The Ability of Central Leptin Administration to Induce Phosphorylation of STAT3 in Lactating Rats

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ABSTRACT

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Leptin secreted from white fat acts in the brain to decrease food intake and increase energy expenditure. Although the discovery of leptin promised a powerful way of combating obesity, subsequent research suggested that most obese individuals were actually resistant to the effects of leptin. High circulating levels of leptin are most commonly associated with leptin resistance but evidence is accumulating that sensitivity to leptin differs between sexes and across reproductive states. For example, earlier studies suggest that peripheral administration of leptin is less effective in decreasing food intake in lactating than in cycling rats. Leptin's effects on food intake are believed to be mediated primarily through activation of the JAK/STAT and the resulting phosphorylation of STAT3. In the present study the ability of central leptin injections to induce PSTAT3-ir in the VMH, PVN, and ARC was compared among cycling rats (Cyc) and lactating females on either postpartum day 4 or 16. Results show that in all hypothalamic areas examined there were more PSTAT3 positive cells in rats receiving leptin than those receiving saline. Within the PVN, this response was attenuated in the lactating groups and was most reduced on Day 16 postpartum. These results further support previous findings that there is a reduction in leptin sensitivity that occurs during the lactational period.

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INTRODUCTION

Leptin, an adipose-derived hormone, that acts in the brain to increase energy expenditure and reduce appetite, was discovered in 1995 (Halaas et al., 1995). This breakthrough was thought to be monumental because it was believed that leptin could be used as a treatment for obesity. However, such hopes were quickly met with disappointment, since it is now understood that most cases of obesity are related to high circulating leptin levels and an inability to respond to the effect of this protein (Friedman & Halaas, 1998). Obesity research has now focused on uncovering the mechanisms underlying leptin resistance. Accumulating evidence suggests that, in many cases, high circulating levels of leptin lead to central resistance, however, in some situations sensitivity to this hormone changes as an adaptive response to particular physiological requirements such as the increase in fat storage that occurs prior to hibernation and during pregnancy (Kronfeld-Schor, Richardson, Silvia, Kunz, & Widmaier, 2000; Ladyman, 2008). In this thesis the possibility that leptin sensitivity is also changed during lactation, a reproductive state associated with a dramatic increase in food intake, was investigated. Because the ability of leptin to induce the phosphorylation of signal transducer and activator of transcription (PSTAT) is a key step through which leptin suppresses food intake, the ability of central leptin to induce PSTAT3 was compared among cycling and lactating rats.

The notion that fat stores play a role in the homeostatic mechanisms controlling food intake and energy balance is not a novel proposal. In 1953, Gordon Kennedy put forward his "lipostasis" hypothesis which suggested that "metabolites" generated by the periphery at levels proportional to fat stores are released into the circulation and

determine the amount of food that an organism consumes and the degree of energy it expends, thus resulting in a relatively consistent body weight (Kennedy, 1953; Langhans & Geary, 2005, chap. 2). Evidence for such a compound(s) first emerged from a parabiosis experiment carried out by Hervey (1959) that involved the surgical union of two rats resulting in a shared circulatory system. Such a manipulation allowed humoral factors to freely flow from one animal to the next. Following the joining of the pair, one of the parabionts received an electrolytic lesion to the area of the hypothalamus. This manipulation resulted in increased food intake and obesity in lesioned animals while the conjoined partner reduced its food consumption and lost weight. If the joined partner with the intact hypothalamus was subsequently lesioned, it too began to overeat and gain weight. This experiment was the first to demonstrate that Kennedy's proposed blood borne factor worked by reducing food intake and weight and that it required an intact hypothalamus to do so (Hervey, 1959; King, 2006).

Investigations involving murine genetic syndromes of obesity provided further insight into candidates for such satiety factors(s). One of these syndromes was discovered at Roscoe B. Jackson Memorial Laboratories in 1949 when it was observed that in comparison to their wild type counterparts, certain mice were hyperphagic and as a result were heavier by four-fold, diabetic (type II), and infertile. Furthermore, these animals exhibited hypogonadism, hypothermia, as well as increases in circulating corticosterone (Ingalls, Dickie, & Snell, 1949; Elias, Kelly, Lee, Ahima, Drucker, Saper, & Elmquist, 2000). It was eventually determined that these mutants were homozygous for a recessive gene that would come to be known as the obese (ob) gene. Another model, discovered in the same lab in 1966, known as the diabetes mouse, is recessive for the db

gene, and resembles the ob/ob mouse with the exception that its symptom onset is earlier and it has a shorter life expectancy (Hummel, Dickie, & Coleman, 1966).

Using the parabiosis procedure, Coleman and Hummel (1969) connected the circulation of the db/db mouse to a wildtype mouse, and demonstrated that the non-mutant partner had a reduction in blood glucose and lost weight at an accelerated rate, with most subjects succumbing to these effects within 7 weeks of being attached. This suggested that a satiety compound circulating in the db/db mouse had potent effects on the wildtype mouse while being unable to exert similar effects on the db/db mouse, exhibiting a type of resistance (Coleman & Hummel, 1969). When the ob/ob mouse was subjected to this same procedure, however, the effect was very different. The mutant parabiont did not exhibit its typical phenotype, but instead increased weight at a reduced pace and did not become obese. These results pointed to the existence of an appetite reducing compound in the wildtype mouse that was able to exert an effect on the ob/ob mouse (Coleman 1973).

Armed with the evidence from the research described above, the lipostatic signal that Kennedy had proposed over 40 years earlier was awaiting its discovery. In 1994, Friedman and his colleagues went on to clone and sequence the gene of the ob mouse and the homologue in hominoids (Zhang et al., 1994). From their work, they were able to determine that the ob gene codes for a protein that acts as a circulating factor to produce satiety. In the following year, the team was able to express the ob gene in bacteria and purify its product. They named the resulting protein leptin, originating from Greek word *leptos*, meaning thin (Halaas et al., 1995).

Using a recombinant form of the OB protein, Halaas et al. (1995) administered the product to C57BL/6J ob/ob mice, and C57BL/Ks db/db mice intraperitoneally. The researchers discovered that after daily injections for 33 consecutive days, ob/ob mice were able to lose approximately 40% of their original weight when compared to vehicle controls. This reduction was driven primarily by a reduction in hyperphagia.

Furthermore, there was a marked improvement in the diabetes condition typically seen in this murine model. In sharp contrast, db/db mice failed to respond to the OB protein with a reduction in food intake or body weight when compared to subjects receiving a vehicle injection. On the other hand, wild-type mice receiving exogenous leptin responded with a decrease in body weight. From these results the authors concluded that ob/ob mice suffer from a lack of leptin production, while the db/db mice are resistant to the biological effects of the OB protein (Halaas et al., 1995). Similar results were found by Pelleymounter and colleagues (1995) and by Campfield and colleagues (1995), both suggesting that the OB protein is involved in the regulation of fat deposition and body weight by affecting appetite and energy balance (For review see Castracane & Henson, 2006, chap. 1).

Since its initial discovery, much information has been gleaned regarding the nature and function of leptin. Leptin is a protein consisting of 167 amino acid residues that are synthesized principally by the white adipose tissue (WAT) and to a lesser extent by the brown adipose tissue (BAT), the placenta and stomach (Zhang et al., 1994; Masuzaki et al., 1997; Bado et al., 1998). This hormone is released in the periphery and circulates in the blood stream at levels that are primarily determined by the amount of stored triglycerides (Maffei et al., 1995). Leptin binds to specific receptors (ObR), which

fall into the family of class I cytokines. ObR are themselves a product of the db gene, which was found to be dysfunctional in the obese db/db mouse (Tartaglia et al., 1995). The db gene has been localized to chromosome 1p in humans and 4 in mice (Chung, Kehoe, Chua, & Leibel, 1996). To date, six isoforms of the leptin receptor have been discovered, ObRa-ObRf. ObRa and ObRc, localized to the choroid plexus and epithelial cells, are candidates for mediating the active transport mechanism that is involved in allowing leptin to reach the brain from the circulation (Cioffi, 1996; Bouret, 2008; Kastin, Pan, Maness, Koletsky & Ernsberger, 1999).

ObRe acts as a soluble binding protein as opposed to a receptor and has not been found in humans (Li et al., 1998). The ObRf subtype is unique to the rat, while ObRd receptor subtype exists only in mice (Wang, Zhou, Newgard, & Unger, 1996). Only two of these receptor subtypes have been shown to have intracellular signaling capabilities: the long ObRb and short ObRa leptin receptor isoforms (Uotani, Bjorbaek, Tornoe, & Flier, 1999). The ObRb is unique in its ability to fully engage signaling of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway and it is richly expressed in the hypothalamus (For review see Schulz & Widmaier, 2006, chap. 2).

JAK/STAT pathway activation is a critical step in the control of food intake and ObRb is coupled to this pathway by leptin. When leptin attaches itself to ObRb, it results in the addition of a phosphate to the JAK proteins that are associated with the receptor. In turn, this leads to the phosphorylation of several tyrosine sites on the receptors which include amino acid residues 1138 and 985 (McCowen, Chow, & Smith, 1998). This then provides a site to which signal transducer and activator of transcription 3 (STAT3)

proteins can bind, causing them to become phosphorylated by JAK2. These phosphorylated STAT3 (PSTAT3) proteins then detach and go on to form homodimers in the cytoplasm, translocate to the nucleus and control the transcription of genes (Bjorbaek & Kahn, 2004; Leonard & O'Shea, 1998). Of the immediate early genes that are transcriptionally regulated by PSTAT3, the suppressors of cytokine signaling (SOCS) molecules are important in a negative feedback loop that operates intracellularly thereby stopping the JAK/STAT pathway from being stimulated excessively (Bjorbaek, El-Haschimi, Frantz, & Flier, 1999). Phosphotyrosine phosphotase 1 B (PTP1B) is another negative regulator of leptin signaling which follows the activation of ObRb (Zhang & Scarpace, 2006). Although activation of the JAK/STAT pathway has been most strongly linked to leptin's effects on food intake, this hormone also acts through other intracellular signaling pathways including the phosphatidylinositol 3 (PI3) kinase and mitogen-activated protein (MAP) kinase pathways (Berti, Kellerer, Capp, & Häring, 1997; Takahashi et al., 1997).

Leptin receptor messenger ribonucleic acid (mRNA) has been found in a number of tissues throughout the body including the placenta (Lea et al., 2000), skeletal muscle (Muoio, Dohn, Fiedork, Tapscott, & Coleman, 1997), liver (Reyes, Lazalde, Posadas del Rio, & Escalante, 2008), stomach (Bado et al., 1998), pancreas (Kieffer, Heller, & Habener, 1996), brown and white adipose tissues (Sarmiento et al., 1997), and the brain (Li, Wang, & Yeh, 1999). Despite the abundance of these receptors, it appears as though leptin exerts most of its effects on energy balance in the brain, and most researchers have focused on its effects in the hypothalamus.

Within the hypothalamus, several nuclei have been found to express ObRb mRNA including the arcuate nucleus (ARC), lateral hypothalamus (LH), dorsomedial nucleus (DMH), ventromedial hypothalamus (VMH), ventral premamillary nuclei (PMV), and the paraventricular nucleus (PVN) (Mercer et al., 1996; Fei et al., 1997; Elmquist et al., 1998). Recent evidence suggests that leptin is one of a multitude of neural, hormonal, and metabolic communications originating from the peripheral system that are received and integrated by these nuclei (Smith & Grove, 2002).

The ARC contains two populations of "first order" neurons which are responsive to leptin (Coll, Farooqi, & O'Rahilly 2007). One set of neurons colocalize neuropeptide Y (NPY) and agouti-related protein (AgRP) which are both orexigenic (Hahn, Breninger, Baskin, & Schwartz, 1998). AgRP acts as an antagonist at melanocortin receptors (MCR) types 3 and 4, while NPY, which is the most potent orexigenic agent yet discovered, has its effects on food intake by acting at the Y1 and Y5 receptors. NPY/AGRP neurons project from the ARC to other hypothalamic nuclei such as the PVN, DMH, and LH where they regulate ingestive behavior (Smith & Grove 2002). Electrophysiological studies have shown that leptin treatment increases inhibitory postsynaptic potentials in the NPY/AGRP neurons of the ARC (Riediger, Traebert, Schmid, Scheel, Lutz & Scharrer, 2003) and it has been reported that this hormone actually increases the number of inhibitory synapses on these neurons (Horvath, 2006). In the absence of leptin, NPY expression in the ARC is increased, and these neurons are more active (Cohen et al., 2001).

By contrast, when leptin binds to its receptor on proopiomelanocortin peptide (POMC) neurons, their membrane potential is reduced and firing rates of the axons are

increased (Cowley et al., 2003). Both CART (cocaine and amphetamine-regulated transcript) (Kristensen et al., 1998) and alpha melanocyte-stimulating hormone (α MSH), a cleavage product of POMC, reduce food intake and increase energy expenditure; but the latter appears to be more potent (Seeley et al., 1997). The increased activity of POMC neurons following leptin administration results in increased release of α MSH which acts through MCR-3 and MCR-4 activation to decreased food intake (Hsiung et al., 2005). Dense groupings of MCR-3 can be found in the ARC, VMH, and the preoptic nucleus, while MCR-4 is found in abundance in the PVN, DMH, and the LH (Shimizu, Inoue, & Mori 2007). The activation of POMC/CART neurons by leptin has widespread effects since they project both within the hypothalamus (PVN and LH) and beyond to areas such as the amygdala, septum, and the brainstem (Chen, Williams, Grove, & Smith 2004).

The ARC is not the only site of leptin action in the hypothalamus or the only site at which leptin plays a critical role in the control of body weight. Leptin receptors have been detected on the steroidogenic factor 1 (SF-1) neurons in the VMH and deletion of these cells leads to obesity (Dhillon et al., 2006). Activation of PVN neurons, which release corticotrophin releasing hormone (CRH) can be blocked with the administration of leptin (Huang, Rivest, & Richard, 1998) and the longform of the leptin receptor has also been localized to oxytocin (OT) neurons in the PVN (Kokay, Bull, Davis, Ludwig, & Grattan, 2006). ObRb is also present in LH neurons expressing melanin concentrating hormones (MCH) (Stepanyan et al., 2003).

Assays that have examined activation of c-Fos and STAT3 have also identified extra-hypothalamic brain areas that are responsive to leptin including the parabrachial

nucleus (PBN), periaquductal gray (PAG), the dorsal raphae (DR) of the midbrain, the nucleus of the solitary tract (NTS) in the caudal brainstem and the ventral tegmental area (VTA) (Muenzberg, Huo, Nillni, Hollenberg & Bjorbaek, 2003). Evidence has so far failed to implicate any of these leptin responsive non-hypothalamic regions in food intake or energy balance directly, with the exception of the VTA, in which leptin administration has been found to reduce the consumption of food (Hommel et al. 2006).

In summary, leptin is generally considered to provide a signal of long-term energy stores to the neural circuitry sub-serving food intake and energy expenditure. Usually when there is a high degree of adipose tissue, there is an increase in leptin which acts at the level of the ARC to inhibit the NPY/AgRP neurons and hence reduces feeding, while exciting the POMC/CART neurons to release anorexic neuropeptides. In contrast, when there is a loss of leptin signaling, there is a decrease in energy expenditure and an increase in food intake (van de Wall et al., 2008).

When leptin was discovered, it was hoped that it might lead to the development of a therapeutic agent that could prevent or even reverse obesity (Henry & Clarke, 2008). Experiments which exhibited the effectiveness of leptin administration to reverse the ob/ob mouse condition and to reduce food intake in normal weight rodents provided evidence that this hormone might in fact be an effective anti-obesity agent. However, attempts of the pharmaceutical industry to develop leptin as a therapy for obesity were met with disappointment when it was found that weight reduction only occurred in a small proportion of obese individuals (approximately 5-10% have low leptin production). Closer investigation of obese non-responders revealed that unlike the ob/ob model, in which the lipostatic signal is not produced, overweight subjects have increased plasma

levels of leptin in comparison to controls. This suggests a condition of leptin resistance in most forms of obesity whereby the periphery provides accurate signals with regards to the proportion of fat stores; however this signal fails to exert a central effect at the level of the brain (Friedman & Halaas, 1998). These findings led obesity researchers to change their focus to uncovering the mechanisms underlying this leptin resistant state.

Several hypotheses have been forwarded to explain the causes of leptin resistance. One posits that there is a defect in the transport system of leptin from the periphery into the brain. For example, F344xBN rats that exhibit an increase in adiposity with age have been found to have a decreased response to peripheral versus central infusions (Scarpace, Matheny, & Shek, 2000). Another suggested mechanism involves a decrease in the number of leptin receptors within the hypothalamus. Decreased ObR mRNA has been found to be related to age related obesity (Scarpace, Matheny, Moore, & Tumer, 2001). Finally disruptions in intracellular signaling have also been suggested as a mechanism behind leptin resistance (Sahu, 2003). It has been shown that leptin activation of STAT3 is reduced in normal mice that become obese through exposure to high fat diets (Haschimi, Pierroz, Hileman, & Bjorbaek, 2000). It is also possible that SOCS3 may indirectly contribute to leptin resistance. It has been found that in mice with diet induced obesity (DIO), within the ARC there is an increase in SOCS3 expression (Henry & Clarke, 2008)

Since human obesity involving leptin receptor gene or leptin gene mutations are very rare, research has focused on developing models that more closely approximate the most common origin of this condition (Bell & Considine, 2006, chap. 3). The recent rapid of obesity suggests that genes alone cannot provide a full explanation (Archer & Mercer

2007). Environmental conditions that include a lifestyle of low physical activity combined with the consumption of palatable food that is dense in energy increases the risk of the development of obesity substantially, and are the cause of the majority of these cases. Thus investigators examining the mechanisms of leptin resistance have primarily utilized animal models of DIO, considered to be physiological responses that are generally maladaptive (Ladyman, 2008).

Although not directly analogous to the typical diet of Westerners, these models provide rodents with access to a highly palatable solid or liquid supply of sugar or fat in addition to their standard commercial pellet food diet. Generally it has been reported that vulnerable rodents exposed to these obesogenic diets for an extended period of time are hyperphagic and have a 10% to 20% gradual increase in overall weight compared to controls. These effects are highly dependent on the type of rodent and strain that are employed, although employing rats in this type of research is most advantageous since their size allows for more endpoints to be examined such as catheter techniques (Buettner, Scholmerich, & Bollheimer, 2007).

Close investigation of DIO models has revealed important clues to the nature of this condition. For example, in one experiment obesity was induced in mice by prolonged exposure to a high fat diet. Prior to becoming obese these animals were responsive to leptin administered peripherally, however after 8 weeks of high fat feeding they no longer reduced food intake in response to this treatment. Interestingly, these animals reduced food intake in response to central administration (Van Heek et al., 1997). Furthermore, in DIO models, phosphorylation of STAT3 does not occur after peripheral administration, but does after intracerebral ventricular administration (Vaisse, Halaas, Horvath, Darnell

Jr, Stoffel, & Freidman, 1996). These findings provide some support for the idea that there may be a problem with transport of leptin from the periphery to the brain. Although a defect in leptin transport through the Ob-Ra and Ob-Rc might contribute to these results reduction in Ob-Ra and Ob-Rc mRNA in DIO could explain the findings. Data from other models of diet induced obesity suggest leptin resistance of central origin. For example, DIO Wistar rats that received an infusion of leptin into the lateral ventricles exhibited a reduced anorexic effect compared to controls (Levin & Dunn-Meynell, 2002; Widdowson, Upton, Buckingham, Arch, & Williams, 1997). Similar findings were obtained with DIO Sprague Dawley rats, AKR/J mice, and AY mice (Sahu 2004).

More recently, alternative models in which leptin resistance is apparently physiologically adaptive have gained increasing attention. Using a model of an adaptive response and its return to a "normal" state helps us understand how a normally adaptive state can become maladaptive. Furthermore the mechanisms that control its return to a "normal" state can be used for therapeutic purposes in diet induced leptin resistance. Mammalian preparation for hibernation is one such example. Little brown bats increase their food intake prior to winter and exhibit a reduction in their sensitivity to leptin during this time (Kronfeld-Schor, Richardson, Silvia, Kunz, & Widmaier, 2000). Another example of adaptive leptin resistance is apparent in Siberian hamsters. Studies with this species have revealed that their sensitivity to leptin is dependent on the season. For example those hamsters that where exposed to photoperiods that mimicked shorter days for eight weeks and received two weeks of chronic leptin treatment depleted their fat stores. In contrast, there were no changes in food intake, body weight or fat stores in

those hamsters exposed to a long-day light cycle in addition to the leptin treatment (Atcha et al., 2000).

It also appears that there are sex differences in leptin sensitivity. For example, icv administration of leptin to age and weight matched males and females led to a reduction in 24 hour food intake in the female rats only (Clegg, Riedy, Smith, Benoit, & Woods, 2003). It has been proposed that one of the mechanisms regulating this difference may be estrogen since females have higher concentrations of this hormone. Clegg (2006) and her associates tested this hypothesis by comparing male and female animals that had their estrogen levels manipulated to varying degrees with castration, ovariectomy and subsequent administration of estradiol. The authors reported that intact females, castrated males and estradiol treated males all responded with reductions in food intake and body weight when treated centrally with leptin compared to those treated with a vehicle while intact males were not responsive to the same 3.5 microgram dose (Clegg, Brown, Woods, & Benoit, 2006). Thus these results suggest high estrogen levels increase sensitivity to leptin.

Pregnancy has also been investigated as a model of leptin resistance. Increases in circulating leptin have been documented in both rodents and humans during gestation and reflect increased fat stores and/or production of this hormone by the placenta. In the rat, Chien et al. (1997) reported a 1.8 fold increase in serum leptin concentrations (Chien et al., 1997). Despite higher circulating levels of this satiating peptide, the consumption of food either remains the same or increases. For instance, Rocha and colleagues have reported that by the second and third weeks of gestation, rats consume 20% to 27% more food than non-pregnant animals (Rocha, Bing, Williams, & Puerta, 2003). Thus pregnant

rats and humans show an increased food intake in the presence of high circulating levels of leptin. Increase in NPY mRNA expression in the ARC has also been reported during pregnancy (Garcia, Gualillo, Seoane, Dieguez, & Senaris, 2003) consistent with a reduction in leptin signaling.

Insensitivity to the effects of exogenous leptin on food intake in late pregnancy has been reported in ob/ob mice. Ob/ob mice are typically infertile, but fertility can be restored and pregnancy maintained with leptin treatment. In spite of daily leptin treatment, however, pregnant ob/ob mice increased their food intake from day 10 of pregnancy onward. From this it was concluded that a leptin resistance state in the ob/ob mice had emerged (Mounzih, Qiu, Ewart-Toland, & Chehab, 1998).

Similar results have been reported in pregnant rats. A single icv injection of leptin decreased food intake and body weight in cycling and pregnant rats tested on day 7 post conception while being ineffective in changing the food intake of rats on Day 14 of pregnancy. This lack of a behavioral response to leptin was associated with a reduction in the phosphorylation of PSTAT3 in the VMH of the D14 pregnant group (Ladyman & Grattan, 2005). Reductions in expression of the mRNA for the long form of the leptin receptor within the hypothalamus have also been reported in pregnant rats, however they do not appear to relate to the time course of the appearance of leptin resistance in pregnancy (Szczepankiewicz, Wojciechowicz, Kaczmarek, & Nowak, 2006).

The period during pregnancy when leptin resistance is observed coincides with a major change in hormonal profile: a switch from the bi-daily surges of prolactin (PRL) typical of the first half of pregnancy to a chronic increase in circulating placental lactogens (Rosenblatt, Siegel, & Mayer, 1979). Since placental lactogens can cross the

blood brain barrier and bind to central prolactin receptors (PRLR), this results in chronic PRLR stimulation. In both birds (Koch, Wingfield, & Buntin, 2004) and rats (Noel & Woodside, 1993; Sauve & Woodside, 2000), PRL has been shown to act in the brain to stimulate food intake. Most recently Naef & Woodside (2007) found that virgin Wistar rats receiving chronic central infusions of PRL increased their food intake and moreover did not show a decrease in either food intake or body weight following central leptin administration. Leptin induction of STAT3 phosphorylation was reduced in both the VMH and PVN of PRL infused rats. Further evidence for a role for PRL in the induction of leptin resistance comes from a study by Augustine et al (2008). These authors pseudoimpregnanted rats by mating them with vasectomized rats and found that pseudopregnancy alone was not sufficient to induce leptin resistance. Combining pseudopregnancy with chronic PRL infusion did prevent central leptin administration from decreasing food intake and body weight, however. Together, these studies suggest that leptin sensitivity is reduced during mid-late pregnancy and that this state is produced through exposure to chronically high levels of PRL.

In the current study leptin sensitivity in another reproductive state, lactation, was examined. Lactation, like the latter half of pregnancy, is associated with high circulating levels of PRL (Ota and Yokoyama, 1967) accompanied by a dramatic increase in food intake (Cole & Hart, 1938; Malabu, Kilpatrick, Ware, Vernon, & Williams 1994). Unlike pregnancy, however, fat stores and leptin levels actually decrease during lactation.

Because energy stored as fat during pregnancy is utilized to meet the energetic demands of milk production (Naismith, Richardson, & Pritchard, 1982), as fat stores are decreased, circulating leptin levels also decrease (Woodside, Abizaid & Walker, 2000;

Woodside, 2007). Interestingly, however, there is a significant increase in food intake prior to a significant decrease in circulating leptin levels (Woodside et al, 2000).

Some studies have suggested that there is a reduction in the behavioural response to leptin in lactating rats. Woodside, Abizaid, & Walker (2000) exposed lactating dams to continuous subcutaneous infusions of leptin or a vehicle via osmotic minipumps. They found that although leptin treatment decreased body weight, it had no effect on food intake from postpartum days 8 to 22 when compared to vehicle animals. In contrast, leptin icv infusions reduced feeding from postpartum days 8 to 10, however this effect was transient so that by day 11 postpartum it had disappeared (Woodside et al., 2000) Crowley et al. (2004) found similar results when investigating the effects of the administration of subcutaneous infusions of leptin via osmotic minipumps in lactating females on orexigenic neuropeptide expression. The investigators reported that although they were successful at restoring serum leptin concentrations in lactating rats to levels observed in non-lactating rats, resulting in a decrease in levels of NPY mRNA expression, there were only minimal, inconsistent effects on the consumption of food (Crowley, Ramoz, Torto, & Kalra 2004).

Finally, Roy and colleagues carried out an in vitro study in which Western blot analyses for PSTAT3 were carried out on the ARC of virgin and lactating rats following incubation in the presence or absence of 1ug of leptin. Following this treatment, the authors found that leptin resulted in a significant induction of PSTAT3 in the ARC of virgin females, while there was a complete absence of this same protein in lactating females (Roy et al., 2007).

Given the evidence suggesting some decrease in responses to leptin in lactating rats, the current work was carried out to compare the ability of central leptin administration to activate the JAK/STAT pathway among female rats that were cycling, in early lactation (Day 4 postpartum) or late lactation (Day 16 postpartum). To relate responses to leptin with lactational hyperphagia, in Experiment 1 we compared food intake among groups of cycling rats, rats on Day 4 postpartum and rats on Day 16 postpartum. It was predicted that lactating rats would consume significantly more food than cycling females. In experiment 2, the ability of central leptin administration to induce phosphorylation of STAT3 in the VMH, PVN and ARC was compared among cycling and lactating rats on Day 4 or Day 16 postpartum. It was hypothesized that lactating animals would show a reduction in PSTAT3 activated cells in the VMH, PVN, and the ARC in response to central administration of leptin when compared to cycling females.

GENERAL METHODS

Experiment 1: *Effect of Reproductive State on Food Intake Subjects*

28 female virgin Wistar rats were obtained from Charles River Breeding farms, St. Constant, Quebec. Rats weighed between 220 - 240 g upon arrival and were housed in clear plastic cages (20 X 45 X 50 cm). All subjects had ad libitum access to powdered rat chow (Agway Ltd; Syracuse, NY) and water. They were maintained on a 12-hour light/dark cycle with lights on at 08:00 and off at 20:00 h, at a room temperature of 20 $\pm$ 2 °C throughout the experiment.

Procedure

Animals were housed in groups (4-5). Rats assigned to the lactating groups were mated by introducing a stud male into the group cage. Approximately 18 days later, all subjects, including virgins, were separated into clear individual plastic cages (20 X 45 X 50 cm). At this time, food intake was monitored daily for all subjects. Vaginal smears of virgins were taken daily beginning 4 days prior to the first possible day of parturition for the lactating group, and this continued until the day of sacrifice. The day of parturition was designated day 0 postpartum (PP0). All litters of the dams were culled to 8 pups.

Experiment 2: *Effect of Central Leptin Administration on PSTAT3-ir Induction during Different Reproductive States*

Subjects

Twenty-three female virgin Wistar rats were obtained from Charles River Breeding farms, St. Constant, Quebec. Rats weighed between 220 - 240 g upon arrival and were individually housed in clear plastic cages (20 X 45 X 50 cm). All subjects had ad libitum access to food and water and were maintained on a 12-hour light/dark cycle with lights on at 08:00 and off at 20:00 h, at a room temperature of 20 $\pm$ 2 °C throughout the experiment.

Surgeries

All subjects underwent surgery to implant a cannula into the lateral ventricle. On the day of surgery, subjects were anaesthetized using ketamine-xylazine (5.7 mg ketamine and 0.86 mg xylazine/ 100 g body weight). The head was shaved covering an area slightly posterior to the eyes to the base of the skull. The subjects were then placed in a stereotaxic apparatus. A sagittal incision approximately 2.5 cm in length was made

to expose the skull, which was subsequently cleaned. 4 holes were drilled and 3 jeweler screws were placed into the skull. A 22 gauge cannula was implanted into the lateral ventricle at the following co-ordinates AP: - 0.2 mm; L: -1.6 mm; DV: -5.0 mm from the surface of the skull (Paxinos & Watson 1996), and then fixed to the skull using dental cement. Following a minimum of 3 days of recovery from surgery, subjects were injected with angiotensin (50 ng in 2 μl) and water intake was monitored. If the injection resulted in at least 1 minute of rapid drinking, it was considered as evidence for accurate placement of the cannula.

Procedure

Subjects were randomly assigned to either the cycling or the lactating group. Subjects in the cycling group were housed individually in clear plastic cages until the day of infusion. Those subjects assigned to the lactating group were mated by pairing the subject with a sexually experienced male rat. Vaginal cytology of this group was monitored daily until sperm were detected. At this time, subjects were separated from the males and housed individually in clear plastic cages (20 X 45 X 50 cm) until parturition. The day of parturition was designated day 0 postpartum (PP0). At this time the litters of the dams were culled to 8 pups.

Effect of stage of lactation on leptin signaling

The ability of leptin to induce PSTAT3 was compared amongst 6 groups of rats: cycling rats treated with either leptin (CYC/LEP, N=4) or saline (CYC/SAL, N=3); lactating rats on day 4 postpartum treated with either leptin (DAY4/LEP, N=5) or saline (DAY4/SAL, N=4) and lactating rats on Day 16 postpartum treated with either leptin (DAY16/LEP, N=4) or saline (DAY16/SAL, N=3). Leptin (Peprotech, NJ) was dissolved

in dH2O and buffered with a 1 M tris solution to a ph of 7.4 and administered at a dose of 4 μg in a volume of 4 μl. Rats in saline groups were injected with a similar volume of saline. 30 minutes following the infusion, subjects were given an overdose of approximately 0.7 ml of Euthanyl (sodium pentobarbital) and transcardially perfused with saline followed by 2% paraformaldehyde. After brains were extracted, they were post-fixed for approximately 18 hours at room temperature in 2% paraformaldehyde. Brains were then placed in a 30% sucrose solution until they sank or for a maximum of 72 hours and then stored at –80 °C until sectioned on a cryostat. Three sets of 40 μm sections were obtained throughout the hypothalamus and stored in Watson's cryoprotectant until processed for immunohistochemistry.

PSTAT3 immunohistochemistry

Sections through the hypothalamus were washed (3 X 5 min in PBS), incubated in 1% H2O2 and 1% NaOH in PBS for 20 min, and then 0.03% sodium dodecyl sulphate for 10 min. Following 3 X 5-min washes in PBS, sections were placed in blocking solution (4% NGS in 0.25% Triton X-100 and 0.02% sodium azide in PBS) and incubated at room temperature for 1 h. Overnight, sections were placed in primary antibody directed against PSTAT3 (1:1000 dilution; Cell Signaling Technology, Inc. Beverly, MA) in blocking solution at 4 °C. The next day, sections were washed incubated in 3% NGS and secondary antibody (biotinylated goat-antirabbit; Vector) in blocking solution without sodium azide for 1 h, and washed once more. Sections were incubated in ABC reagent (Vector) for 2 h and developed with a DAB Vector kit. The mounting of sections onto gelatin slides was completed and these were then coverslipped using Permount (Fisher Scientific, Quebec, Canada), allowing for subsequent image analysis.

Image Analysis

A Sony XC77 camera (Sony Corp., Kanagawaken, Japan) mounted upon a light microscope (Labolux Leitz GMBH, Wetzlar, Germany) was used to visualize the brain sections obtained in Experiment II. National Institutes of Health Image Analysis Software (1.60 b) (Bethesda, MD) installed on a Power Macintosh computer (G4; Apple Computer, Inc., Cupertino, CA) was used to capture images of the sections and these were saved as Tagged Image File Format picture files.

For estimates of PSTAT3 expression in PVN, only sections in the medial area of the nucleus, (-1.80 mm from bregma) were used (Paxinos and Watson, 1986) and an average over all these sections was obtained. To obtain an estimate of PSTAT3 in the ARC and VMH, sections from -2.56 mm to -2.80 mm from bregma, were counted and the mean number of stained cells/section was calculated. To identify stained cells; sections from each experimental group across each assay that appeared to be stained were selected and a relative density greater than 150 was taken as proof of cell staining and recorded. An average of the density of the cells was then calculated to establish the criterion for each brain region across all experimental groups. The observer counting cells was blind to group membership.

Statistical Analysis

A univariate analysis of variance (ANOVA) was conducted on mean food intake (Experiment 1). A 3 (group: cycling, D4 lactation, D16 lactation) x 2 (treatment: saline or leptin) univariate analysis of variance (ANOVA) was carried out on PSTAT3 immunoreactive (IR) cells (Experiment 2). Where appropriate, pair wise Fisher's least significant difference (LSD) *post hoc* tests were used to compare groups.

RESULTS

Experiment 1

There was a significant main effect of group on food intake; $F(2, 25) = 167.044$, $p<0.001$. Fisher's LSD post hoc analysis revealed that on Day 4 of lactation, animals had significantly higher food intake than cycling rats ($p<0.001$), yet significantly less than Day 16 lactating females ($p<0.001$; see Figure 1).

Experiment 2

As can be seen in Figure 2, leptin administration produced a robust increase in PSTAT3 -IR in the VMH that resulted in a significant main effect of treatment $F(1,15) = 20.982$; $p < 0.001$). Neither the group effect nor the group x treatment interaction was significant.

Figure 3 shows the number of PSTAT3-IR cells/section in the ARC. As in the VMH, leptin administration increased PSTAT3-IR in all groups resulting in a significant main effect for treatment $F(1,15) = 23.011$, $p < 0.001$. There was no significant main effect of group, nor a significant interaction.

As Figure 4 shows, although leptin administration also resulted in an increase in PSTAT3-IR in the PVN (significant main effect for treatment in the PVN; $F(1,15) = 76.069$; $p < 0.001$), the strength of this effect was lower in both lactating groups resulting in significant effects for both group $F(2,15) = 8.531$; $p < 0.003$) and group x treatment interaction $F(2, 15) = 5.425$; $p < 0.017$). Pairwise posthoc comparisons using Fisher's LSD revealed that the cycling group had a higher number of PSTAT3-IR cells than both Day 4 ($p < 0.001$) and Day 16 ($p < 0.001$) groups. Day 4 animals had higher PSTAT3 + cells compared to day 16 ($p < 0.05$) animals.

DISCUSSION

The goal of the current work was to compare the ability of central leptin injections to induce PSTAT3-ir in hypothalamic nuclei of lactating and cycling rats. As expected, leptin injections induced a robust increase in PSTAT3-ir in the VMH, ARC and PVN in all groups. This response was attenuated in the PVN of lactating rats and the reduction in PSTAT3-ir was greater in the Day 16 than the Day 4 postpartum group. Together, these data suggest that lactation, like mid- to late pregnancy, is associated with a change in leptin sensitivity. Whether the reduction in leptin signaling through the JAK/STAT pathway in the PVN reflects a change in behavioral sensitivity to leptin remains to be determined but it has been suggested that the JAK/STAT pathway represents the major route through which leptin exerts its behavioural effects on food intake (Munzberg et al., 2003).

The decrease in leptin signaling that we observed is associated with increased food intake in lactating rats which, as the results of experiment 1 show, reaches approximately 200% of cycling levels by Day 4 postpartum and 325% of cycling levels by Day 16 postpartum. Leptin levels fall dramatically during lactation and reach a nadir at the time of peak milk production (Day 16 postpartum). Thus it is unlikely that leptin resistance is necessary to facilitate the extreme hyperphagia seen at the end of the second week of lactation. Rather, reductions in leptin sensitivity probably make their greatest contribution to lactational hyperphagia in the first week postpartum when fat stores and circulating leptin levels are similar to those of virgin rats.

Although a decrease in leptin sensitivity appears to be common to both pregnancy and lactation there are some differences in these phenomena. One distinguishing feature

between the decrease in leptin sensitivity observed in the current study and that reported by Ladyman & Grattan (2005) is that during pregnancy reductions were localized in the VMH, whereas no effects in this area were observed in the current study. Ladyman & Grattan (2005) did not examine the PVN, but in previous work by Naef and Woodside (2007), chronic PRL infusions were found to decrease leptin-induced PSTAT3-ir in both the VMH and PVN. None of these studies found effects in the ARC although it has been reported that in DIO there is a reduction in PSTAT3 signaling in the DMH, VMH and ARC. It is not clear what effects DIO has on signaling within the PVN since the authors did not examine this area (Levin, Dunn-Meynell, & Banks 2004). It would be useful to confirm the current results using Western blotting techniques. The immunohistochemical technique used here provides good spatial resolution, giving a measure of the number of cells showing detectable immunoreactivity for the protein in question. However, Western blotting would further bolster our findings since it provides an indication of the total amount of protein present in the cell and could detect more subtle differences between groups.

The current study, like those of Ladyman & Grattan (2005) and Levin et al. (2004) focused on one index of leptin signaling: the phosphorylation of STAT3. This focus is an important one since PSTAT3-ir indicates signaling activation within the JAK/STAT pathway and has been most strongly implicated in the anorectic effects of leptin. This hormone, however, also acts through both the MAP kinase (Bjorbaek et al., 2001) and PI3 kinase pathways (Zhao, Huan, Gupta, Pal, & Sahu 2002). In order to paint a complete picture of changes in leptin sensitivity during lactation it will be necessary in future studies to investigate whether there are any changes in leptin signaling through

these pathways during lactation by comparing the ability of central leptin to induce phosphorylated AKt and phosphorylated ERK in lactating and cycling rats.

The lack of any apparent effect of lactation on the ability of leptin to induce pSTAT3-ir in the ARC is consistent with earlier results of Crowley et al. (2004). These authors showed that leptin administration to lactating rats from Days 8 to 10 postpartum reduced NPY expression in the ARC, presumably reflecting a local effect of leptin, but had only a small effect on food intake. The fact that leptin can have expected effects in one hypothalamic area but not produce the expected effects on food intake is indicative of the complexity of signals and neural circuits that control ingestive behavior. Leptin receptors have been localized to a number of hypothalamic nuclei implicated in the control of food intake including the PVN, VMH and ARC as well as the LH (Rosenblum et al., 1996; Mercer et al., 1996) that are all interconnected (Gutwiller & Beglinger, 2005, chap. 1) suggesting that leptin can act at multiple sites to modulate energy balance. For example, previous work has suggested that lesions to the PVN, VMH and ARC all result in a reduction in leptin's ability to decrease food intake and body weight (Choi et al., 1999). Moreover, although investigations of direct administration of leptin into the PVN on food intake have not been carried out, microinjections into the VMH (Jacob, et al 1997) and the ARC (Satoh et al., 1997) independently serve to reduce food intake.

Recently most attention has focused on the roles of the ARC and VMH in the control of energy balance. However, the role of the PVN in the control of food intake is well-established. The PVN serves as an integrative site for many of the pathways involved in regulating energy balance (Gutzwiller & Beglinger, 2005, chap. 1). It receives projections from NPY/AGRP and the POMC/CART neurons in the ARC as well

as terminals of axons projecting from the LH. This nucleus is particularly sensitive to the effects of a variety of neurotransmitters including NPY, galanin, alpha-MSH, serotonin, norepinephrine, and opioid peptides; all of which have been implicated in appetite and energy regulation (Sahu, 2004). Furthermore, as with lesions of the VMH, damage to the PVN by way of electrolytic lesions or knife cuts result in hyperphagia and subsequent obesity (Leibowitz, Hammer, & Chang 1981). The PVN also contains a number of neuropeptides including CRH, TRH and oxytocin that are implicated in the control of food intake and there is some evidence that CRH and oxytocin neurons contain leptin receptors (Hakensson et al., 1998; Nishiyama, Makino, Asaba, & Hashimoto, 1999). Whether some specific subset of these cell populations are primarily affected by the changes in leptin signaling observed in the current study will be examined in the future using double immunohistochemistry.

Although the current data show a clear reduction of leptin sensitivity in lactating rats, they do not shed light on the mechanisms through which such changes in sensitivity are produced. Perhaps the simplest explanation for these effects would be a down regulation in leptin receptor density in lactating rats (Scarpace, Matheny, Moore, & Tumer, 2000). Another possibility is that the energetic demands of milk production and delivery give rise to signals that themselves modulate the response of leptin. A final alternative is that the hormonal state of the lactating rat modulates leptin responsivity. The finding that leptin sensitivity is reduced in early lactation prior to an appreciable loss of body fat or decrease in circulating leptin levels and when the loss of energy to the milk, although appreciable, is not at its peak, suggests that a simple increase in energy demand although possible, is not a likely explanation for changes in leptin sensitivity

(Woodside et al., 2000). There is, however, considerable evidence to suggest that the hormonal state of lactating rats and, in particular, the high plasma levels of PRL and low levels of estrogen typical of lactation may play an important role in these effects (Woodside, 2007; Smith & Grove, 2002).

PRL, which is a peptide hormone released from the pituitary in response to suckling stimulation, enters the brain via an active transport mechanism and has been shown to act centrally to increase food intake (Noel & Woodside, 1993; Sauvé & Woodside, 1996; Naef & Woodside, 2007). Moreover, Naef and Woodside (2007) showed that chronic intracerebroventricular PRL infusions in virgin rats both increased food intake, suppressed the behavioral response to a central injection of leptin, and attenuated leptin-induced increases in PSTAT3-ir and Fos in both the VMH and PVN. In addition, Augustine et al. (2008) showed that the leptin resistance of mid-late gestation was likely to result from the high circulating levels of placental lactogen that activate PRLR in the brain. Given the results of the current study, it is interesting that Woodside and Sauvé (1996) found that the PVN was a particularly important site for PRL in the control of food intake after central infusions of PRL resulted in an increase in feeding in virgin cycling rats.

The way in which PRL might modulate leptin sensitivity is not clear. PRL receptors, like leptin receptors belong to the cytokine family (Roy et al. 2007) and PRL, like leptin, activates the JAK/STAT signaling pathway. However PRL induced activation of this pathway results in the phosphorylation of a number of STAT molecules including STAT3 as well as STAT5, which is considered to be the most functionally important for PRL's actions in the brain (Grattan & Kokay, 2008). Similar to leptin activation of the

JAK/STAT pathway, PRL induced activation of its receptor leads to the production of a number of signals of cytokine suppression (SOCS) proteins including SOCS3 (Anderson et al., 2006). It has been suggested by Bjorbaek (1999) that an up regulation in SOCS3 could be the factor causing leptin resistance since it results in an inhibition of leptin signaling. However, in order for an intracellular SOCS3 increase to have an effect on leptin PSTAT3 cell activation, both leptin and PRL receptors must be colocalized on the same neuron. Recently, evidence of the coexpression of ObRb and PRLR was found to occur in the same neurons in the PVN (Roy et al., 2007) suggesting that at least in some brain areas the condition exists for this kind of interaction between PRL and leptin.

Estrogen levels, which are low during lactation, generally act as an anorectic agent (Ladyman, 2008). Administration of this hormone results in reductions in food intake and fat stores, while opposite effects are observed with the removal of the ovaries (Morin & Fleming, 1978). One way in which estrogen exerts its effects on food intake is by potentiating the satiating effects of peptides such as CCK and leptin (Asarian & Geary, 2006). For example, Clegg et al (2006) have shown that female rats are more sensitive to effects of leptin on food intake than male rats and that this increased sensitivity depends on circulating estradiol levels. The recent demonstration (Gao et al., 2007) that estrogen administration results in a reorganization of synaptic inputs to POMC neurons in the ARC are similar to that seen following leptin administration and represents another pathway through which estrogen may modulate energy balance. Interestingly, recent evidence suggests that in addition to genomic effects, estrogen administration can also lead to the phosphorylation of STAT proteins including STAT3 (Gao et al., 2007). Given the data suggesting estrogen potentiates leptin's actions, the absence of estrogen

during lactation might be expected to reduce leptin sensitivity. Evidence for the possibility of cross talk between both leptin and estrogen receptors has also emerged since the finding of colocalization of both sets of receptors in the neuronal perikarya in a number of hypothalamic nuclei including that of the parvicellular PVN (Diano, Kalra, Sakamoto, & Horvath, 1998).

In conclusion, our finding of a reduction in leptin sensitivity during lactation provides some insight into the possible mechanisms underlying the ability of mothers to meet the high energetic demands that are typically associated with lactation. The results offer support to the idea that this adaptive form of reduction in leptin sensitivity is related to a disruption of the intracellular signaling of the JAK/STAT pathway specifically within the PVN, while not affecting the ARC or the VMH as observed in other models of resistance. Although distinct from DIO models, this adaptive model of reduced leptin sensitivity can be useful in providing further understanding into the signaling pathways of food intake and energy balance, and offer insight into the mechanisms underlying obesity.

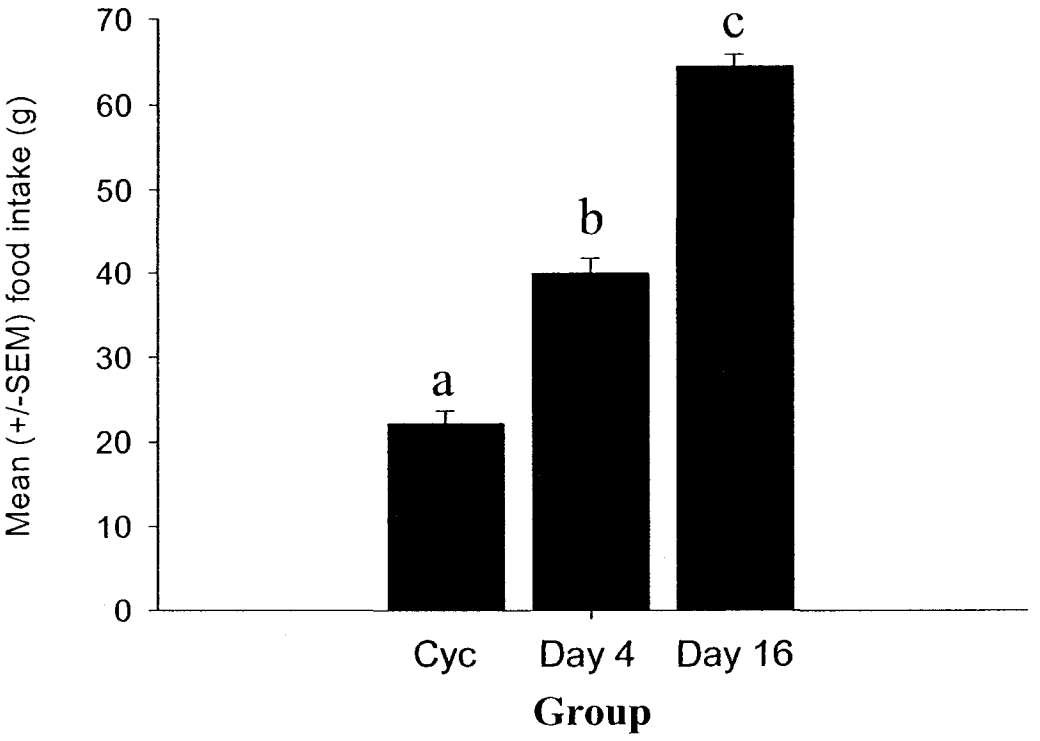

Figure 1. Mean (+/-SEM) food intake of animals in grams. Cycling animals ate less food than Day 4 lactating subjects. Furthermore Day 16 animals ate more food than Day 4 subjects. Groups having different letters are significantly different at the $p<0.001$ level.

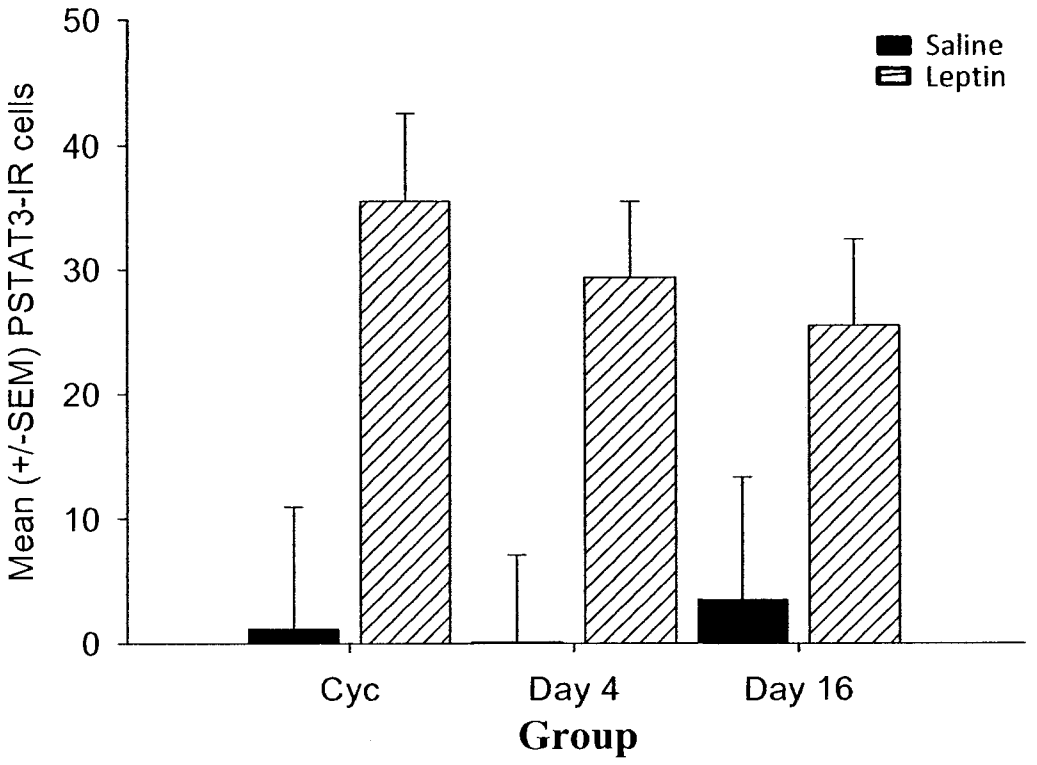

Figure 2. Mean (+/-SEM) number of PSTAT3 + cells in the VMH. Rats that received leptin had an increased number of PSTAT3 + cells $p<0.05$ level.

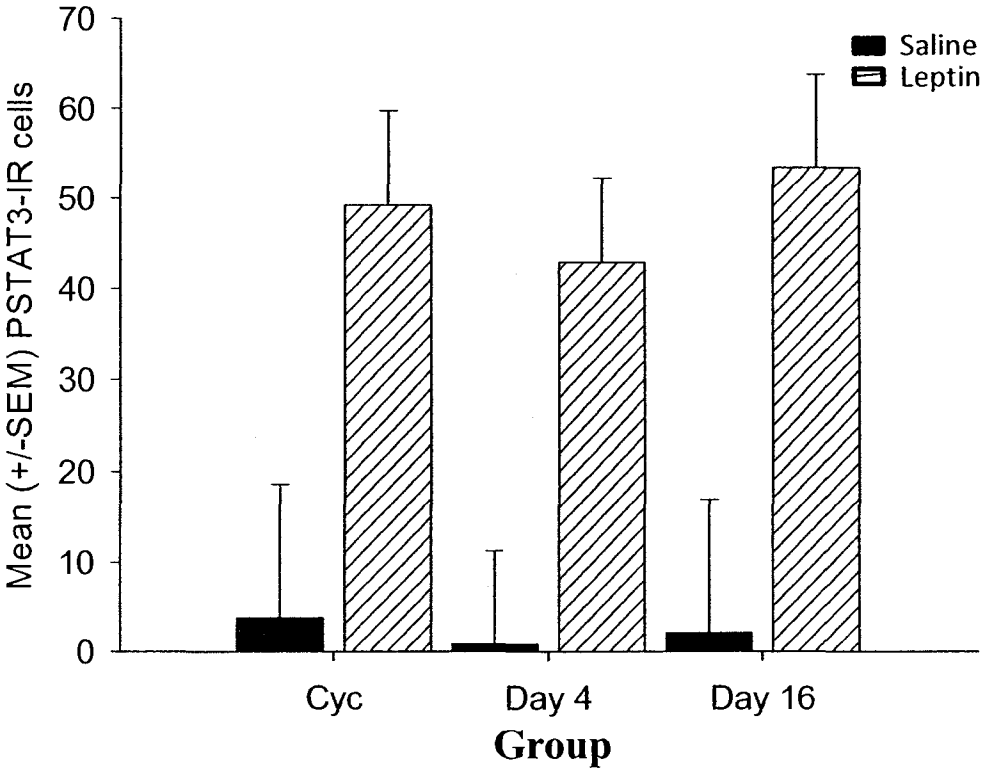

Figure 3. Mean (+/-SEM) number of PSTAT3 + cells in the ARC. Animals that received leptin had an increased number of PSTAT3 + cells $p<0.05$ level.

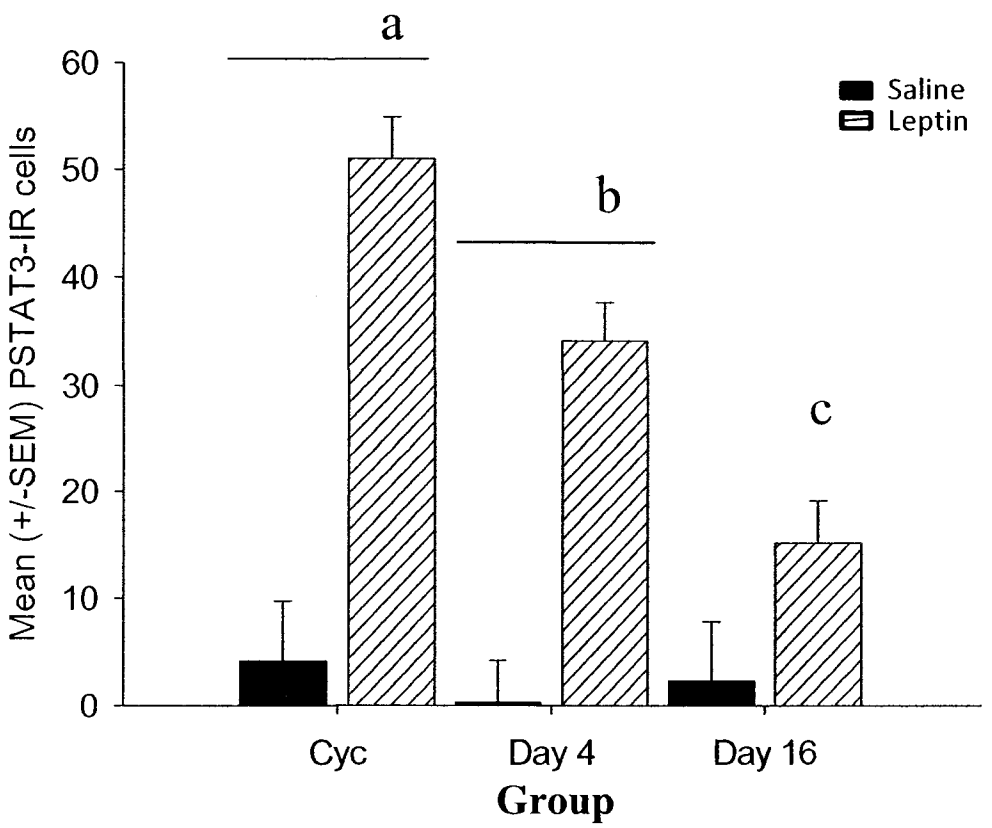

Figure 4. Mean (+/-SEM) number of PSTAT3 + cells in the PVN. Rats that received leptin have an increased number of PSTAT3 + cells $p<0.05$ level. Among leptin treated groups, cycling rats had a higher number of PSTAT3 + cells than both Day 4 and Day 16 rats and Day 4 animals had higher PSTAT3 + cells than Day 16 animals $p<0.05$ level. Groups having different letters are significantly different at the $p<0.05$ level.

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