Changes in NPY Staining in the Arcuate Nucleus During and After Food Restriction in Lactating Rats

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ABSTRACT

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Alfonso Abizaid

This study investigated the effects of food restriction on Neuropeptide Y (NPY) staining in the arcuate nucleus of lactating rats to evaluate a role for NPY in the maintenance of lactational diestrus in food restricted rats. NPY staining was compared on days 15, 20 and 25 of lactation between animals fed ad libitum (AL) and animals food restricted on days 8-14 postpartum (FR). To determine the contribution of nursing underfed pups to any differences observed, litters were switched daily between an additional AL and FR group from day 15 postpartum and dams were sacrificed on day 20 postpartum. Twenty four hrs. prior to sacrifice animals received 1µg of colchicine icv. Animals were perfused and their brains were processed for NPY immunocytochemistry. FR dams had a higher number of NPY stained cells in the arcuate nucleus on day 15 of lactation than AL dams, and this effect persisted for five days after refeeding ($p < .05$). Increases in NPY staining in food restricted rats were unrelated to the nutritional status of their litters. Finally, data revealed that dams that remained in lactational diestrus on day 25, tended to have more NPY-stained cells in the arcuate nucleus than dams that had shown an estrous smear ($p = .08$). These results indicate that food restriction affects NPY immunoreactivity in the arcuate nucleus and the time course of these changes suggest that NPY may play a role in prolonging lactational diestrus.

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The environment has a profound influence on the reproductive outcome of all organisms. Trivers (1972) has proposed that the parental effort of animals varies as a function of environmental conditions. Good environments accelerate the rate of reproduction whereas poor environments delay new reproductive efforts and thus, decrease reproductive rate.

Important environmental factors affecting reproduction in mammals include life expectancy, food availability, temperature, humidity, density of population, photoperiod and predatory pressure (Bronson, 1989). Of these, Bronson (1989) suggests that food availability has the greatest effect on reproduction. Indeed, the common belief that food availability affects reproduction in humans is illustrated by the mythical association of fertility of the land with reproductive fertility made by many ancient cultures. The notion that food availability does influence reproduction is evident in populations of wild mammals whose reproductive seasons coincide with the time of the year in which food is abundant and are depressed or inhibited during the seasons in which food sources are scarce (Bronson, 1989). In human populations, statistics show that the age of menarche is significantly higher in developing countries than in first world countries, possibly as a result of poorer nutritional standards (Eveleth and Tanner, 1976). Further, famine conditions such as the Dutch famine at the end of World War II are followed by a significant drop in the rate of fertility (Stein, Susser, Saenger and Marolla, 1975). In the clinical setting, anorexic and non-anorexic women that undertake severe dietary regimens undergo a cessation of their menstrual cycle (Falk, Halmi, Eckert and Casper, 1983; Frisch, 1982).

Energy shortages are not only imposed on the organism by reducing the amount of food available in the environment. Increases in energy expenditure are also associated with infertility in female mammals. For example, female athletes often show disrupted menstrual cycles during periods of intense exercise (Ronkainen, Pakarinen and Kauppila, 1984).

A prime example of a period having high energy demands is lactation. During the first six months of lactation, women experience an increase in metabolic demands in response to milk production (Whitehead, 1988). Lactation is also associated with a period of anovulation that can vary in length according to the nutritional status of the mother (Frisch, 1982). Thus, if the energetic demands incurred during lactation are enhanced, the period of lactational anovulation is prolonged (Delgado, Martorell and Klein, 1982).

The mechanisms through which energy availability extends the period of lactational anovulation have not yet been determined. It has been suggested that lactational anovulation is mostly dependent on suckling stimulation; the effects of a restricted diet on the length of lactational anovulation could be mediated primarily by changes in suckling intensity from hungry infants (Bongaarts, 1980). On the other hand, Frisch has suggested that the length of lactational anovulation also changes in response to direct effects of nutritional deficiencies on the mother (Frisch, 1982). The solution to this problem would be of important scientific, clinical, economic and political consequences, but answers remain elusive. The study of dietary influences on lactational anovulation in humans is difficult because there are behavioral factors such as differences in physical activity and cultural background that cloud the issue (Bongaarts, 1980; Frisch, 1982). Thus, animal

models have been used to assess the physiological mechanisms underlying nutritional influences on the length of lactational anovulation.

In the rat, as in most rodents, the period of lactational anovulation is referred to as lactational diestrus (Lamming, 1968). Rats nursing eight pups in laboratory conditions have a lactational diestrus period that lasts an average of 18-20 days. This period is prolonged if the energetic demands on the dam are increased by increasing the number of pups in her litter, and is shortened if the energy demands on the dam are reduced by decreasing the number of pups in her litter (Rothchild, 1960). Food availability also has a powerful effect on the reproductive effort of lactating rats. When female rats are allowed only 60% of their normal food consumption during the first two weeks of lactation, the length of lactational diestrus is prolonged by about a week (Woodside, 1991a). The same food restriction regimen given only during the second week of lactation yields similar results, whereas food restriction during the first week of lactation has a somewhat smaller effect (Woodside and Popeski, 1996).

Possible mechanisms through which food restriction prolongs the length of lactational diestrus are described below. The first section describes the contribution of changes in afferent stimulation caused by food restriction, particularly the contribution of altered mother/litter interaction to prolong the length of lactational diestrus. The second section describes those aspects of the reproductive axis that are changed by food restriction during lactation. The third section reviews the different neural and neuroendocrine pathways that could mediate the effect of food restriction on the length of lactational diestrus. Of these pathways, the present thesis explored the possible role that

neuropeptide Y (NPY) may play in prolonging the period of lactational diestrus in rats after food restriction.

Changes in afferent stimulation associated with food restriction.

Clearly the presence of suckling young is necessary for lactational anovulation to occur, although milk delivery is not (Woodside, 1991a). If litters are removed immediately after birth ovulation is not suppressed (Rothchild, 1960), and when litters are removed on day 9 postpartum, lactating rats typically show vaginal estrus by day 12 postpartum indicating that ovulation has occurred (Hansen, Södersten and Eneroth, 1983). It has been argued that the effects of malnutrition on the length of lactational anovulation are mediated by changes in the behavior of the suckling young, or by changes in mother-young interaction (Bongaarts, 1980; Delgado et al., 1982). In addition, the frequency of nursing episodes has been correlated with lactational infertility (McNeilly, Glasier, Howie, Houston, Cook and Boyle, 1983)

Food restriction during the first two weeks of lactation is known to change the behavioral patterns of lactating rats. When dams are food restricted, they spend more time in their nest than the ad lib fed dams, especially during the dark phase of the light/dark daily cycle (Woodside and Jans, 1995). This food restriction regimen also results in litter undernutrition, and retarded development (Woodside, 1991b; Woodside and Jans, 1995). There is some evidence from the rat that increased suckling induced by hungry pups can prolong the lactational diestrus period (Jakubowski and Terkel, 1986). Therefore, it is possible that the prolonged lactational diestrus of

hungry litters.

Woodside and Jans (1995) carried out a series of studies to determine whether nursing underfed pups prolonged the length of lactational diestrus in food restricted rats. In their study, they measured the length of lactational diestrus of dams that were either food restricted or given free access to food during the first two weeks of lactation; at the end of the food restriction period half of the food restricted dams had their litters exchanged with those previously nursed by ad lib fed dams matched for day of parturition, while the rest of the dams raised their own litters throughout the rest of the experiment. Consistent with Jakubowski and Terkel results (1986), ad lib fed lactating rats that were given a litter raised by food restricted dams had a longer period of lactational diestrus. In contrast, food restricted dams given pups raised by ad lib fed rats failed to show a decrease in their length of lactational diestrus. These results suggested that the nutritional state of the litters could affect the length of lactational diestrus, but it is not required for prolonging the period of lactational diestrus seen in food restricted dams.

It then seemed possible that the length of lactational diestrus was affected by changes that occur during the food restriction regimen. As reported by Wiener, Fitzpatrick, Levin, Smotherman and Levine (1978), early in lactation dams tend to spend more time nursing underfed litters than well nourished ones. Therefore it was possible that during this time food restricted dams obtained more suckling stimulation from their malnourished pups, and this change in suckling stimulation mediated the effect of food restriction on the length of lactational diestrus. Woodside and Jans (1995) reduced the time food restricted dams spent with their litters by increasing the nest

temperature so it would be similar to the time ad lib fed dams spend with their litters (Leon, Croskery and Smith, 1978). Results showed that increasing the temperature of the nest or the temperature of the room did not reverse the effect of food restriction on the length of lactational diestrus. Therefore, changes in mother litter interaction during the food restriction period were not necessary to prolong the length of lactational diestrus.

To summarise, changes in the interaction between dams and their litters may prolong the lactational diestrus of ad lib fed dams. In food restricted dams, however, the extended period of lactational diestrus seen after food restriction reflects a physiological effect on the dam herself, rather than an effect that is mediated solely through changes in the suckling young or in maternal behavior.

Effects of food restriction on the reproductive axis.

Ovulation is the result of a series of events occurring at several levels within the hypothalamic-pituitary-gonadal axis. In the rat, gonadotropin releasing hormone (GnRH) secreting neurons release pulses of GnRH into the hypophyseal portal system. At the pituitary, GnRH stimulates the pulsatile release of luteinizing hormone (LH) and follicular stimulating hormone (FSH) into the blood stream (Schally, Arimura, Kastin, Matsuo, Baba, Redding, Nair, Debeljuk and White, 1971). Both these hormones act at the ovary to promote follicular development, and as the follicle develops, increasing amounts of estrogen are secreted into the blood stream (Bronson,1988). Elevated levels of circulating estrogen activate a positive feedback loop leading to a rise in the frequency of GnRH pulses and a surge of

LH release (Freeman, 1988). The surge in LH levels causes the follicles to rupture and to release the mature ova (Feder, 1981). It is this process that is disrupted during lactation and the resumption of which is delayed by food restriction.

During lactation, suckling stimulation from the young maintains a hormonal profile characterised by low levels of circulating estrogen (Smith and Neill, 1977) and the absence of pulsatile LH release (Fox and Smith, 1984), although normal levels of FSH are maintained after the first few days of lactation (McNeilly, 1988; Smith, 1978). Smith and colleagues have determined that pituitary GnRH receptor levels are low in lactating rats (Smith 1984), but that exogenous administration of GnRH to lactating animals leads to a surge of LH release (Fox and Smith, 1984). Thus, it seems that the primary factor leading to the suppression of ovulation during lactation is a lack of GnRH release.

Although some aspects of endocrine function are decreased during lactation, others are increased. Prolactin (PRL) levels rise during lactation and act on the ovary to stimulate progesterone release from the corpora lutea (Tomogane, Ôta and Yokohama, 1969). Circulating progesterone levels rise early in lactation peaking at around day 12 postpartum and decline thereafter (Grota and Eik-Nes, 1967). Lactation is also accompanied by increased circulating corticotropin releasing hormone (CRF) and adrenocorticotropin hormone (ACTH) that is dependent on the suckling stimulus (Riskind,Millard, and Martin, 1984; Walker, Lightman, Steele and Dallman, 1992).

Food restriction prolongs the length of lactational diestrus by altering

the hormonal environment that is characteristic of lactation. Walker, Mitchell and Woodside (1995) compared circulating levels of LH in food restricted and ad lib fed dams before and after a bolus injection of GnRH. Results suggested that food restriction prolonged the period of lactational diestrus of rats by suppressing LH levels. Both ad lib fed dams and food restricted dams showed low levels of LH release on day 4 of lactation, but food restricted rats showed lower circulating levels of LH by day 15 and day 20 of lactation. This result was particularly interesting because it demonstrated a long lasting suppressive effect on LH release that persisted beyond the period of food restriction. Furthermore, both food restricted and ad lib fed rats showed an increase in LH release in response to exogenous GnRH challenge demonstrating that the suppression of LH was not mediated by a reduction in pituitary responsiveness to GnRH.

Taken together these results suggest that food restriction during lactation prolongs the period during which hypothalamic GnRH release is suppressed even after food restricted dams are refed. The next section addresses the mechanisms through which food restriction may modulate GnRH release in the lactating animal, and introduces NPY as a modulator of these mechanisms.

Role of NPY in suppressing GnRH in food restricted dams

GnRH release is known to be suppressed by hormones that are abundant during lactation such as PRL, progesterone, and ACTH (Smith, 1978; De Greef and Van Der Schoot, 1983; Walker et al, 1992). At first it was suspected that the food restriction effect on lactational diestrus was mediated

by changes in the levels of these hormones. The present section reviews evidence suggesting that neither PRL, progesterone, nor ACTH are directly involved in the prolonged suppression of LH and GnRH that is seen in food restricted dams. The section then presents the hypothesis that NPY is a likely candidate in directly mediating the food restriction effect on the reproductive axis.

In ad lib fed lactating females, PRL and/or progesterone play an important role in suppressing GnRH release. Both suckling stimulation and elevated levels of PRL are thought to work synergistically to suppress GnRH during lactation (Smith and Neill, 1977). In addition, given that PRL maintains progesterone release from the corpora lutea, changes in PRL result in changes in progesterone secretion (Tomogane, Ôta and Yokoyama, 1975). Although PRL levels are not higher in food restricted dams than in ad lib fed dams (Walker et al., 1995), progesterone levels are. Woodside (1991b) reported that progesterone levels of food restricted animals were increased compared to progesterone levels of ad lib fed rats until at least day 20 of lactation. Progesterone levels remain elevated after refeeding suggesting that increased progesterone could play an important role in mediating the effect of food restriction on GnRH (Walker et al., 1995). However, decreasing progesterone levels by removing the ovaries does not increase LH release from the pituitary of food restricted lactating rats (Walker et al., 1995).

The hypothalamic-pituitary-adrenal (HPA) axis has also been implicated in the suppression of LH release, but the role that this system plays in mediating the food restriction effect on the length of lactational diestrus remains unclear. It is known that stress-induced CRF release activates the

synthesis and release of ACTH from the pituitary (Rivier, Rivier and Vale, 1986) and central administration of CRF suppresses LH levels in cycling female rats via a suppression of GnRH (Rivier and Vale, 1985). Moreover, the CRF-induced suppression of LH in cycling female rats may be mediated through the opioidergic system because μ-opioid receptor antagonists reverse the effect of CRF on circulating LH levels (Petraglia, Vale and Rivier, 1986). Further, food restriction elevates the levels of circulating corticosterone levels in cycling female rats (Honma, Honma and Hiroshige, 1984). Although ACTH levels are elevated during lactation (Walker et al., 1992), ACTH levels of food restricted dams are not different from those of ad lib fed dams (Walker et al., 1995). Furthermore, the food restriction effect on the length of lactational diestrus remains present after treatment with the opioid receptor antagonist naltrexone (Woodside, unpublished data). Therefore, it seems that the HPA axis plays a minor role, if any, in the suppression of LH seen after food restricting lactating rats.

NPY may mediate the effects of food restriction on the length of lactational diestrus because of its anatomical distribution, and its effects on metabolic and reproductive function. NPY, a 36 amino-acid peptide belonging to the pancreatic polypeptide family, was discovered in 1982 (Tatemoto, Carlquist, and Mutt, 1982). Immunocytochemical studies have determined that NPY is widely distributed in the brain of a large variety of animals (see Danger, Tonon, Jenks, Saint-Pierre, Martel, Fasolo, Brenton, Quirion, Pelletier and Vaudry, 1990 for a review). These studies have found that NPY is particularly abundant in the hypothalamus, with a large concentration of NPY cell bodies in the arcuate nucleus and a dense network of fibres in the

PVN, as well as in brain stem regions that receive metabolic information from the blood steam and the liver (Allen, Adrian, Allen, Tatemoto, Crow, Bloom and Polak, 1983).

NPY has dramatic effects on food intake. When NPY is injected in the ventricles or the PVN of satiated rats, it causes a robust dose-dependent increase in food intake (Clark, Kalra, Crowley and Kalra, 1984; Stanley, Chin and Leibowitz, 1985). Animals given intracerebroventricular (icv) injections of NPY do not show tolerance to its orexigenic effects and they become obese unless injections are stopped (Stanley, Kyrkouli, Lampert and Leibowitz, 1986). In addition, NPY levels are elevated in the PVN and in the arcuate nucleus in response to food deprivation, and remain elevated in the arcuate nucleus for up to one day after refeeding (Sahu, Kalra and Kalra, 1988).

NPY may not simply increase feeding but it may also act as a general energy balance modulator. NPY icv injections elevate plasma concentrations of insulin (Moltz and McDonald, 1985), inhibit plasma levels of growth hormone (McDonald et al., 1985), decrease body temperature (Jolicoeur, Michaud, Rivest, Ménard, Gaudin, Fournier and Saint-Pierre, 1991) and increase white fat deposition (Billington, Briggs, Harker, Grace and Levine, 1994). Rats that are trained to exercise to get food show elevated levels of hypothalamic NPY that are similar to those seen after food restriction (Lewis, Shellard, Koeslag, Boer, McCarthy, McKibbin, Russell and Williams, 1993). Thus NPY appears to be elevated in situations of negative energy balance.

Unless the steroid environment is conducive for an LH surge, NPY has a suppressive effect on LH release. NPY icv injections have a suppressing effect on LH levels in cycling female rats and ovariectomized rats, unless NPY

is administered on the morning of proestrus or after ovariectomized rats are treated with estradiol to mimic proestrus levels (Kalra and Crowley, 1984; McDonald, Lumpkin, Samson and McCann, 1985). Further, chronic central administration of NPY via osmotic minipumps suppresses cyclicity and reduces pituitary weight, number of pituitary GnRH receptors and the ovarian weight of cycling female rats (Catzeflis, Pierroz, Rohner-Jeanrenaud, Rivier, Sizonenko and Aubert, 1993). Chronic infusions of NPY via osmotic minipumps also delay the onset of the first estrous cycle in prepubertal rats (Pierroz, Gruaz, d'Alleves and Aubert, 1995).

The arcuate nucleus contains the highest density of NPY cell bodies in the hypothalamus (Danger et al., 1990). The arcuate nucleus receives afferent input from areas known to convey metabolic information such as the nucleus of the solitary tract in the brain stem, and the ventromedial hypothalamus (Zaborski, 1982), and projects fibers to areas that control energy homeostasis and reproduction such as the PVN, median eminence and MPOA (McDonald, 1993; Billington et al., 1994; Guy, Li and Pellertier, 1988). Lesion and retrograde labelling techniques have shown that the NPY cell bodies in the arcuate nucleus project fibres to numerous sites within and outside the hypothalamus. Within the hypothalamus, the main target for these fibres is the PVN. When the arcuate nucleus is lesioned, NPY fiber staining in the PVN is significantly diminished (Kerkérian and Pelletier, 1986). Furthermore, arcuate nucleus NPY cells project fibres to the MPOA, where NPY immunostained fibers are in direct contact with GnRH neurons (Tsuruo, Kawano, Kagotani, Hisano, Daikoku, Chihara, Zhang and Yanaihara, 1990). NPY cell bodies in the arcuate nucleus also send projections

to the median eminence, an area rich in luteinizing hormone-releasing hormone (LHRH) terminals (Danger et al, 1990) . Moreover, NPY cell bodies in the arcuate nucleus may play a primary role in modulating the effects of NPY on food intake and reproductive function because neural transection of the brain stem afferent inputs to the arcuate nucleus has little impact on feeding behavior and gonadotropin secretion (Sahu, Kalra, Crowley and Kalra, 1989). These studies provide anatomical evidence for a role of NPY neurons in the arcuate nucleus in modulating reproductive function and metabolic control.

During lactation, several hypothalamic nuclei show elevated levels of NPY. Among them, the PVN, the arcuate nucleus, the ventromedial nucleus and the dorsomedial nucleus showed the greatest differences on day 15 of lactation compared with non-lactating controls (Malabu, Kilpatrick, Ware, Vernon and Williams, 1994). Smith (1993) compared NPY and proopiomelanocortin gene expression in the median eminence and in different portions of the arcuate nucleus of cycling rats on the day of diestrus and of lactating rats on day 10 postpartum. Results showed that whereas proopiomelanocortin gene expression decreased during lactation, NPY expression was elevated in the arcuate nucleus but only in its medio-dorsal part. Smith (1993) also found that arcuate nucleus NPY content was elevated in lactating rats and suggested that these changes in arcuate nucleus NPY gene expression and content could be associated with both the increased appetite and the suppression of hypothalamic GnRH release seen during lactation. In addition, Ciofi and associates (1991) found a group of cells that expressed NPY and its carboxyterminal precursor (prepro NPY)-associated

peptide (CPON) in the external median eminence of rats during lactation but not during the estrus cycle, pregnancy nor after pup removal. Therefore, NPY content in the arcuate nucleus and the median eminence is possibly increased as a response to the energetic demands imposed by lactation.

In summary, NPY is abundant in hypothalamic centers such as the arcuate nucleus, MPOA and the PVN, that regulate homeostatic responses. NPY synthesis and release at these sites are activated in situations in which energetic homeostasis is challenged as when rats are food deprived or during lactation. NPY also serves as a signal that interacts with circulating steroid levels to regulate the reproductive axis. When NPY levels are chronically elevated, they trigger mechanisms that suppress the reproductive axis. NPY would seem, therefore, to be a prime candidate to serve as a mediator between energetic state and reproductive function.

Given that NPY modulates reproductive function and responses to energetic challenges, NPY could directly mediate the GnRH suppression seen in food restricted dams. High levels of hypothalamic NPY seen during lactation may be elevated further by food restriction. If the increased NPY levels were shown to persist beyond the period of refeeding it is possible that they could cause the prolonged suppression of GnRH and LH release.

The present study investigated the effects of food restriction on NPY staining in the arcuate nucleus of lactating rats using immunocytochemical methods. NPY had to fulfill four requirements before being considered a good candidate in mediating the effect of food restriction on the length of lactational diestrus. First, it was expected that there would be more cells stained for NPY in food restricted animals after food restriction (Day 15

postpartum) than in ad lib animals. Second, it was expected that the NPY staining in the arcuate nucleus of food restricted females would remain elevated after refeeding when LH levels are still suppressed (day 20 postpartum). Third, NPY staining in the arcuate nucleus of food restricted dams was expected to decrease towards the expected termination of lactational diestrus (day 25 postpartum). Finally, given that the effect of food restriction on the length of lactational diestrus is not mediated by changes in the mother/litter interaction, no changes on the intensity of staining for NPY were expected in food restricted rats that received pups previously nursed by ad lib fed rats compared to food restricted rats that nursed their own litter.

METHOD

Subjects

Forty-two virgin female Wistar rats obtained from Charles River Breeding Farms (St Constant, Québec) weighing between 220-250 grams were subjects in this experiment. Animals were mated by housing them with a male (four females and one male per cage) in stainless steel cages (50 cm x 20 cm x 15 cm). In late pregnancy, rats were housed individually in plastic maternity cages (45 cm x 25 cm x 20 cm) in which they remained throughout the rest of the experiment. Animals were maintained on a 12 hr light/dark cycle (lights on at 08:00 hr, lights off at 20:00 hr) at a room temperature of 20 ± 2° C. On day 1 postpartum (day 0 = day of parturition), all litters were culled to eight pups.

Surgery

On day 2 postpartum, rats were anaesthetized with a ketamine-xylazine mixture (5.7 mg ketamine and .86 mg xylazine/100 g of body weight) and implanted with a stainless steel guide-cannula (22 gauge; Plastic One Products) into the right lateral ventricle (AP: .2 mm: L: 1.6 mm; DV: 4 mm below dura; Pellegrino, Pellegrino and Cushman, 1979). Cannulae were held in place with dental acrylic moulded around four stainless steel screws driven into the skull. All guide-cannulae were protected with a cannula obturator

(Plastic One Products) extending 1 mm beyond the tip of the guide-cannulae. To test the accuracy of cannulae placements animals were given a 2 µl icv injection of Angiotensin II (25 µg/ml; Sigma) on day 7 postpartum. If animals showed immediate drinking behavior, cannulae placements were accepted as accurate (Hulsey and Martin, 1992). Animals that failed to drink were removed from the experiment.

Procedure

Animals were assigned to one of two diet regimens (ad libitum (AL) vs. food restricted (FR)) and subdivided into groups according to the day of lactation on which they were sacrificed (day 15, day 20 or day 25). The groups were as follows: 1) Ad lib day 15 (AL15; n=5); 2) Ad lib day 20 (AL20; n=7); 3) Ad lib day 25 (AL25; n=5); 4) Food restricted day 15 (FR15; n=5); 5) Food restricted day 20 (FR20; n=4) ; and 6) Food restricted day 25 (FR25; n=5). Starting on day 8 postpartum, food restricted animals received 50% of a previously ascertained ad lib food ration until day 15 postpartum. Ad lib fed animals had free access to food throughout the experiment, and all animals had ad lib access to water throughout the experiment. Food consumption was recorded daily from day 1 to day 15 postpartum, and dam and pup weights were monitored daily throughout the experiment. Daily vaginal smears were obtained from day 4 postpartum until animals were sacrificed to monitor vaginal cyclicity. Two additional groups, a food restricted (FRSW; n=6) and an ad lib fed (ALSW; n=5) group, were matched for day of parturition and had

their litters switched daily from day 15 until day 20 postpartum, when they were sacrificed.

Immunocytochemistry

To visualise NPY stained cell bodies in the arcuate nucleus, all animals were given a 2 µl icv infusion of colchicine (1 mg/ml; Sigma) 24 hours before being sacrificed. Animals were given an overdose of sodium pentobarbitol and then perfused transcardially with 300 ml of cold isotonic saline (.09%; Fisher) and 500 ml of cold paraformaldehyde (4% paraformaldehyde in .1 M PBS, ph 7.3-7.4; Sigma). Brains were post-fixed in 4% paraformaldehyde overnight at 4°C. The next day, brains were sectioned on a sliding vibratome. Forty micron sections were taken throughout the rostro-caudal extent of the arcuate nucleus and every second section was retained for staining. Sections were transferred to wells and washed in Tris buffer 3 times in 15 min. (.1 M tris in .9% saline, ph 7.3-7.5; Sigma). The tissue was then incubated for 1 hour in blocking serum (.25% Triton X (TTX, Sigma), 3% normal goat serum (NGS, Vector), and Tris buffered saline (TBS, Sigma) mixed with powder milk (1 gm in 20 ml of TBS)) and then incubated in the primary antibody solution (aNPY, Peninsula Laboratories diluted 1:10 000 in .25% TTX, 1% NGS and TBS and powder milk) at 4°C overnight. The addition of powder milk to the blocking and the primary antibody solutions was recommended to reduce non-specific background staining. The 50% ethanol and the 3% hydrogen peroxide were used to block peroxidase activity that may produce excessive background

staining (Hsu, 1993).

The next morning, sections were washed in TBS (3 times in 15 min.) and then washed in 50% methanol (Sigma) followed by a 30 min. incubation in 3% hydrogen peroxide solution (Sigma), and another 5 min. wash in 50% methanol . After being washed three times with TBS, sections were incubated in the secondary antibody (biotinylated rabbit anti-goat) for 1 hour followed by another 1 hour incubation in ABC reagent (Vector). Tissue staining was developed using the Vector DAB kit (Diaminobenzene, nickel intensified). The stained sections were then mounted on gelatin coated slides and cover slipped with mounting medium (Permount, Fisher).

Image Analysis

Sections were visualised using a Sony XC77 camera mounted on a light microscope (Labolux Leitz GMBH, model 1.58). Images were captured using the NIH image 1.58 analysis system installed on a Power Macintosh 6100 computer.

The arcuate nucleus was divided into three areas on the basis of the plates from the stereotaxic atlas of Paxinos and Watson (1986): 1)ARC 1 or rostral arcuate nucleus (Plates #26-28; -2.12 to -2.56 mm posterior to Bregma) ; 2) ARC 2 or medial arcuate nucleus (Plates #29-30; -2.80 to -3.14 mm posterior to Bregma); and ARC 3 or medio-dorsal arcuate nucleus (Plates #31-33; -3.30 to -3.60 mm posterior to Bregma: see Appendix A). Only sections that contained one of these three portions of the arcuate nucleus were selected. The sections

were viewed at 100X magnification on the microscope, and captured into the image analysis system. Then, the boundaries of the arcuate nucleus were manually drawn onto the images, and densely stained cells within these boundaries were counted by two raters. The inter-rater correlation was high ($r = .91$, $p < .05$), therefore individual cell counts from the two raters were averaged to obtain a single final cell count for each section. The cell counts for each section were averaged to obtain a mean number of cells stained for NPY within ARC 1, ARC 2, and ARC 3 for each animal.

Data Analysis

The effects of food restriction on NPY staining in the arcuate nucleus of lactating rats were evaluated using a three-way repeated measures analysis of variance (ANOVA) with diet (ad lib fed vs food restricted) and day of lactation (Days 15, 20 and 25) as the between subjects factors, and part of the arcuate nucleus (ARC1, ARC 2 and ARC 3) as the within subject factor.

A second three-way ANOVA with food regimen (ad lib fed vs. food restricted) and type of litter (switched vs not switched) as the between subjects variables and part of the arcuate nucleus (ARC 1, ARC 2, and ARC 3) as the within subjects variable was conducted to determine whether suckling differences affected the number of NPY- stained cells in the ARC on day 20 of lactation.

An independent measures *t*-test was used to determine the differences in NPY staining between dams that had shown vaginal estrus by day 25 of

lactation and those that had not.

Daily recordings of food intake were summarized to obtain an average daily intake for the first week of lactation (days 1-7) and one for the second week of lactation when experimental animals were food restricted (days 8-14). A two tailed *t*-test was used to used to compare food intake between ad lib and food restricted dams in week 1.

A two-way (diet X day of lactation) repeated measures ANOVA was used to analyse the changes in body weight from day 1 of lactation of ad lib and food restricted dams at the beginning (day 8) and at the end of the food restriction regimen (day 14).

A two-way (diet X day of lactation) repeated measures ANOVA was performed to compare the mean overall pup weight gain of litters nursed by ad lib fed dams versus litters nursed by food restricted dams at the beginning (day 8) and at the end of the food restriction regimen (day 14).

RESULTS

Mean number of cells stained for NPY

Figure 1 shows the overall mean number of cells per section stained for NPY in the arcuate nucleus of dams on days 15, 20 and 25 of lactation. Overall, food restricted rats had a higher mean number of NPY-stained cells in the arcuate nucleus ($F_{(1,25)}= 5.81$, $p < .05$; significant main effect for diet). No significant differences in staining were seen across days 15, 20 and 25 of lactation ($F_{(2,25)}.= .64$, $p > .05$; non significant main effect for day of lactation), and the diet X day of lactation interaction was also insignificant ($F_{(2,25)}= .38$, $p > .05$). As shown in Figure 2, there were differences in NPY staining within the arcuate nucleus ($F_{(2,50)}= 18.89$, $p < .05$; significant main effect for area of the arcuate nucleus). Post hoc tests (Tukeys HSD) determined that the ARC 2 and ARC 3 divisions had a significantly larger mean number of cells per section stained for NPY than the ARC 1 division ($p < .05$). No significant diet X area of the arcuate nucleus nor Day of lactation X part of the arcuate nucleus interactions were found (see Table 1 in Appendix B). Figures 3 and 4 show sample pictures of NPY staining in the three portions of the arcuate nucleus of ad lib fed and food restricted dams on days 15 and 20 of lactation respectively.

Figure 5 shows the differences in the number of NPY-stained cells between dams that had shown vaginal estrus and those that remained in lactational diestrus by day 25 of lactation. Animals that remained in lactational diestrus had a higher mean number of NPY-stained cell bodies in

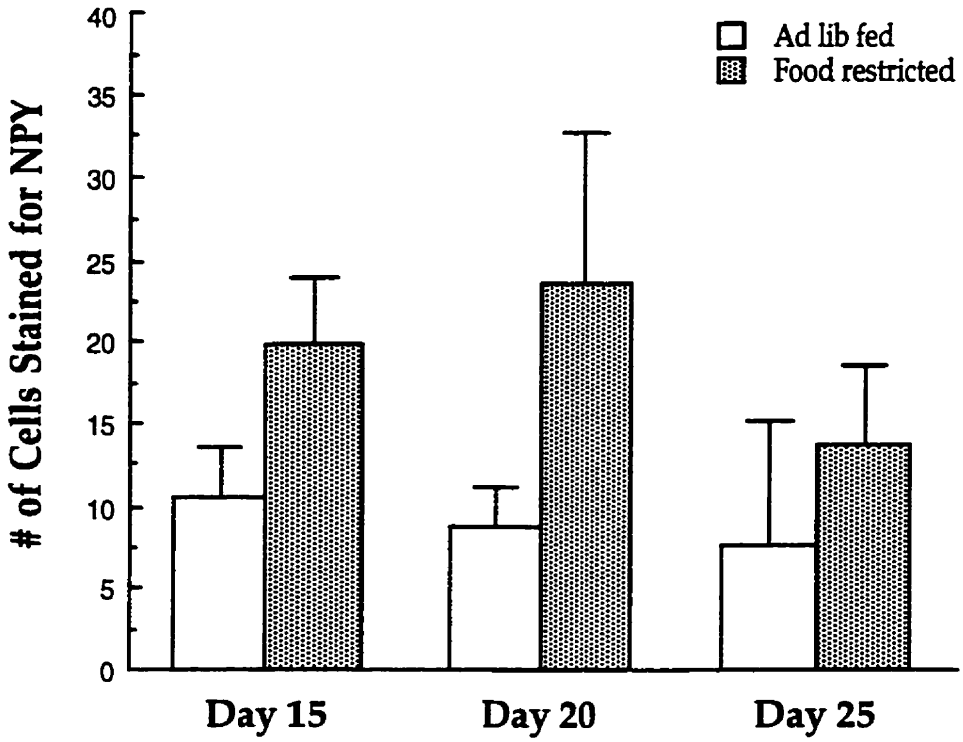

Figure 1: Mean number of cells per section stained for NPY in the arcuate nucleus of food restricted and ad lib fed dams on days 15, 20 and 25 of lactation. Error bars represent standard errors.

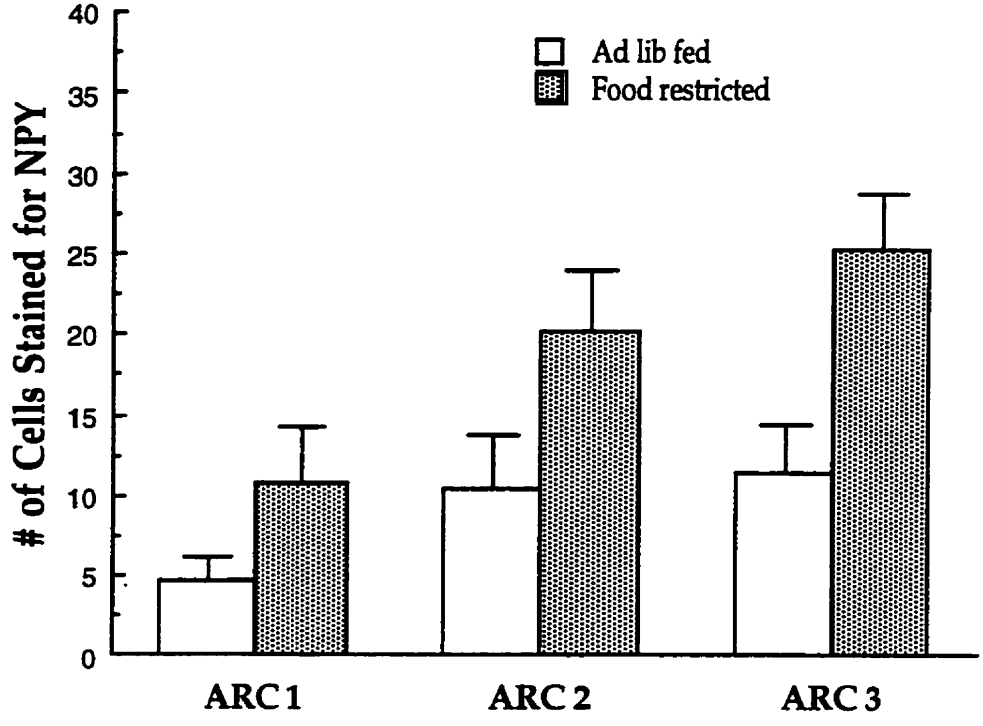

Figure 2: Overall average number of cells per section stained for NPY in different portions of the arcuate nucleus of ad lib and food restricted dams. Error bars represent standard errors.

Figure 3: Sample sections of the ARC 1, ARC 2 and ARC 3 of ad lib (Top) and food restricted (Bottom) dams sacrificed on day 15 of lactation. Arrow heads point to densely stained NPY cell bodies

Figure 4: Sample sections from the ARC 1, ARC 2 and ARC 3 portions of the arcuate nucleus of ad lib (Top) and food restricted (Bottom) dams sacrificed on day 20 of lactation. Arrows point to densely stained NPY neurons.

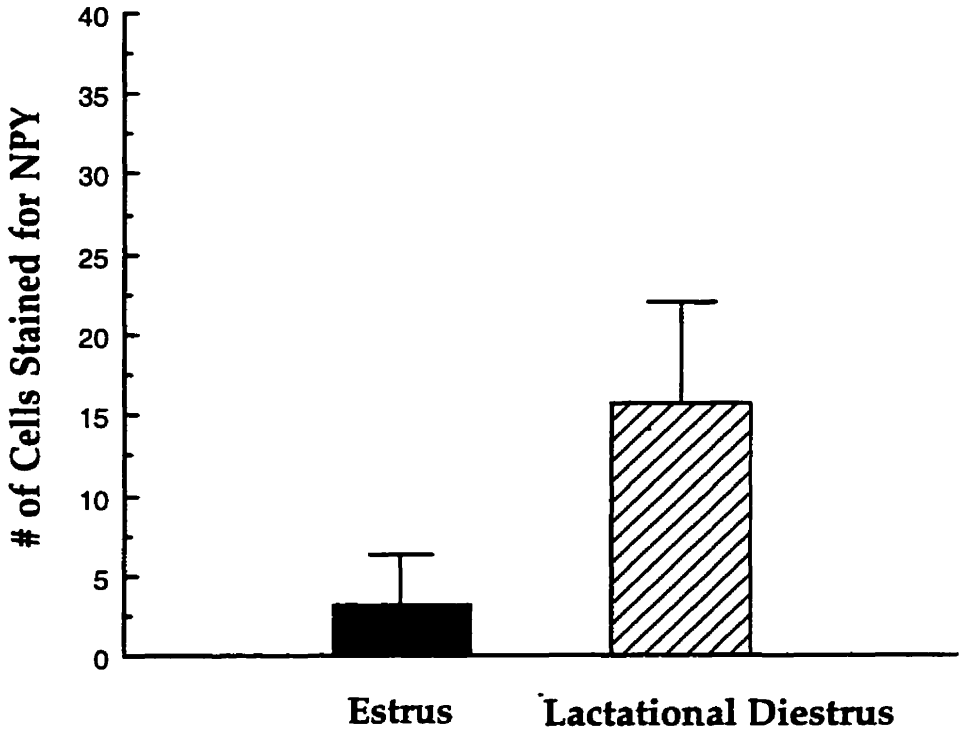

Figure 5: Mean number of cells per section stained for NPY in the arcuate nucleus of dams that had shown vaginal estrus (n=4) versus dams that remained in lactational diestrus (n=6) by day 25 of lactation. Error bars represent the standard errors.

the arcuate nucleus than those that had shown vaginal estrus, although this difference did not attain statistical significance (t (8)= -1.51, p= .08; one tailed test). Figure 6 shows sample pictures of ARC 1, ARC 2 and ARC 3 sections from animals that showed vaginal estrus and those that remained in lactational diestrus by day 25.

Finally, figure 7 shows the mean number of cells per section stained for NPY in the arcuate nucleus of food restricted dams that nursed ad lib pups, and that of ad lib dams that nursed food restricted pups. A significant main effect for diet demonstrated that food restricted animals showed a significantly higher mean number of NPY stained cells across the arcuate nucleus than did ad lib fed animals ($F(1,18)= 5.34, p < .05$). Neither the main effect for litter nor the diet X litter interaction were statistically significant (see Table 2 in Appendix B).

Figure 8 shows a sample photograph of the different portions of the arcuate nucleus stained for NPY of dams that had their litters switched and those who nursed their own. There were significant differences in staining within the arcuate nucleus ($F(2,36)= 11.01, p < .05$), where ARC 2 and ARC 3 had a higher mean number of NPY-stained cells per section than ARC 1 ($p <$ .05; Tukeys HSD). No other effects reached statistical significance (see Table 2 in Appendix B).

Control Measures

Figure 9 shows the mean daily food intake of ad lib and food restricted

Figure 6: Sample sections of the ARC 1, ARC 2 and ARC 3 portions of the arcuate nucleus from animals sacrificed on day 25 of lactation. The top panels show sections obtained from dams that had shown vaginal estrus. The bottom panels are sections obtained from animals that remained in lactational diestrus. Arrows point to cells densely stained for NPY.

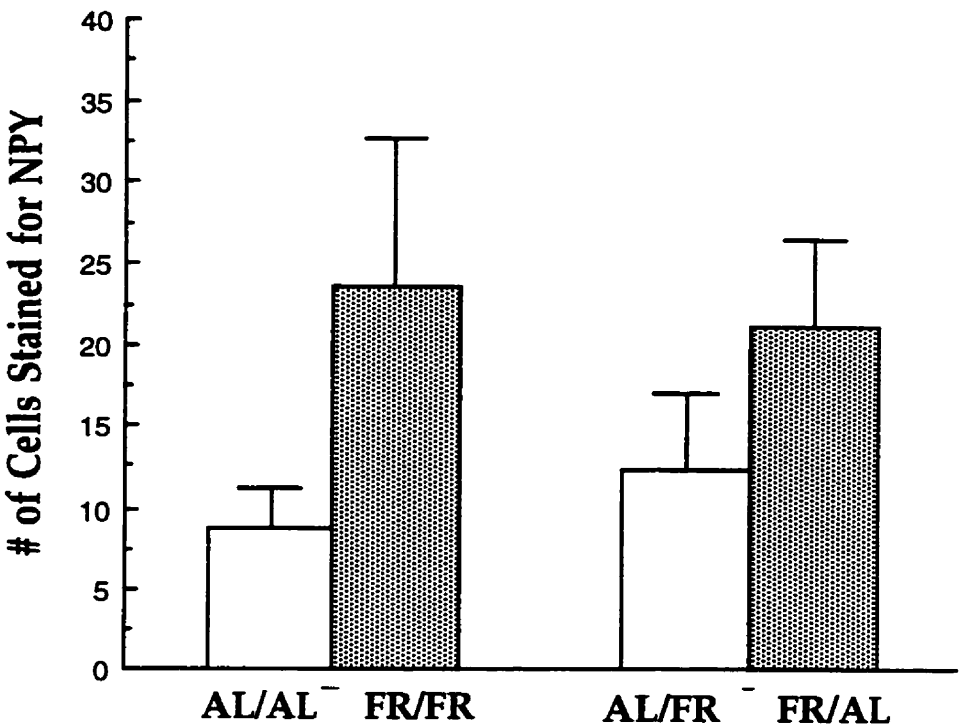

Figure 7: Mean number of cells per section stained for NPY in the arcuate nucleus of ad lib and food restricted dams which nursed their own litters (AL/AL, FR/FR) versus dams whose litters were switched daily starting on day 15 of lactation (AL/FR, FR/AL). All dams were sacrificed on day 20 of lactation. Standard errors are represented by the error bars.

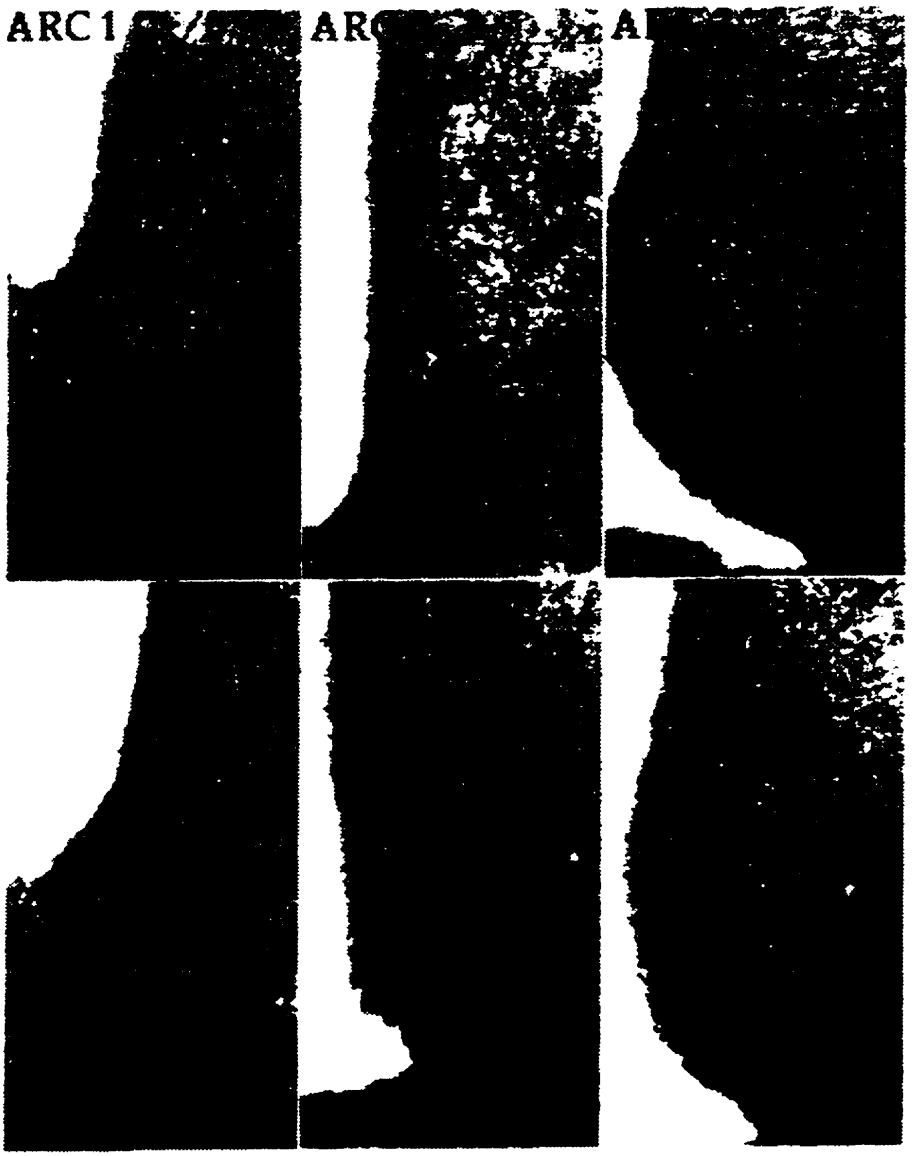

Figure 8: Sample sections from the ARC 1, ARC 2 and ARC 3 of ad lib (top) and food restricted (bottom) dams whose litters were switched daily from day 15 to day 20 of lactation. Arrows point to densely stained NPY cell bodies.

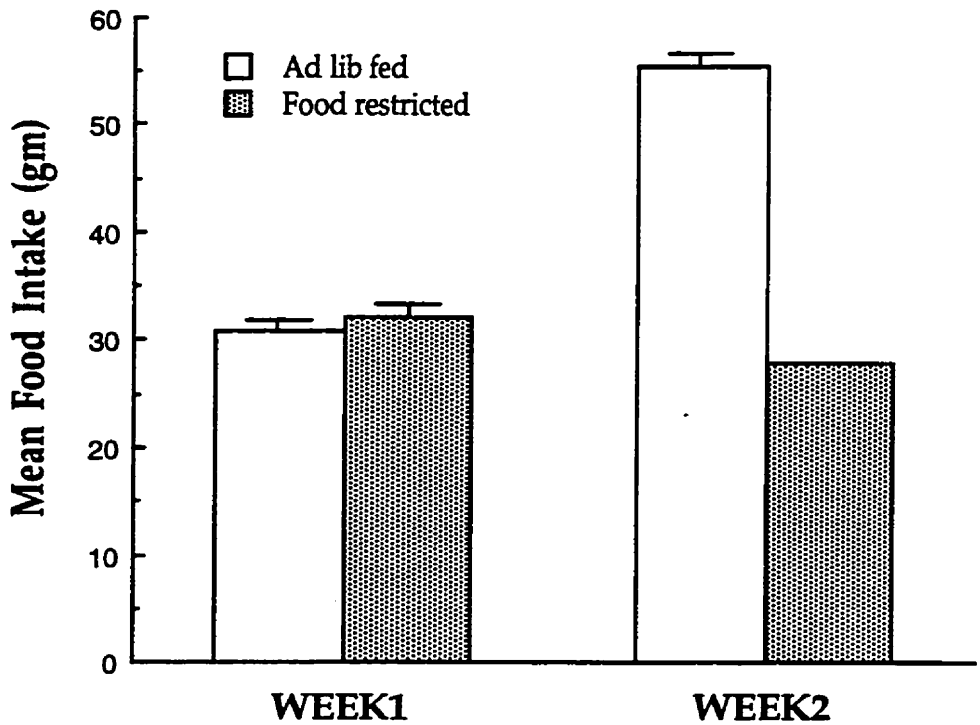

Figure 9: Mean daily food intake before the food restriction regimen was applied (WEEK 1) and at the end of the food restriction regimen (WEEK 2). Error bars represent the standard errors.

dams for the first and second week of lactation. Both groups were eating similar daily amounts during the first week of lactation (t (40)= -.90, p > .05, two-tailed test), but at the end of the second week of lactation food restricted dams had only been allowed to eat 50.23% of the food consumed by the ad lib fed controls.

Figure 10 shows the change in dam weight between day 1 and day 8 of lactation when all animals were fed ad lib and between day 1 and the end of the food restriction regimen (day 14). The ANOVA yielded significant main effects for diet (F (1,40)= 86.15, p < .05) and day of lactation (F(1,40)= 121.48, p < .05), and a significant diet X day of lactation interaction (F(1,40)= 391.60, p < .05). Simple effects analyses indicated that prior to the food restriction regimen all dams showed comparable changes in body weight (F(1,40)= .44, p >.05). Food restricted animals, however, underwent a significant drop in body weight by the end of the food restriction regimen (F(1,40)= 271.29, p <.05).

Figure 11 depicts the mean individual pup weight gain between days 1-8 of lactation and between days 1-14 of lactation. A two-way ANOVA (diet X phase) revealed significant main effects for diet (F(1,40)= 11.35, p < .05) and phase (F(1,40)= 1965.08, p <.05), and a significant diet X phase interaction (F(1,40)= 203.87, p < .05). Simple effect analyses showed that the mean weight gain of pups that were nursed by ad lib fed and food restricted animals was similar before the diet regimen was applied (F(1,40)= 2.95, p >.05); food restriction, however, decreased the mean pup weight gain (F(1,40)= 48.17, p <.05).

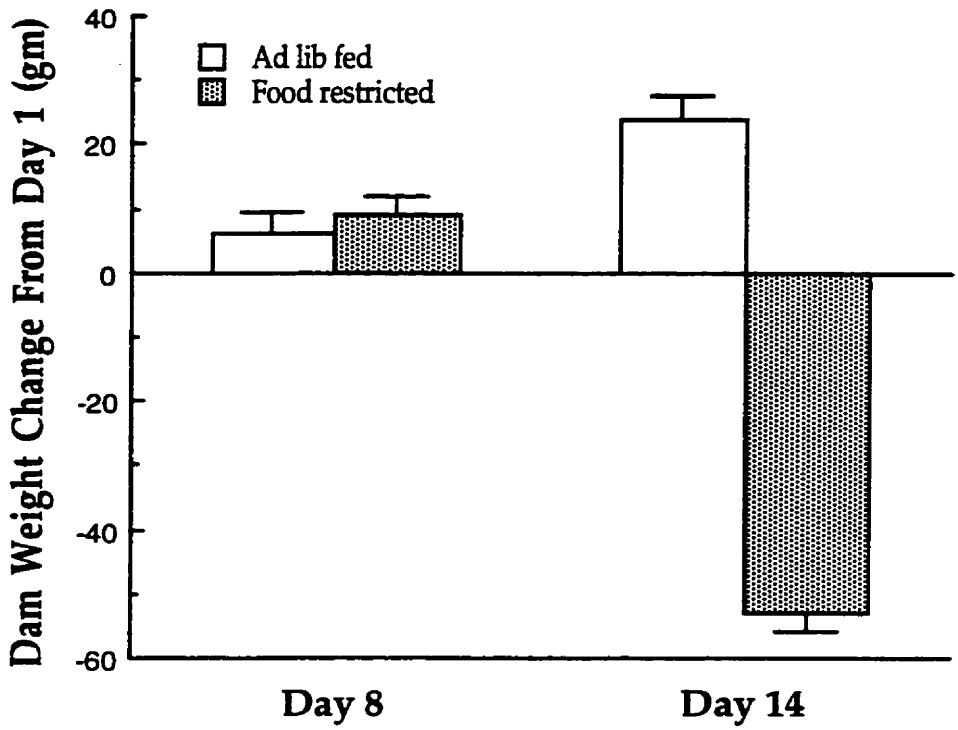

Figure 10.- Mean dam weight change from day 1 postpartum to day 8 postpartum, and from day 8 postpartum to day 14 postpartum in ad lib fed and food restricted dams. Error bars represent standard error.

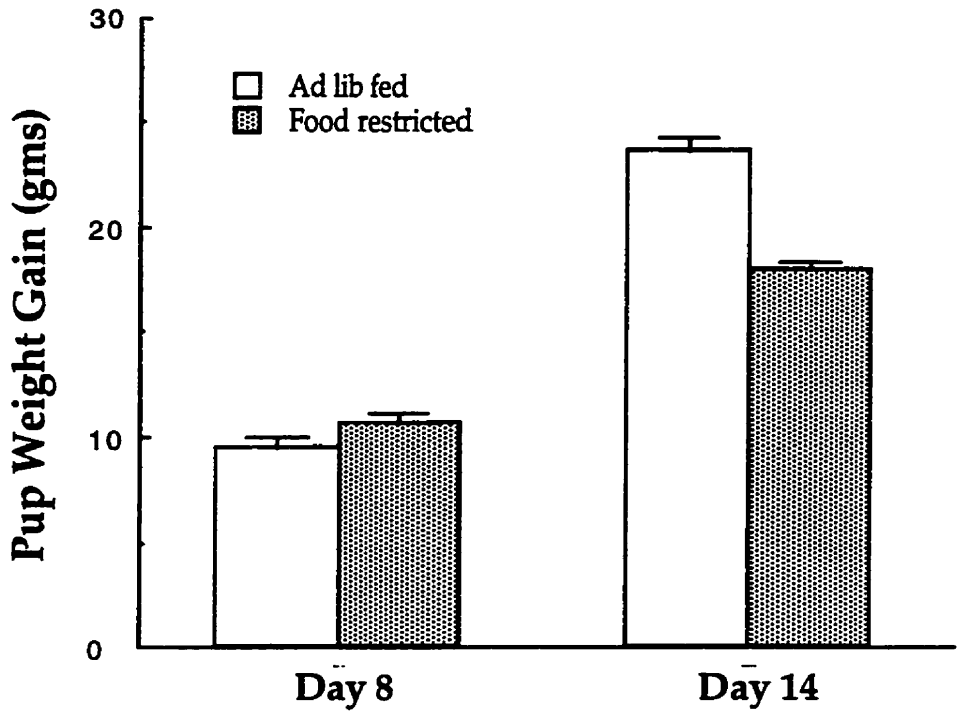

Figure 11: Mean Pup weight gain before the food restriction regimen (Day 8), and by the end of the food restriction regimen (Day 14). Error bars represent standard errors.

DISCUSSION

NPY had to fulfill four requirements to be considered a primary candidate in the mechanism mediating the effects of food restriction on the length of lactational diestrus in rats. First, NPY staining had to be affected by the food restriction regimen. Second, NPY staining had to be elevated at times in which LH release has been reported to be suppressed. Thus, if LH release is suppressed at day 20 of lactation in food restricted rats, then NPY staining should remain high in food restricted dams on day 20 of lactation. Third, NPY staining had to decrease as dams began cycling again. And fourth, NPY staining in food restricted dams had to remain unaffected by changes in suckling stimulation.

Results from this study show that NPY meets these four requirements. Food restriction during the second week of lactation increased the average number of cell bodies stained for NPY in the arcuate nucleus. Food restricted dams showed an overall higher mean number of NPY-stained cells per section than did the ad lib fed animals on day 15 of lactation. This finding was important because it showed that the elevated levels of arcuate nucleus NPY seen in lactating rats (Malabu et al. 1994; Smith, 1993) could be further increased by food restriction. Smith (1993) reported increased NPY gene expression in the medio-caudal portion of the arcuate nucleus. Consistent with this report, the number of cells stained for NPY in the ARC 2 and ARC 3 portions corresponding to the medial and medio-caudal portion of the arcuate nucleus, was greater than in the rostral ARC 1 portion in both food restricted and ad lib fed dams.

The data also revealed that the staining in the arcuate nucleus of food restricted dams remained elevated for at least five days after refeeding. The increased NPY staining seen in food restricted dams on day 15 was still present by day 20, and in some animals by day 25 of lactation. This pattern of increased staining closely correlates with the time during which LH is suppressed in previously food restricted dams (Walker et al., 1995).

The mean number of NPY-stained cells per section varied greatly in both the ad lib fed and food restricted dams on day 25 of lactation. But when these animals were regrouped according to vaginal cytology, there was a trend for dams that had shown vaginal estrus to have a lower number of NPY stained cells per section than those that remained in lactational diestrus by day 25 of lactation. Albeit these differences failed to reach statistical significance, they suggest a relationship between reproductive status and NPY staining in the arcuate nucleus.

The increase in arcuate nucleus NPY staining seen in food restricted rats was not related to the fact that they were suckling hungry pups. Food restricted dams that received pups previously nursed by ad lib fed dams showed the same elevated pattern of NPY staining in the arcuate nucleus as food restricted dams that nursed the same litter throughout the experiment. This was consistent with the idea that the food restriction effect is not primarily the result of suckling hungry pups (Woodside and Jans, 1995). As reported by Woodside and Jans (1995), ad lib fed dams prolonged their period of lactational diestrus by 2-3 days if they are given pups previously nursed by a food restricted dam. In the present study, however, ad lib fed rats that received hungry pups did not show an increase in NPY staining in the

arcuate nucleus, suggesting that the suckling-induced extension of the lactational diestrus period is unrelated to changes in NPY levels in the arcuate nucleus.

Control measures support previous data showing that food restriction during lactation resulted in a dramatic drop in dam weight and rate of growth of their litters (Woodside, 1991b). As expected, ad lib fed dams increased their food intake across the first two weeks of lactation (Ôta and Yokohama, 1967).

Results from the present study are consistent with those in which energetic demands increase NPY levels in the arcuate nucleus (Sahu et al., 1988; Malabu et al., 1994; Smith, 1993; Lewis et al., 1993; Schwartz, Sipols, Marks, Sanacora, White, Scheurinck, Kahn, Baskin, Woods, Figlewicz and Porte, 1992). The current data are also consistent with the data of Catzeflis et al. (1993) and Pierroz et al. (1995) showing that chronic elevation of NPY suppresses reproductive function.

There are three different sites on the hypothalamic-pituitary pathway at which NPY may affect LH levels. NPY could modulate GnRH release by directly acting on GnRH cell bodies located in the MPOA (Guy et al., 1988; Tsuruo et al., 1990). NPY fibers are also numerous in the median eminence, where they could modulate LHRH release into the portal blood system (Danger et al., 1990). Finally, NPY may directly modulate LH release from the pituitary, where it may change GnRH binding to its receptor (Knox, Bauer-Dantoin, Levine and Schwartz, 1995). Perhaps direct administration of NPY into these three sites concurrent with measures of gonadotropin release would provide an insight into which of these sites is more responsive to the effects of NPY.

effects of NPY.

The factors that maintain elevated NPY in the arcuate nucleus of food restricted dams even after five days of ad libitum feeding are not known. As reviewed above, situations of negative energy balance result in elevated NPY synthesis and content in the arcuate nucleus. The brain receives constant metabolic feedback from two main sources: the vagal afferents stemming from the liver, and from humoral signals such as glucose or insulin, that cross the blood-brain barrier (Wade and Schneider, 1992). These signals also seem to play a role in regulating the reproductive axis (Wade, Schneider and Li, 1996). There is some evidence suggesting that NPY synthesis is regulated by metabolic signals such as glucose, insulin and leptin (Akabayashi, Zaia, Siva, Chae and Leibowitz, 1993; Schwartz et al., 1992; Stephens, Bashinski, Bristow, Bue-Valleskey, Burgett, Craft, Hale, Hoffmann, Hslung, Kriauclunas, MacKellar, Rosteck, Schoner, Smith, Tinsley, Zhang and Heiman, 1995). Increased energetic demands that result in elevated NPY gene expression and content, are also accompanied by a decrease in plasma levels of glucose and insulin (Malabu et al, 1994; Lewis et al., 1993). Perhaps these signals remain altered after the food restriction period and maintain arcuate nucleus NPY levels elevated. This hypothesis could be easily tested by measuring circulating glucose and insulin levels in lactating rats after the food restriction regimen.

Signals from adipose tissue have recently been implicated in the control of NPY synthesis, food intake and reproduction. Leptin, a protein synthesized in adipose tissue of normal mice but absent in the blood of genetically obese (ob/ob) mice, seems to modulate food intake and body

weight (Pelleymounter, Cullen, Baker, Hecht, Winters, Boone and Collins, 1995). Daily peripheral and central leptin treatment results in a marked decrease of body weight and food intake in genetically obese (ob/ob) mice (Halaas, Gajiwala, Maffei, Cohen, Chait, Rabinowitz, Lallone, Burley and Friedman, 1995; Campfield, Smith, Guisez, Devos and Burn, 1995). Moreover, daily leptin treatment stimulates LH and FSH release, and promotes gonadal development in both female and male ob/ob mice (Barash, Cheung, Weigle, Ren, Kabigting, Kuijper, Clifton and Stainer, 1996). It has been suggested that the effects of leptin on food intake and reproduction are mediated by changes in arcuate nucleus NPY because leptin treatment for 30 days decreases arcuate nucleus NPY gene expression in ob/ob mice (Barash et al.; 1996; Stephens, Bashinski, Bristow, Bue-Valleskey, Burgett, Craft, Hale, Hoffmann, Hslung, Kriauclunas, MacKellar, Rosteck, Schoner, Smith, Tinsley, Zhang and Heiman, 1995). This idea has gained momentum with results indicating that serum leptin levels in female mice fall rapidly while hypothalamic NPY gene expression increases after 48 hrs of fasting. These changes are followed by a dramatic suppression of LH levels and a disruption of cyclicity that is ameliorated by leptin injections (Ahima, Prabakaran, Mantzoros, Qu, Lowell, Maratos-Flier and Flier, 1996) . These results are consistent with the idea that NPY levels remain elevated until fat stores are replenished and the leptin signal is increased.

In sum, the present study used immunocytochemical staining to quantify differences in arcuate nucleus NPY between food restricted and ad lib fed lactating rats. The results open the doors to at least two lines of research. First, although immunocytochemistry may provide an estimate of NPY

content in the arcuate nucleus, more quantitative measures of NPY content are needed to ensure that the increased staining seen after food restriction translates into more NPY synthesis, or more NPY release at the terminals. Therefore, the results from this study should be followed by studies using radio-immuno-assays for NPY in sections of the arcuate nucleus, and of areas enervated by the arcuate nucleus such as the MPOA and PVN of ad lib fed and food restricted dams.

The second line of research should assess directly whether NPY suppresses LH release in food restricted lactating rats. One approach would be to manipulate the action of NPY directly by using selective NPY antagonists. NPY has a wide variety of effects throughout the nervous system which may be mediated by its interaction with at least five receptor subtypes. NPY-induced feeding and reproductive functions were thought at first to be mediated by the action of NPY on the Y1 receptor subtype since [Leu^{31},Pro^{34}]-NPY, a Y1 receptor agonist, stimulated food intake in satiated male rats (Kalra et al., 1993) and potentiated LHRH release from the median eminence of ovariectomized rats (Stanley, Magdalin, Seirafi, Nguyen & Leibowitz, 1992). More recently, however, the effects of NPY on food intake have been associated to its actions on an "atypical" Y1 receptor subtype (Wahlestedt and Reis, 1993) and on the Y5 receptor (Gerald, Walker, Criscione, Gustafson, Batzi-Hartmann, Smith, Vaysse, Durkin, Laz, Linemayer, Schaffhauser, Whitebread, Hofbauer, Taber, Branchek and Weinshank, 1996); the Y4 receptor may also play a significant role in mediating the orexigenic and neuroendocrine effects of NPY because, in contrast to the Y1 receptor, the Y4 receptor is abundant in the hypothalamus (Bard, Walker, Branchek and

Weinsbank, 1995). The recent cloning of NPY receptor subtypes has resulted in the development of more potent and selective NPY receptor antagonists (Larhammar, Blomqvist, Yee, Jazin, Yoo and Wahlestedt, 1992; Balasubramaniam, Sheriff, Johnson, Prabhakaran, Huang, Fischer and Chance, 1994; Serredeil-Le Gal, Valette, Rouby, Pellet, Oury-Donat, Brossard, Lespy, Marty, Neliat, Cointet, Maffrand and Le Fur, 1995; Daniels, Matthews, Slepetis, Jansen, Viveros, Tedepalli, Harrington, Heyer, Landavazo, Leban and Spaltenstein, 1995). If sustained elevated levels of NPY after food restriction suppress LH and prolong lactation, perhaps NPY antagonists given during this period would lift the LH suppression and terminate lactational diestrus.

The use of colchicine in the present study was necessary to visualise NPY immunostained neurons. A disadvantage of this procedure is that colchicine may have neurotoxic effects that, on their own, could affect hypothalamic NPY staining (Mundy and Tulson, 1990). This seems unlikely in the present study because all animals were treated with colchicine. Furthermore, the pattern of staining seen was similar to that in which NPY mRNA was assessed using *in situ* hybridization, a procedure that does not require colchicine (Smith, 1993).

In conclusion, lactation is characterized by a profound suppression of GnRH and LH release that is prolonged if the dam is food restricted during the first two weeks or the second week of lactation (Woodside, 1991b; Woodside and Popeski, 1996; Walker et al., 1995). Food restriction during the second week of lactation also results in elevated NPY staining in the arcuate nucleus that may be associated with the prolonged LH suppression. These

results make NPY a likely candidate in the mechanisms that extend lactational diestrus in food restricted rats.

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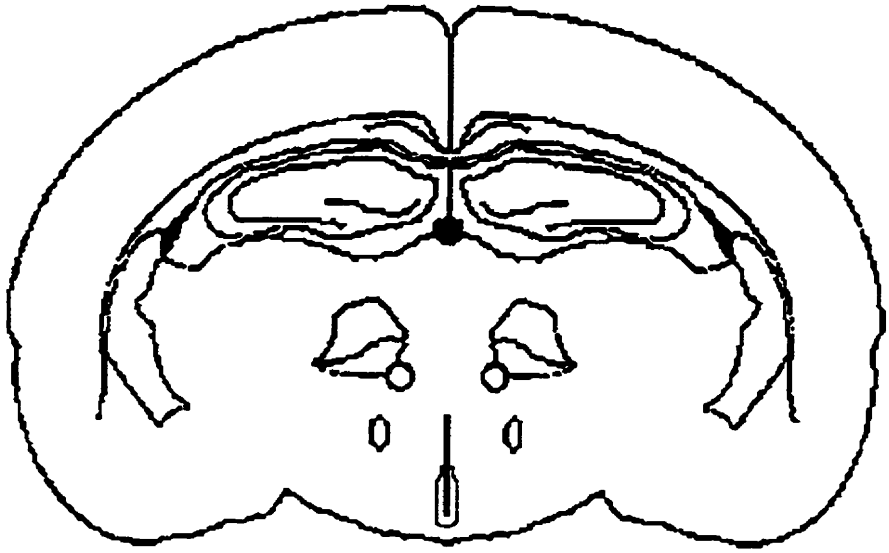
APPENDIX A

ARC 1, ARC 2, and ARC 3 Portions of the Arcuate Nucleus



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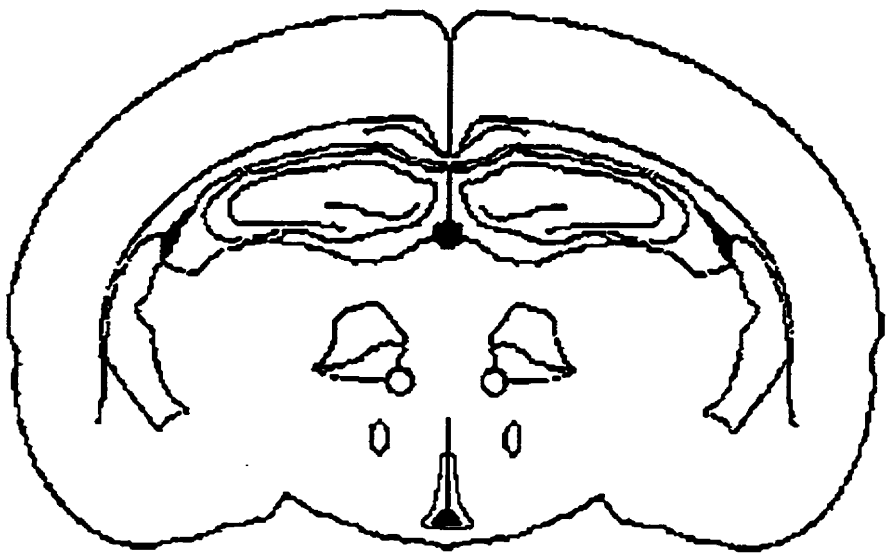

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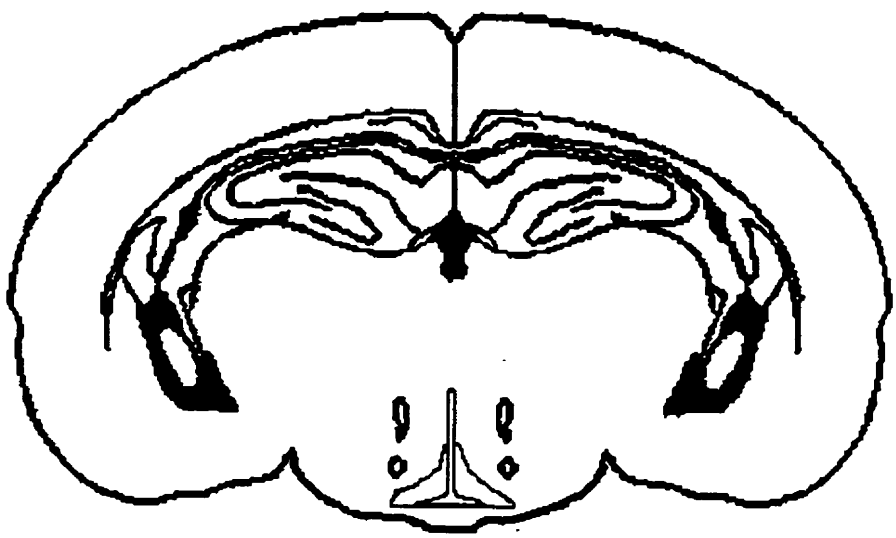

ARC 3

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APPENDIX B

Source Tables of Analyses of Variance and Post hoc Analyses

Table 1

Source table for the ANOVA of mean number of cells per section stained for NPY in the arcuate nucleus of ad lib fed and food restricted rats on days 15, 20 and 25 of lactation.

Source	SS	df	MS	F	P
Between groups					
Diet	2325.50	1	2325.50	5.81	.024
Day of lactation	510.30	2	255.22 .64	.547	
Diet x day of lactation	306.45	2	153.22	.38	.686
Error	10000.37	25	400.01		
Within groups					
Portion of arcuate	1811.15	2	905.57	18.89	.000
Diet x portion of arcuate	186.69	2	93.34	1.95	.153
Day of lactation x Portion of arcuate	393.48	4	98.37	2.05	.101
Diet x day of lactation x portion of arcuate	136.67	4	34.17	.71	.587
Error	2397.12	50	47.94		

Table 2

Source table for the ANOVA of mean number of cells per section stained for NPY in the arcuate nucleus of ad lib fed and food restricted rats that had their litters switched from day 15 to day 20 of lactation versus dams that nursed the same litter throughout the experiment.

Source	SS	df	MS	F	P
Between groups					
Diet	2261.28	1	2261.28	5.34	.033
Type of litter	3.80	1	3.80	.01	.926
Diet x type of litter	141.39	1	141.39	.33	.570
Error	7621.18	18	423.40		
Within groups					
Portion of arcuate	1403.86	2	701.93	11.02	.000
Diet x portion of arcuate	170.94	2	85.47	1.34	.274
Day of lactation x Portion of arcuate	257.56	2	128.78	2.02	.147
Diet x day of lactation x portion of arcuate	225.73	2	112.86	1.77	.184
Error	2293.20	36	63.70		

Table 3

Source table for the ANOVA of mean dam weight change before the food restriction regimen (day 8 of lactation) and at the end of the food restriction regimen (day 14 of lactation).

Source	SS	df	MS	F	P
Between groups					
Diet	28803.39	1	28803.39	86.15	.000
Error	13373.41	40	334.33		
Within groups					
Day of lactation	10348.38	1	10348.38	121.48	.000
Diet x day of lactation	33358.30	1	33358.30	391.60	.000
Error	3407.38	40	85.18		

Table 4

Source table for the ANOVA of mean pup weight change before the food restriction regimen (day 8) and at the end of the food restriction regimen (day 14).

Source	SS	df	MS	F	P
Between groups					
Diet	115.50	1	115.50	11.35	.002
Error	406.97	40	10.17		
Within groups					
Day of lactation	2370.09	1	2370.09	1965.08	.000
Diet x day of lactation	245.88	1	245.88	203.87	.000
Error	46.24	40	1.21		

Table 5

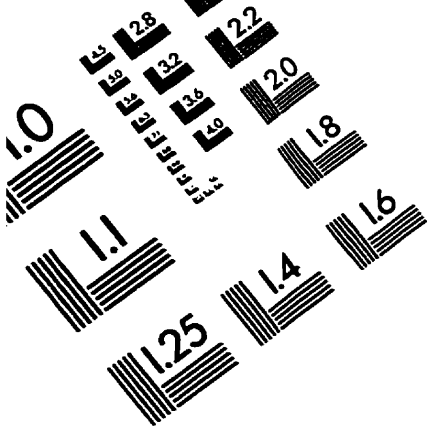
Source table for the simple effect analyses of dam weight change from day 1 of lactation before the food restriction regimen (day 8 of lactation) and at the end of the food restriction regimen (day 14 of lactation).

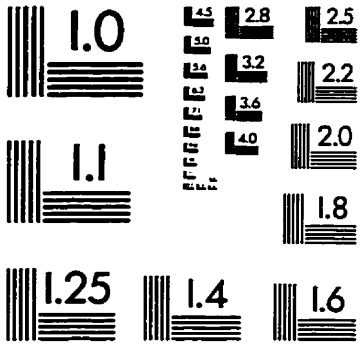
Source	df	MS	F	P
Simple Effects for Diet				
Day 8	1	83.55	.44	.512
Error	40	190.69		
Day 14	1	62078.14	271.29	.000
Error	40	228.83		
Simple Effects for Day of Lactation				
Ad Lib fed	1	3437.35	40.35	.000
Error	40	85.18		
Food Restricted	1	38595.16	453.08	.000
Error	40	85.18		

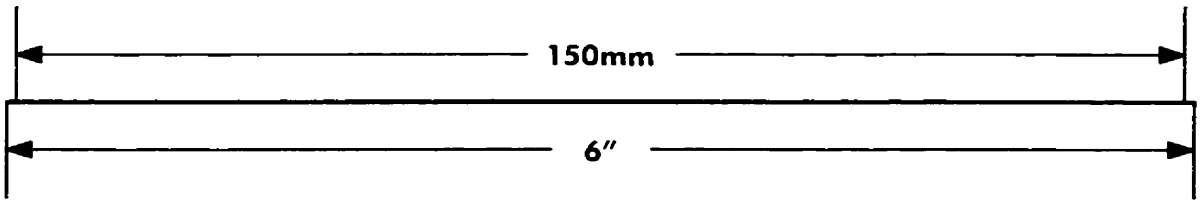
Table 6

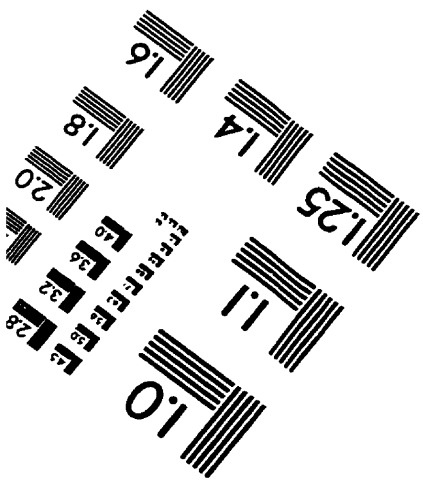
Source table for the simple effect analyses of mean pup weight gain before the food restriction regimen (day 8) and at the end of the food restriction regimen (day 14).

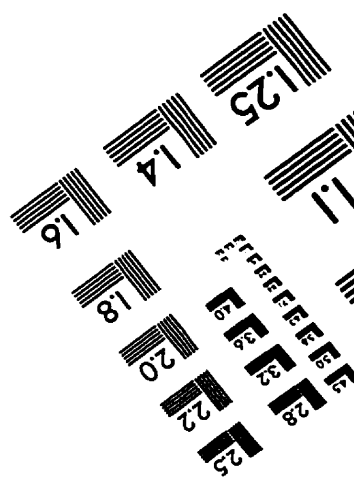
Source	df	MS	F	P
Simple Effects for Diet				
Day 8	1	12.17	2.95	.094
Error	40	4.13		
Day 14	1	349.22	48.17	.000
Error	40	7.25		
Simple Effects for Day of Lactation				
Ad Lib fed	1	2174.94	1803.28	.000
Error	40	1.21		
Food Restricted	1	519.84	431.01	.000
Error	40	1.21		











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