as well as decrease CRF expression under stress conditions in mice (Richard, 1998). In our earlier experiments, we demonstrated that pups fed a high fat diet resulting in increased plasma leptin levels had reduced ACTH responses to stress. In spite of this, acute injection of leptin failed to have any significant effect on ACTH or B levels in rat pups at either 10 or 21 days of age. The circulating leptin concentration in these acutely injected animals was approximately 55 ng/ml at day 10, when measured 3 hours after injection; almost 10 times above the normal range at this age, indicating that the HPA axis appears to be resistant to influence by short-term increases in plasma leptin It could be argued that the leptin receptor (Ob-R) system is not fully developed in 10 day-old pups, or may not be functional, but we refuted this hypothesis by showing significant effects of chronic leptin treatment on body weight and fat deposition, as well as down-regulation of the leptin receptor.

Because rats experience naturally a high fat diet during the entire suckling period, the

fact that an acute leptin injection does not affect HPA activity comparable to a high fat diet does not necessarily indicate that leptin is not responsible for mediating some of the effects of a high fat diet on this neuroendocrine axis.. It may indicate that leptin treatment needs to be chronic rather than acute in order to see a significant treatment effect. Indeed, we found that exogenous leptin did not modify the peak ACTH response to ether stress in 10-day old pups, but induced a significantly faster return of plasma ACTH to baseline after termination of the stressor. When compared in terms of total ACTH secreted by analyzing area under the curve, it was clear that leptin administration markedly reduced the amount of plasma ACTH over the course of 1 hour following the administration of stress. Chronic leptin administration also caused significantly reduced CRF expression in the PVN 60 min. after the stress, while not affecting basal levels of CRF. Comparing the results of acute and chronic leptin treatment, we believe that the expression of the effect of leptin on HPA activity is not necessarily related to the circulating levels of leptin at the time of the stress, but are more dependent upon a chronic elevation in circulating levels over at least 4-7 days prior to testing. In one series of experiments, we used the high dose of leptin and started injection on Day 6 through D10. In this case we still observed a decrease in ACTH response. The action of leptin to modulate ACTH secretion was consistent in the chronic paradigm (beginning on Day 2) whether leptin was administered at a low (1mg/kg) dose or a high (3mg/kg) dose, and was also consistent whether an injection was given on the day of testing (Day 10) or not (where the last injection was 24 hours earlier). This is in spite of the fact that the high dose produced levels significantly higher than the low dose (see Figure 5) and that an injection on the day of stress testing produced extremely high values for plasma leptin at the time of stress.

An increase in the return of plasma ACTH secretion to baseline levels after exposure

to stress is generally indicative of changes in glucocorticoid (GC) feedback sensitivity Meaney, 1989). In particular, Walker et al have previously demonstrated an increase in sensitivity to GC's during the developmental period in rats (Walker et al, 1986), due to a greater proportion of free steroid in neonatal rats. Alternatively, the work of Sapolsky et al has described changes in GC feedback which take place with aging and result in prolonged stress responsiveness (Sapolsky, 1991). Other examples of altered GC feedback have been described by Plotsky et al. who have noted that obese *fa/fa* rats exhibit impaired feedback suppression of ACTH secretion with high levels of leptin, but have a severely impaired leptin biological signal. (Plotsky et al, 1992). Therefore, one possible mechanism mediating the changes in stress-induced ACTH response in leptin-treated animals might be an enhanced glucocorticoid feedback system. If these pups are indeed in a state of negative energy balance due to leptin-induced increases in energy expenditure, it is reasonable to suppose that a more efficient glucocorticoid feedback system would be adaptive in preventing prolonged corticosterone release and exacerbation of this negative energy balance condition. Such an adaptation of the HPA axis would compensate for the metabolic effects of leptin. Although we found no differences between treatment groups with respect to basal or stimulated corticosterone levels, preliminary results have demonstrated that circulating levels of corticosterone-binding-globulin tended to be lower in leptin-injected compared to vehicle-injected groups. While not statistically significant, such a difference would mean a higher concentration of circulating free B in leptin treated pups, which would effectively increase the availability of the biologically active B to the glucocorticoid receptor for feedback. CBG may be subject to more rapid turnover during the neonatal period, so further characterization of this interaction is required to determine the true significance of this effect.

Alternatively, GC sensitivity can be modified by a change in the number or sensitivity

of glucocorticoid receptors in the brain and/or pituitary (Meaney, 1993). This could represent another mechanism by which leptin impinges on the HPA axis. Our analysis of the glucocorticoid receptor (GR) expression in the brain showed a significant increase in receptor mRNA in the CA2 region of the hippocampus as well as a trend towards a similar increase in the CA1 region and the PVN. Upregulation of the GR could amplify the feedback of GC's on the HPA axis and serve to limit the duration of the stress response. Of interest is the observation that while leptin modified GR expression, mRNA levels for MR were unchanged. This suggests not only differential regulation of GR and MR, but that basal levels of ACTH and B (which are controlled by MR) would not be altered even though stress induced levels of these hormones (regulated by GR occupancy) would be modified by leptin. In fact, this is exactly what we observed. Furthermore, leptin may have a direct effect on CRF via its own receptor, and the decrease in stimulated CRF expression is also be consistent with an enhanced GR system. Thus, inhibition of stress responsiveness in neonates may have more to do with an alteration of feedback mechanisms caused by continuous exposure to leptin.

D. MATERNAL BEHAVIOUR

One important finding of our studies is that leptin treatment of the entire litter elicited significantly more pup ano-genital licking by the mother compared to litters injected with vehicle. The reasons for changes in this particular aspect of maternal behaviour are unclear. As demonstrated for the gender-related differences in maternal licking (Moore, 1982), it is possible that olfactory cues or altered locomotion in leptin treated pups might enhance maternal licking behaviour towards these pups. Alternatively, increased ultrasonic vocalizations in leptin-treated pups might signal increased sympathetic activity and

thermogenesis in these animals and stimulate maternal attention (Hofer, 1993). Maternal licking and grooming behaviour has long been thought to be critical in developmental processes including the development of the HPA axis (Rosenfeld, 1994). More recently, it was shown that dams who display more licking / grooming behaviour towards their litters have pups whose HPA response to stress is decreased as adults (Liu, 1997). Thus, one of the beneficial effects of elevated levels of leptin during development might be to elicit specific behavioral responses in the mother which are directed towards reducing stress responsiveness in the offspring.

This could be one reason for the high fat content of milk provided by mothers to their young. It seems reasonable to suggest that one purpose of feeding on milk (which is naturally rich in fat) might be to allow optimal maternal-pup interaction, leading to normal, rather than exaggerated, stress responsiveness in adulthood. If so, increasing the fat content of milk further should heighten the protective effect on the stress response. In fact this is what we have observed in our experiments using various diets during gestation and lactation. We previously reported changes in peak ACTH response to stress in pups when the maternal diet was altered to a high fat composition during lactation only. Here, we extended this paradigm to include the feeding of the various diets during gestation as well, to ensure consistency of the high fat diet nursed on by the pups over the entire course of the neonatal period. We demonstrate a blunting of the ACTH response to stress in this model, despite no significant differences in ano-genital licking / grooming between dams on high fat versus control diets. The reasons for this lack of difference in maternal behaviour are not known. It is possible that our relatively small sample size may not allow detection of subtle changes in grooming / licking behaviour.

E. IMPACT OF LEPTIN ON OB-R AND NPY EXPRESSION

Very little is known about the developmental profile of the leptin receptor, Ob-R and its various isoforms or about the precise hormonal regulation of these receptors. It is becoming increasingly clear that the isoforms are physiologically In our study, we demonstrated that rat pups chronically treated with leptin over the first 10 days of life had significantly reduced Ob-R mRNA in the arcuate nucleus of the hypothalamus compared to vehicle-treated pups. This is consistent with the effects of leptin on its receptor that have been seen demonstrated in adults. Ligand induced down-regulation of Ob-R has been demonstrated *in vitro* (Uotani, 1999) and *in vivo*, it has also been shown that systemic administration of leptin decreases expression of the long form of the receptor by 30% in the arcuate nucleus of mice (Baskin, 1998). Although we detected all forms of the Ob-R in our experiments, it appears that in neonates, as in adults, elevated leptin causes down-regulation of its own receptor. Studies in adult rodents have demonstrated that the leptin receptor is up-regulated when there is a decreased amount of leptin available such as in conditions of fasting (Baskin, 1998; Huang, 1997).

Because the arcuate nucleus is involved in the integrated control of feeding behaviour, it seems likely that the impact of alterations in Ob-R receptor density in this nucleus would have an impact on food intake. Interaction of leptin with its receptor on ARC neurons is thought to directly influence the expression of NPY in these neurons in adult rats (Williams, 1999) and Ob-R mRNA has been shown to colocalize with NPY mRNA in these cells. NPY is yet another important factor regulating food intake and metabolism, representing one of the strongest signals stimulating food intake. When leptin concentrations are reduced, such as in a fasted state, the inhibitory effect of leptin on NPY neurons is released, inducing a net

increase in NPY expression and increased feeding drive (Baskin, 1999). In our studies, we observed a significant reduction in NPY expression in the ARC with chronic leptin injection, consistent with the inhibitory action of leptin demonstrated in adults. However, we observed no differences in the frequency of milk ejection reflex in mothers nursing pups that were injected leptin, indicating that feeding drive and milk consumption may not have been different between the groups. It is therefore unclear what the consequences of decreased hypothalamic NPY production and secretion might be during the development of these rat pups.

Under the conditions of elevated dietary fat, our preliminary analysis indicates that the low carbohydrate diet (which contained the highest proportion of fat) resulted in decreased NPY expression as well. If this result is confirmed by additional experiments, one potential cause of the reduction in NPY expression in these conditions could be an increase in leptin associated with the increased dietary fat content. It has also been reported that, for adult rats, a high fat diet leads to increased expression of the short form of the leptin receptor, Ob-Ra, in the blood brain barrier (Boada, 1998), indicating that the amount of leptin reaching the brain may be increased. However, we did not specifically examine this particular isoform, nor did we measure any parameter of food intake in the pups who were nursing the different diets. The interaction between leptin and NPY during the developmental period is likely different from that in adulthood, since pups must continue to consume food and put and on weight in order to grow and mature. The increase in leptin associated with added weight and adiposity may not signal satiety, but rather transduce one of leptin's other functions, possibly as a growth factor or even to exert control of glucocorticoid activity via the HPA axis, as we have demonstrated in these experiments.

VII. SUMMARY AND CONCLUSION

In summary, we have shown that exogenously injected leptin is biologically active in neonatal rat pups and acts to decrease fat deposition and body weight gain. We suggest that these effects are mediated by an increase in energy expenditure rather than a decrease in energy intake since we detected no differences in milk ejection by the dams. We have also demonstrated that while acute injection of leptin results in increased plasma leptin in neonatal rat pups, chronic elevations in circulating leptin by exogenous administration is necessary to produce effects on the HPA axis. These effects include a decrease in the overall magnitude, but not peak production of the ACTH response, and a decrease in stimulated expression of CRF in the PVN. We have hypothesised that this is due to alterations in the glucocorticoid feedback system in these pups. While we detected no significant changes in CBG levels, we showed significantly increased expression of the glucocorticoid receptor in the CA2 region of the hippocampus to support this suggestion. Since basal levels of ACTH, B and CRF were not changed, the lack of difference in mineralocorticoid levels in any brain region measured lends further weight to our argument. The increase in maternal licking / grooming behaviour we observed is also consistent with these neuroendocrine changes, thus we further suggest that increased leptin in pups somehow signals such changes in behaviour by the dam, although the precise mode of action remains unclear. In support of previous finding by our laboratory, we showed that the high leptin levels in pups consuming high fat milk is associated with decreased stress responsiveness.

These experiments have also shown that both chronic leptin injection and an increase in the proportion of dietary fat is associated with a decrease in NPY expression in the ARC nucleus of the hypothalamus. Since we also showed that leptin injection causes

downregulation of its own receptor in this nucleus, and since these two peptides are known to co-localize, we suggest that leptin may influence the expression of NPY during the developmental period. But, the way in which this occurs with respect to food intake or any other action of NPY remains to be specified.

In conclusion, this series of experiments underlines the importance of the interaction between neuroendocrine systems and energy balance during the developmental period and particularly highlights the significance of leptin as one major communicating link in these processes. With the increase in obesity in our society and the prevalence of metabolic disorders such as diabetes, the implications of a role for leptin in the modulation of these systems are far-reaching. It is our hope that these findings might contribute to a better understanding of human metabolic disorders and in particular to strategies for detection and treatment during early childhood development.

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