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Food Restriction Attenuates the Hormonal and Neuronal Responses to the Positive Feedback Effects of Estradiol to Prolong Lactational Diestrus in Rats

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ABSTRACT

Food Restriction During Lactation Prolongs the Period of Insensitivity to the Positive Feedback Effects of Estradiol on Gonadotropin Release.

Alfonso Abizaid

Food restriction prolongs lactational infertility in rats. The experiments presented in this thesis were done to investigate the possibility that this effect is due to an attenuated response to the positive feedback effects of estradiol (E2) that stimulate the surge in luteinizing hormone (LH) release. Furthermore, the hypothesis that that the hypothalamus is one site affected by food restriction in attenuating E2 induced LH surges was examined. The first series of experiments examined the ability of E2 to induce LH surges in both ad lib fed and food restricted dams at different times of lactation. Results were that on day 20 postpartum (pp) ad lib fed dams showed LH surges after E2 treatment, but food restricted dams did not. Ovariectomy (OVX) or RU486 treatment restored the ability of E2 to induce LH surges in food restricted dams, and chronic progesterone (P) reduced E2-induced LH surges in ad lib fed OVX dams. The second series of experiments showed that food restricted dams had less Fos-like immunoreactive (FOS-ir) cells in the anteroventral preoptic area (AVPV) than ad lib fed dams on day 20 pp. These effects were reflected in a reduced ability of E2 to induce P receptor (PR) immunoreactivity, but not in the number of E2 receptors (ERα) in the AVPV. As with the LH surge, the effects of food restriction on E2 induction of PRs were mediated by P. Results suggest that the lactational diestrus is prolonged by a decrease in sensitivity to E2 in the AVPV, and this effect is mediated by the high levels of P in food restricted dams.

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The work here presented is the result of several years of hard work in combination with the excellent guidance from my graduate student supervisor and mentor Dr. Barbara Woodside. Barbara, I am truly grateful for the opportunity to learn in your laboratory not only about the behavioral neuroendocrinology of lactation, but also about doing solid scientific research. I have cherished the time I have spent working with you, and as I've told previously, every day I came to work I was sure I would be learning something new, and doing something relevant and fun. Thank you from the bottom of my heart.

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CHAPTER 1

General Introduction

Lactation in many species of animals is associated with the suppression of fertility, and the duration of this period of infertility is modulated by changes in food availability. The experiments described in this thesis are designed to elucidate some of the mechanisms underlying the duration of lactational infertility in the rat.

The study of factors regulating fertility is essential for our economic, health and individual well being. A recent report from the National Council for Science and the Environment predicts that if world population continues to grow at the current rate, within the next 50 years food requirements will increase by about 250% (Nowels, 2002). Understanding the physiological mechanisms that regulate reproduction may help us create solutions to promote birth control in human populations and also enhance fertility in livestock. Furthermore, understanding these mechanisms will provide insight into how clinical conditions such as anorexia nervosa and some types of obesity, are often associated with infertility.

Fertility in all living organisms is dependent on the availability of food within the environment (Bronson, 1989). Although scientific studies of the factors affecting fertility are relatively recent in the history of humanity, our ancestors recognized the intimate relationship between fertility and food availability. This is clearly reflected in the concept of beauty depicted in statues and paintings of deities across different cultures. Good examples of such depictions are the Venus of Willendorf and the Venus of Lespugne calculated to be about 30,000 and 25,000 years old respectively. These statues are thought to reflect fertility as embodied in the shape of women who have well developed fat deposits

particularly around the hips and breasts. In pre-Columbian art, deities thought to rule over fertility were depicted as females, and were not only associated with birth but also with water and corn, the main dietary staple for Mesoamerican cultures.

The intimate relationship between food availability and reproduction has also been recorded in studies looking at seasonal or catastrophic changes in food resources and their effect on the reproductive function of animals including humans. It is evident that seasonal variations in food availability coincide with seasonal reproductive patterns in many wild animal populations (Bronson, 1989). This is particularly evident in animals living in the northern or southern hemispheres where food availability varies widely across the year; a similar effect is also seen in regions closer to the equator that receive limited amount of rainfall (Bronson, 1989). In addition, a reduction in the amount and quality of pastures is associated with decreased reproductive capacity in ungulates (Loudon, McNeilly, & Milne, 1983; Asdell, 1949). In humans, famine or rigorous physical training are also related to reduced fertility (Stein, Susser, & Saenger, 1976; Stein, Susser, Saenger, & Marolla, 1972), and in extreme cases, infertility (Falk & Halmi, 1982); Frisch, 1982; Bongaarts, 1980a; Bongaarts, 1980b; Zimmer, Weill, & Dubois, 1944).

The mechanisms by which reduced food availability affects reproductive function in human or wild animal populations is difficult to assess given the multiple variables that might co-vary to alter fertility. In humans, for example, extreme famine results in anovulation and gonadal atrophy (Zimmer et al., 1944; Bongaarts, 1980a; Bongaarts, 1980b). Bongaarts argues that infertility in these circumstances could be brought about simply by the high levels of stress related to these extreme conditions (Bongaarts, 1980b). Moreover, separation from spouse, abstention from sex, and abortion, all factors that are common in these

conditions, could also play a role in the decreased rate of reproduction. Similar problems can be cited in the study of reproduction in wild animal populations, where reproductive function can be hampered indirectly by increased predatory pressure, decreased availability of mates, and increased foraging time (Bronson, 1989).

Laboratory experiments in which nutrient deficiencies are manipulated systematically have established more direct evidence for an effect of reduced food availability on reproductive function in female mammals. Such studies have determined that chronic food restriction or acute food deprivation result in a profound suppression in the reproductive viability of laboratory and farm animals (Foster, Karsch, Olster, Ryan, & Yellon, 1986; Karsch et al., 1984; Bronson, 1989; Wade & Schneider, 1992; Wade, Schneider, & Li, 1996 Kalra & Kalra, 1996). The effects of changes in food availability on reproductive function have been particularly well documented in female rodents (Wade & Schneider, 1992; Wade et al., 1996). For example, decreased food availability delays the onset of puberty, and disrupts the estrous cycle of female rodents, and reproductive function is restored soon after refeeding (Bronson, 1989; Knuth & Friesen, 1983; Morin, 1975; Wade & Schneider, 1992; Wade et al., 1996; Kalra & Kalra, 1996).

Lactation is considered the most energetically demanding period in the life of female mammals (McNeilly, 1994; Canas, Romero, & Baldwin, 1982). In order to compensate for an increase in the daily energy requirements of milk production, lactating rats, for example, increase their food intake, mobilize their energy stores, and decrease their metabolic rate (Canas et al., 1982; Fleming, 1976; Vernon, 1980; Vernon, 1989; Vernon et al., 1995). These metabolic changes are accompanied by a delay in new reproductive efforts. This period of infertility is commonly termed lactational diestrus in mammals that have an oestrus cycle

such as rodents and ruminants (McNeilly, 1994). In humans and other primates there is a similar period of infertility associated with lactational amenorrhea. It is noteworthy, however that females in these species may ovulate during lactation without menstruating, thus the period of amenorrhea does not necessarily define the period of anovulation (McNeilly, 2001; McNeilly, Tay, & Glasier, 1994).

As with puberty and during the ovulatory cycle, the reproductive axis of lactating females is sensitive to shortages in food availability. For example, it has been shown that lactating rats that have restricted access to food (50 % of the ad lib intake during the first two weeks postpartum (pp)) show a prolonged lactational diestrus compared to that of lactating rats that have free access to food and nurse the same number of young (Woodside, 1991; Woodside & Jans, 1995). Furthermore, increasing the number of young within a litter, and presumably the amount of energy that a dam must spend to sustain the energy requirements of the litter also prolongs lactational diestrus (Lindblom, Sodersten, & Eneroth, 1985; Smith, 1984; Woodside & Popeski, 1999). Similar effects of food restriction and number of offspring on lactational infertility have been reported in sows, cows, sheep, monkeys and humans (Delgado et al., 1979; McNeilly, 1994).

Although there may be some differences across species in the mechanisms that regulate the duration of lactational infertility, it is obvious that the degree of variability in the duration of lactational diestrus reflects a mechanism that has evolved to maximize the number of surviving offspring that a female mammal produces throughout her lifespan in different environmental conditions. This argument is supported by parental investment theory (Trivers, 1972). In this theory, Trivers suggested that parental investment - the investment that a parent provides for a set of offspring at the cost of providing for future offspring- is an important factor that determines the reproductive success of an organism. He

suggested that animals that are able to provide the greatest amount of parental investment to their offspring at a minimal cost in terms of future reproductive efforts become more attractive for prospective mates. In the case of the lactating rat, lactational infertility allows the dams to concentrate energy onto a suckling litter without investing in a future litter, and this energetic diversion is prolonged in food restricted dams. One can therefore suggest that a flexible lactational infertility period has evolved through mechanisms of natural and sexual selection to ensure the survival of young offspring given different environmental conditions (Short, 1976a; Short, 1976b; Loudon et al., 1983; Bongaarts, 1980b; Konner & Worthman, 1980).

Considering that lactational infertility appears to be a phenomenom that maximizes reproductive success, it is interesting that most research done on the interaction between food and fertility is done in non-lactating females (i.e. Wade & Schneider, 1992; Wade et al., 1996; Tsukamura & Maeda, 2001). One reason for this could be that lactational anovulation is related to several endocrine and non-endocrine factors that interact to produce an anovulatory state. Although some of these factors have been described for lactating rats under conditions of ad libitum feeding (McNeilly, 2001; Smith & Neill, 1977), little work has been conducted to understand the determinants of lactational diestrus in animals that are unable to increase their food intake to meet their energetic demands (Flint & Vernon, 1998; McGuire, Butler, & Rasmussen, 1992; McGuire, Pachon, Butler, & Rasmussen, 1995). This thesis examines some of the neuroendocrine mechanisms that regulate the duration of the lactational diestrus in the rat, and how they change in response to food restriction. The remainder of this chapter discusses the sensory stimuli that contribute to the suppression of fertility during lactation and discuss their relative contribution in extending lactational infertility during reduced food availability. Subsequently, the neuroendocrine events that lead to

ovulation in the cycling female rat, and what is known about the changes in these events during lactational diestrus is described. Finally, evidence to support the hypothesis that the prolonged period of lactational diestrus seen after food restriction may in part be the result of decreased efficacy of positive feedback signals on the reproductive axis is presented. The remaining chapters of this thesis will describe experiments to test this hypothesis and to elucidate the mechanisms involved.

Stimuli that inhibit reproduction during lactation

The reproductive axis of lactating females is suppressed, and the sensory stimuli that participate in this suppression interact in complex ways that have often led to problems of interpretation in the literature (i.e. Bongaarts, 1980a; Bongaarts, 1980b; Frisch, 1982). Nevertheless, they can be grouped in two general categories: 1) somatosensory stimuli due primarily to the effects of suckling, and 2) metabolic stimuli that reflect changes in energy balance. This categorization, however, is arbitrary and there are many difficulties dissociating these mechanisms from each other. For example, suckling stimulation is essential for the inhibition of the hypothalamic-pituitary-ovarian (HPO) axis during lactation (McNeilly, 1994), but suckling stimulation is also associated with milk production and secretion, which alter energy balance. Given that the metabolic changes associated with milk production can also inhibit the HPO, it is difficult to dissociate the effect of these changes from those of suckling stimulation per se on the HPO. Studies that have attempted to do this are described below.

Suckling stimulus. The suckling stimulus provided by the young during nursing is necessary for lactational infertility, and removal of the young results in the

reinstatement of ovulation. In humans and non-human primates, increases in the duration and number of nursing bouts are associated with prolonged periods of lactational amenorrhea (Delgado et al., 1979;McNeilly, 1994; McNeilly, 2001; Konner & Worthman, 1980). One striking illustration of the relationship between suckling and the duration of lactational infertility is that of the !Kung, one of the last hunter-gatherer populations around the world. !Kung women are known to have an average inter birth interval that lasts over three years, and this interval is correlated with a nursing frequency of approximately once every 13 minutes throughout the day and night (Konner & Worthman, 1980; Stern, Konner, Herman, & Reichlin, 1986). The association between nursing frequency and infertility is also seen in farm animals. In sheep, lactational infertility is prolonged in direct relationship with the number of young an ewe nurses, and fertility is rapidly restored if the young are taken away (Schirar et al., 1989; Schirar et al., 1990). Beef cows - which are allowed to nurse their young constantly throughout the day- usually show a longer lactational diestrus than dairy cows who are milked only in the mornings and evenings (Lamming, Wathes, & Peters, 1981).

In the rat, as in other species, suckling stimulation is necessary for maintaining lactational diestrus. Removal of litters at any point during lactation results in a resumption of follicular development and ovulation within four days (Taya & Sasamoto, 1980). In addition, dams that nurse large litters have a longer lactational diestrus than those nursing small litters, and lactational diestrus can be prolonged if an old litter is replaced with a litter of young pups (Lindblom et al., 1985).

Suckling and food restriction.- Food restriction of the dam to 50% of the ad lib intake decreases milk yield (McGuire et al., 1992; Woodside, 1991), and results in growth retarded, hungry pups (Woodside, 1991; Woodside & Popeski, 1999; Woodside & Jans, 1995; McGuire et al., 1992). One could, therefore, argue that

food restriction prolongs lactational infertility simply by increasing the amount of time that the pups spend nursing their mother, and/or by increasing the intensity of their suckling stimulation. Thus, in a way, a food restricted litter may provide a degree of suckling stimulation that is similar to that obtained by a mother nursing a large litter. Other evidence, however, suggests that changes in the frequency and in the duration of the suckling stimulus do not play a major role in the prolonged lactational diestrus that is seen in food restricted lactating rats. The strongest evidence on this point comes from studies where litters are switched between ad lib fed and food restricted dams. In these studies food restricted dams that are given pups previously nursed by ad lib fed dams still show a longer lactational diestrus (Woodside & Jans, 1995). The extended lactational diestrus of food restricted dams is present even when litters are switched daily between ad lib fed and food restricted dams to control for suckling stimulation (Woodside & Jans, 1995). Although food restricted rats spend more time in contact with their litters, and presumably are nursing more frequently, diminishing contact time by increasing the room temperature does not result in a shorter lactational diestrus (Woodside & Jans, 1995). Interestingly, ad lib fed dams given litters of pups that had been previously nursed by food restricted dams do show a longer lactational diestrus than those who retain their well fed litters. This suggests that well fed dams are sensitive to the effects of variations in suckling duration on the reproductive axis but food restricted females are not.

Metabolic changes during lactation and infertility.

During lactation, rats increase their food intake to meet the energetic demands of their young. In addition, they mobilize their fat depots, increase

their glucose turnover rate, and obtain further resources by increasing ketone body production and utilization (Fleming, 1976; Leon & Woodside, 1983 ; Canas et al., 1982). Therefore, lactation, even in ad lib fed females, results in a metabolic state similar to that seen following food restriction or food deprivation of virgin animals. This state is exacerbated if the animals are food restricted during the first two weeks of lactation, which results in loss of up to 20% of their muscle and almost all their fat stores (Woodside, Kyriazis, & Abizaid, 2001; Glore & Layman, 1985).

Food restriction or food deprivation of cycling rodents affects both the pulsatile and surge release of luteinizing hormone (LH) resulting in anovulation. In rats, for example, a 48-hour fast leads to a suppression of LH pulses in intact females (Nagatani, Tsukamura, & Maeda, 1994). Chronic food restriction of 50% ad libitum intake seems to also enhance negative feedback effects of EB on LH pulses but it does not seem to suppress an EB induced LH surge in OVX females (Sprangers & Piacsek, 1988; Sprangers & Piacsek, 1997). One might predict then that lactational infertility arises from the same set of cues through which food restriction suppresses the reproductive axis in cycling females.

A leading theory suggests that the central nervous system regulates food intake primarily by sensing signals that provide information on the amount of metabolic fuels that are available for conversion into energy, and that these signals also regulate the reproductive axis (Ritter, 1986; Wade et al., 1996). Indeed, there is compelling evidence that changes in metabolic fuel availability disrupt cyclicity and rapidly suppress the reproductive axis and behavior of hamsters and rats (Wade & Schneider, 1992; Wade et al., 1996; Abizaid, Jafferali, Pellertier, & Woodside, 2001; Nagatani et al., 1996a). Furthermore, data suggesting that specific macronutrient restriction did not increase lactational diestrus if dams were allowed to compensate calorically (Woodside, Abizaid, &

Caporale, 1998a) led us to propose that food restriction prolonged lactational diestrus by decreasing the availability of metabolic fuels for oxidation. More recently, however, it was found that drugs that interfere with the utilization of metabolic fuels such as 2DG and the inhibitor of fatty acid oxidation mercaptoacetate (MA), do not prolong lactational diestrus (Abizaid et al., 2001). The inability of these agents to prolong lactational infertility may be a function of the low levels of estrogen in lactation because estrogen potentiates the effects of inhibitors of metabolic fuel oxidation on the reproductive axis (Nagatani et al., 1994). In addition, it has been shown that glucoprivic drugs are less effective in states (such as lactation) when the levels of ketone bodies are high (Ritter, 1986). Whatever the cause of the insensitivity to glucoprivation or lipoprivation seen in lactating rats, these data suggest that food restriction during lactation is likely to be associated with changes in long term signals regulating energy homeostasis.

Recently, interest has focused on how the interaction between peripheral signals of adiposity and hypothalamic peptides in particular neuropeptide Y (NPY) modulation of both food intake and the reproductive axis. Leptin, the protein product of the ob gene, is synthesized in white adipose tissue (Campfield, Smith, Guisez, Devos, & Burn, 1995; Halaas et al., 1995; Pelleymounter et al., 1995). Both leptin and insulin circulate at levels proportional to the size of fat stores (Maffei et al., 1995). The production and secretion of NPY is modulated by changes in circulating levels of leptin and insulin to regulate food intake and fertility (Elmquist, 2001; Erickson, Hollopeter, & Palmiter, 1996; Rohner-Jeanrenaud, Cusin, Sainsbury, Zakrzewska, & Jeanrenaud, 1996; Rohner-Jeanrenaud & Jeanrenaud, 1996; Schwartz et al., 1991; Schwartz, Seeley, Campfield, Burn, & Baskin, 1996). Both leptin and insulin receptors are detected within NPY neurons in the arcuate nucleus (ARC) and both act within these neurons to decrease NPY synthesis (Mercer, Moar, Rayner, Trayhurn, &

Hoggard, 1997; Schwartz, Figlewicz, Woods, Porte, & Baskin, 1993). There is considerable evidence to support a permissive role for leptin in reproductive function. For example, leptin levels are reduced by a 48-hour fast that disrupts ovulation (Trayhurn, Thomas, Duncan, & Rayner, 1995) and leptin treatment during food deprivation maintains reproductive function (Ahima et al., 1996). In addition, exogenous leptin administration restores ovulation in leptin deficient mice, and advances puberty in food restricted female rats (Barash et al., 1996). Food shortages are also associated with an increase in the synthesis of NPY within the ARC (Kalra, Sahu, Kalra, & Crowley, 1990). A 48-hour fast results in an increase in NPY content in areas like the medial preoptic area (MPOA) where NPY axons contact gonadotropin releasing hormone (GnRH) neurons (Sahu, Kalra, & Kalra, 1988; Tsuruo et al., 1990). Although NPY potentiates the LH surge after the appropriate steroid hormone treatment, NPY attenuates LH pulses in OVX animals that do not receive such treatment (Kalra & Crowley, 1984). NPY also suppresses ovulation in virgin female rats if delivered chronically into the brain (Catzeflis et al., 1993; Raposinho et al., 1999; Woodside, Beaule, & Lauay, 2002). Therefore, food deprivation or chronic food restriction could produce a state of chronically elevated NPY release into the MPOA and/or median eminence to reduce LH pulsatility and disrupt ovulation.

During lactation, insulin and leptin are reduced in response to a drop in adiposity (Vernon, 1989; Woodside, Abizaid, & Jafferali, 1998b; Woodside et al., 1998b; Brogan, Mitchell, Trayhurn, & Smith, 1999). In food restricted dams, leptin remains low for at least five days after the food restriction regimen has been lifted (day 20 postpartum (pp)) (Woodside et al., 2001). The reduction in circulating leptin levels seen during lactation correlates with increased NPY levels in the ARC suggesting that leptin works as a "brake" for NPY, and in its absence, NPY is chronically elevated to increase food intake and suppress the release of

LH (Li, Chen, & Smith, 1998a; Li, Chen, & Smith, 1998b; Li, Chen, & Smith, 1999b; Smith, 1993; Malabu, Kilpatrick, Ware, Vernon, & Williams, 1994; Pickavance et al., 1996; Abizaid, Walker, & Woodside, 1997). Therefore it could be suggested that a decrease in leptin and/or insulin levels results in chronic NPY secretion promoting a state of lactational infertility, and that food restriction maintains infertility by keeping the levels of leptin and/or insulin reduced.

If this were true then restoring leptin and/or insulin signaling should shorten the lactational diestrus of food restricted dams. In support of this our data show that leptin reverses the effects of a 48-hour fast at peak lactation (days 13-15 pp) on the length of lactational diestrus of rats (Woodside et al., 1998b)). In contrast, leptin does not shorten the lactational diestrus of dams that are food restricted throughout the first two weeks of lactation (Woodside et al., 2001). These differences may reflect the differential effects that fasting has on lactational performance in comparison with chronic dietary restriction. These effects might also reflect the ability of leptin to act peripherally and centrally to enhance metabolic fuel availability (Schneider et al., 1998). For example, a 48-hour fast at peak lactation rapidly reduces milk yield to negligible amounts, whereas chronic food restriction only partially reduces milk yield in rats (Woodside et al., 1998b; Woodside & Jans, 1995). When leptin is administered to food restricted rats, milk yield as reflected in pup growth is increased suggesting that any metabolic fuels liberated by increases in leptin are channeled into milk production (Woodside et al., 2001). Leptin has no effect on milk yield in fasting animals, however, so that any increase in metabolic fuel availability subsequent to leptin treatment may serve as an additional signal of positive energy balance at the hypothalamus and disinhibit the HPO. In support of this schema, we have shown that food restricted lactating rats that are prevented from delivering milk by galactophore transection (GC), show a shorter lactational diestrus when given exogenous

leptin administration (Woodside et al., 2001).

In summary, stimuli associated with both suckling stimulation and negative energy balance are present in both ad lib fed and food restricted lactating females. When the energetic demands of lactation are eliminated by galactophore transection, the fact that suckled rats still show a period of anovulation suggests that suckling alone is capable of suppressing the reproductive axis (Woodside & Jans, 1995). Because the galactophore transection results in both ad lib fed and food restricted dams nursing equally deprived pups, any effects of food restriction on lactational diestrus are independent of the nutritional status of the pups, and can only be ascribed to changes in energy availability (Woodside & Jans, 1995). Moreover, sensitivity to parameters of suckling is overridden by food restriction probably because of the involvement of signals of long term energy stores acting on central mechanisms that control both energy homeostasis and inhibit the reproductive axis. Finally, in contrast to the effects of food restriction in cycling females - which disappear rapidly once the animal is refed, the reproductive axis is suppressed during lactation for a period that extends beyond the duration of food restriction itself. The pathways through which these effects might be mediated are reviewed in the section below.

Hormonal and neural control of ovulation.

In the rat, ovulation occurs every 4-5 days and the mechanisms that control it are orchestrated by the HPO axis. Briefly, the hypothalamus secretes pulses of gonadotropin-releasing hormone (GnRH) that reach the pituitary via the portal blood system. At the pituitary, GnRH acts on the gonadotroph to stimulate the release of LH and follicular stimulating hormone (FSH) into general

circulation. These hormones then promote follicular maturation in the ovaries as well as the release of ovarian steroid hormones such as estradiol (E2) and progesterone (P). In female rats, E2 has a dual effect on the reproductive system. Soon after ovulation, E2 provides negative feedback signals to both the hypothalamus and the pituitary that attenuates gonadotropin pulses. Neither LH nor FSH pulses however, are completely inhibited and both continue to act on the ovaries to produce mature follicles (FSH), and to increase the synthesis of E2 (LH) resulting in a peak in E2 release that precedes ovulation. This peak provides a positive feedback signal that culminates in a surge in GnRH followed by a surge in LH release. The LH surge dramatically increases the production of antral fluid within follicles and causes them to burst, releasing the mature ova. Without a surge in LH, ovulation cannot occur (Everett, 1961a; Feder, 1981; Freeman, 1994; see Figure 1).

The ovulatory cycle is also regulated by P, which like E2, has a dual effect upon the HPO axis. P levels markedly increase soon after the peak in EB release, and these increased P levels enhance and advance the timing of the LH surge through mechanisms that involve the action of P at both the hypothalamus and the pituitary (Everett, 1961; Levine, 1997). These elevated levels of P become inhibitory soon after the peak of the LH surge and may be a limiting factor not only for the surge that is in progress but also to prevent a surge from occurring the following day. In addition, chronically elevated P levels suppress E2 induced LH surges ((Everett, 1961a; Smith, Freeman, & Neill, 1975; DePaolo & Barraclough, 1979).

Hypothalamic-Pituitary-Ovarian Axis (HPO)

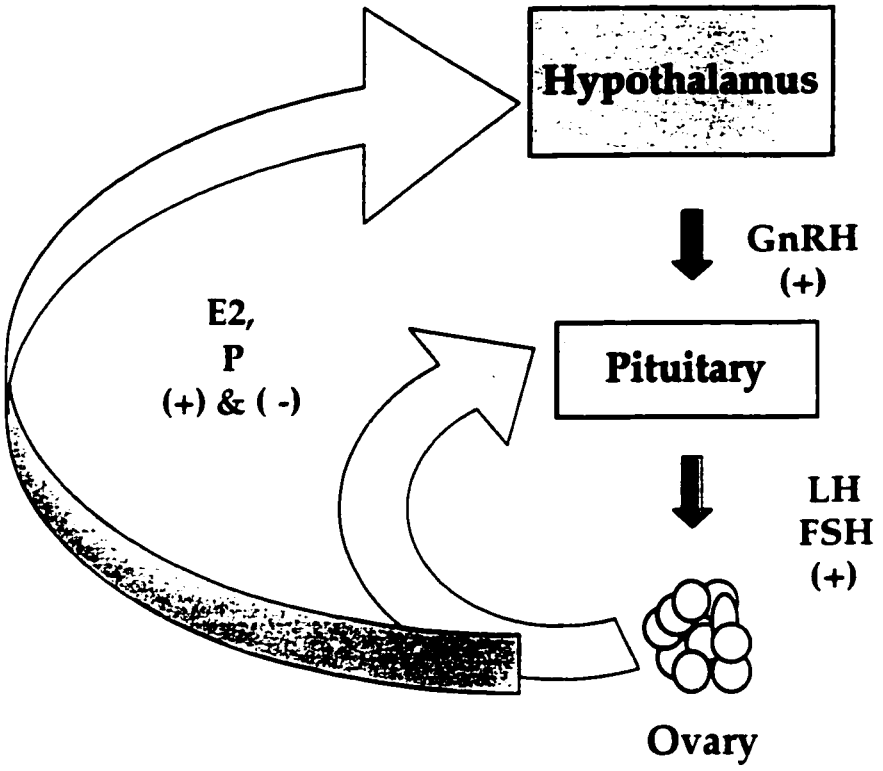

Figure 1.- Schematic representation of the HPO. As seen in this picture, secretion of hypothalamic GnRH induces the release of LH and FSH from the pituitary, which in turn stimulate follicular maturation. As follicles mature, they secrete both E2 and P which generally act to suppress the secretion of gonadotropins. In females, however, E2 acts on the hypothalamus and pituitary to induce a surge in LH release that is essential for ovulation to occur. A peak in preovulatory P secretion facilitates the effect of E2 on LH secretion and prevents the ocurrance of a surge in LH the following day.

In summary, ovulation can be said to occur as a result of two mechanisms: 1) a mechanism that regulates the pulsatile release of LH and FSH to induce follicular maturation; and 2) one that produces an LH surge to induce follicular rupture and egg release. Both of these mechanisms are regulated directly or indirectly by the action of ovarian hormones at the hypothalamic, and pituitary level during the estrous cycle.

The pulsatile release of gonadotropins is regulated primarily by the negative feedback effects of ovarian hormones (Herbison, 1998). This is demonstrated by the increase in the magnitude and amplitude of LH pulses that is seen after ovariectomy (OVX) in female rats (Herbison, 1998). The way in which ovarian hormones regulate the pulsatile release of gonadotropins, however, remains a mystery. Given recent data suggesting that some GnRH neurons contain estrogen receptors of the β subtype (ERβ), it is possible that E2 acts in GnRH neurons to regulate GnRH secretion directly (Herbison & Pape, 2001). Most research evidence suggests, however, that E2 regulates GnRH secretion through its actions on interneurons (Herbison, 1998; Herbison, Chapman, & Dyer, 1991). For example, the magnitude of GnRH pulses may be modulated by the action of steroid hormones on cells that produce inhibitory neurotransmitters like gamma aminobutyric acid (GABA) or opioid peptides.

Anatomical data support the contention that GABA modulates GnRH release. For example, although the majority of GnRH neurons lack steroid hormone receptors, they do possess GABA receptors which when activated by GABA or progesterone metabolites decrease the magnitude of both GnRH and LH pulses (Herbison, 1997). Furthermore, there are considerable numbers of GABA neurons in regions adjacent to GnRH neurons and these cells contain both estrogen and progesterone receptors (Herbison, 1997; Herbison, 1998). In addition, there is evidence showing that ovariectomy decreases GABA turnover

in hypothalamic areas including the anterior preoptic area where the largest number of GnRH secreting neurons are located (Yoo et al., 2000).

The regulation of pulsatile GnRH and LH secretion has also been linked to the inhibitory effects of endogenous opioids. It is well established that endogenous opioids like β-endorphin, dynorphin, and enkephalins suppress LH pulsatility. Endogenous opioids appear to act primarily through the μ opioid receptor to regulate LH release, given that μ opioid antagonists such as naloxone rapidly increase GnRH and LH secretion (Kalra, 1993). The mechanisms by which opioids regulate GnRH and LH pulsatility, however, have not been described in detail although they may involve both direct and indirect actions of these peptides on GnRH cell bodies and terminals. For example, in spite of the fact that GnRH neurons do not contain opioid receptors (Sannella & Petersen, 1997), μ opioid agonists hyperpolarize GnRH neurons *in vitro* by modulating potassium (K) channels, and thus preventing the activation of these neurons by other agents (Lagrange, Ronnekleiv, & Kelly, 1995). The ARC may play an important role in the opioidergic control of GnRH given that it contains a large population of neurons that produce endogenous opioids, and which are also rich in P and E2 receptors both α and β (Shughrue, Lane, & Merchenthaler, 1997; Shughrue, Scrimo, & Merchenthaler, 1998a; Shughrue, Scrimo, & Merchenthaler, 1998b). These neurons project to various brain sites associated with the control of reproductive function (Kalra, 1993). It is therefore possible that the negative feedback effects of steroid hormones are mediated through the activation of opioidergic neurons within the ARC (Kalra, 1993). Alternatively, endogenous opioids may modulate GnRH release at the level of the median eminence where GnRH and opioid containing terminals coexist (Kalra, 1993). It has also been suggested that, endogenous opioids may serve as a brake to neurotransmitters such as norepinephrine, glutamate and nitric oxide which increase GnRH pulses

(Gore & Terasawa, 2001; Bhat et al., 1998; (McCann & Rettori, 1996; Prevot, Bouret, Stefano, & Beauvillain, 2000)).

In contrast to the tonic release of GnRH and LH secretion, the surge in GnRH and LH release in female mammals is attained by the action of stimulatory factors coupled with the removal of inhibitory signals. The events that lead to an LH surge include the coupling of the positive feedback effects of steroid hormones with the stimulatory effects of a circadian signal, and the withdrawal of inhibitory signals such as endogenous opioids and GABA (Everett, 1961a; Kalra, 1993; Kimura, Jinnai, & Sano, 1995; Levine, 1997).

Several lines of converging evidence implicate the anteroventral preoptic area (AVPV), a periventricular region within the anterior preoptic area, as an important site of the integration of these signals, and ultimately to the generation of an E2-induced LH surge. The AVPV has a large concentration of estrogen receptors both α and β (Greco, Allegretto, Tetel, & Blaustein, 2001; Shughrue, Scrimo, & Merchenthaler, 1998b; Simerly, Carr, Zee, & Lorang, 1996a), and has reciprocal connections with the suprachiasmatic nucleus (SCN; (van der Beek et al., 1994), the site of the circadian clock, as well as with neurons in the ARC. The AVPV is also connected to neurons near the vascular organ of the lamina terminalis (OVLT), where a large proportion of GnRH neurons are located (Le, Berghorn, Rassnick, & Hoffman, 1999; Le, Wise, Murphy, Coolen, & Hoffman, 2001). Furthermore, lesions to the AVPV disrupt the production of LH surges (Le et al., 2001; Wiegand & Terasawa, 1982; Wiegand, Terasawa, & Bridson, 1978; Wiegand, Terasawa, Bridson, & Goy, 1980). Finally, E2 treatment with or without P, to virgin OVX rats results in a marked increase in the expression of Fos-like immunoreactivity (FOS-IR) within the AVPV around the time of the LH surge (Insel, 1990; Auger & Blaustein, 1995; Le et al., 1999). Interestingly, FOS-IR in the AVPV correlates with the activation of GnRH

neurons and with the magnitude of the steroid-induced LH surge (Le et al., 1999).

Recently, it has been proposed that P receptors in the AVPV constitute the limiting factor in the induction of LH surges (Levine, 1997; Levine, Chappell, Schneider, Sleiter, & Szabo, 2001). According to Levine, E2 binds to estrogen receptors in the AVPV to increase progesterone receptor transcription. Once progesterone receptors are synthesized, they are transactivated by cyclic adenosine monophosphate (cAMP) a second messenger that can be produced after activation of G-protein coupled receptors. Cyclic AMP levels within the AVPV are assumed to be increased by the daily neuronal signal, which could come directly from neurons within the SCN (Krajnak, Kashon, Rosewell, & Wise, 1998a; Krajnak, Kashon, Rosewell, & Wise, 1998b; Krajnak, Rosewell, & Wise, 2001; van der Beek et al., 1994) or indirectly from noradrenergic or dopaminergic neurons in the mid and hind brain (Etgen, Chu, Fiber, Karkanias, & Morales, 1999; Sanghera, Anselmo-Franci, & McCann, 1991). In either case, the transactivation of progesterone receptors by cAMP ultimately leads to an increase in GnRH output which in turn is potentiated by the preovulatory peak in P secretion acting on these same progesterone receptors (Levine, 1997; Levine et al., 2001). The rising levels of P, however, also work to downregulate progesterone receptors to reduce GnRH output, and finally, to terminate the current LH surge and prevent its re-occurrence the following day (Freeman, 1994). Levine also proposed that if progesterone receptors are not present in the AVPV when cAMP is synthesized during the morning of an expected LH surge, the surge does not occur. In support of this, progesterone receptor knockout (PRKO) female mice do not show EB induced LH surges (Chappell, Lydon, Conneely, O'Malley, & Levine, 1997), and progesterone receptor blockers like RU486 given early on the morning of proestrus, also block E2-induced LH surges

(Chappell & Levine, 2000).

In rats as well as several other species of mammals, the LH surge is also associated with the activation of a subgroup of GnRH neurons just caudal to the OVLT. Both FOS-IR and JunB-like immunoreactivity are expressed in these GnRH neurons at the time of the preovulatory LH surge in intact animals, or after E2 or E2 + P treatment in OVX animals (Lee, Abbud, Smith, & Hoffman, 1992; Lee, Smith, & Hoffman, 1990a; Lee, Smith, & Hoffman, 1990b). As with FOS-IR in the AVPV, the proportion of OVLT GnRH neurons activated at the time of the surge correlates with the magnitude of the LH surge (Le et al., 1999; Le et al., 2001), and destruction of the OVLT results in the alteration of LH surges (Wiegand & Terasawa, 1982). Whether the induction of immediate early gene expression in this subgroup of GnRH neurons is necessary for a surge to occur remains undetermined but this neural event remains a good correlate of EB-induced LH secretion (Hoffman & Lyo, 2002)(see Figure 2).

Lactational diestrus: endocrine patterns

Changes in the HPO.- It is clearly established that in most species of mammals, including humans, pulsatile release of LH as well as the LH surge mechanism are suppressed for most of lactation (McNeilly, 1994). In contrast, FSH is suppressed only early in lactation. For example, FSH levels in lactating rats are comparable to those of cycling females after day 5 pp (Taya & Greenwald, 1982a; Taya & Greenwald, 1982b). As a result of the suppression of LH release, follicular maturation is retarded, resulting in lower E2 secretion from thecal cells in the ovary (Smith & Neill, 1977; Taya & Greenwald, 1982a) and low circulating levels of E2 in rats (Ota and Yokoyama; Smith & Neill, 1977).

In lactating rats, E2 levels gradually increase so that by end of the second

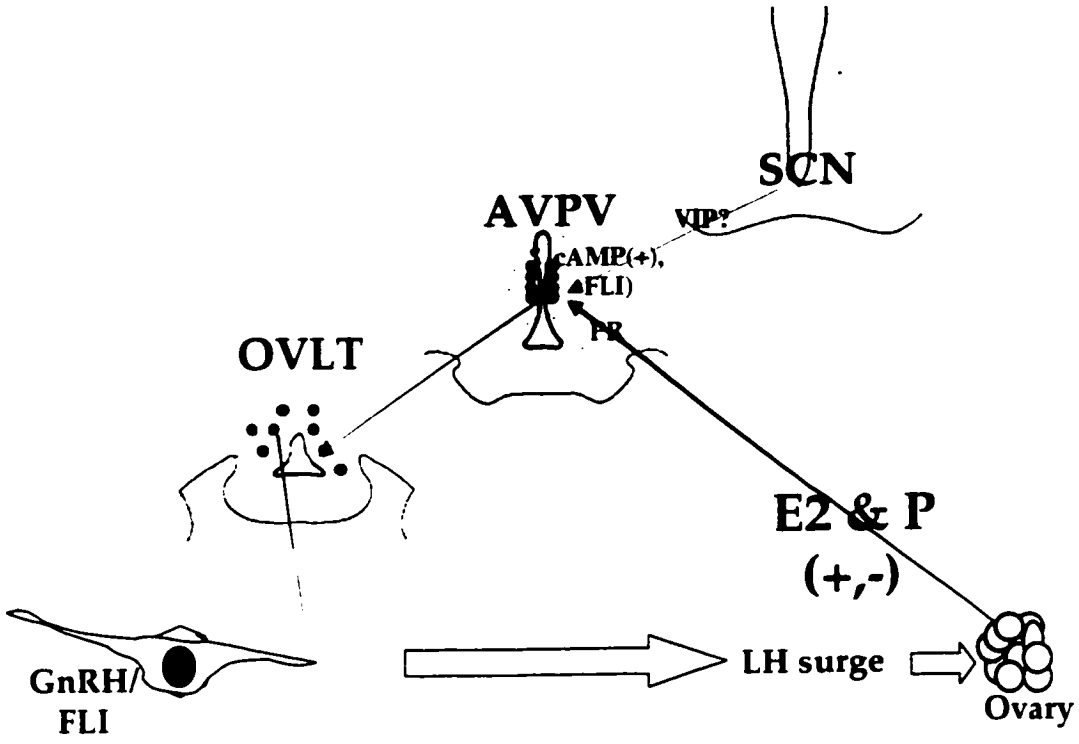

Figure 2.- Neuronal mechanisms underlying the LH surge. As seen in this figure, E2 induces the transcription of P receptors in the AVPV. These receptors are transactivated by the activation of second messengers systems such as cAMP which are produced by the coupling of signals from the SCN. Activation of neurons in the AVPV is accompanied by activation in a subgroup of GnRH neurons just caudal to the OVLT and a surge in LH release from the pituitary. The surge is advanced and potentiated by direct activation of P on AVPV P receptors. These receptors, however, are soon degraded by the preovulatory peak in P so as to prevent another surge the following day.

week pp they resemble those of cycling females on the (morning) of diestrus (Smith & Neill, 1977; Taya & Greenwald, 1982b). In addition, functional corpora lutea are formed after the postpartum ovulation. These secrete high amounts of P that peak at around day 10-13 pp and decrease thereafter (Smith & Neill, 1977; Grota & Eik-Nes, 1967). The corpora lutea are responsible for the increase in P release maintained by prolactin (PRL), a hormone that is secreted from the pituitary in response to suckling stimulation and that, in addition to its luteotropic effects, acts on the mammary gland to stimulate milk synthesis (Grattan, 2001; Ben-Jonathan, Arbogast, & Hyde, 1989; Jakubowski & Terkel, 1986; Sodersten & Eneroth, 1984). PRL levels are high early in lactation and gradually drop so that by the end of the third week of lactation they are similar to those of cycling females (Jakubowski & Terkel, 1986).

As noted above, the low levels of LH observed in lactating animals do not seem to reflect the inability of the pituitary to synthesize or secrete LH. Rather, evidence suggests that the primary alteration in the reproductive axis during lactation is a reduction in GnRH secretion (Smith, 1982). Because GnRH upregulates its own receptor, the low levels of GnRH release characteristic of lactation are associated with reduced GnRH receptor number in the pituitary of lactating rats (Smith, 1982). A full LH response can be elicited from the pituitary of a lactating rat if the rat is given GnRH pulses every 50 min for about 48-72 hours (Lee, Paul, & Smith, 1989b).

Maeda (Maeda et al., 1995) has proposed that nuclei within the medio-basal hypothalamus interact to generate pulsatile GnRH and hence pulsatile LH release, and that these nuclei are inhibited during lactation. He was able to restore LH pulsatility in lactating rats by severing afferent projections to the medio-basal hypothalamus (Maeda et al., 1995). The source of these afferent pathways has not yet been identified, but apparently they do not arise from

anterior hypothalamic regions because knife cuts separating the anterior hypothalamus from the medio-basal hypothalamus do not restore LH pulsatility. In fact, LH pulsatility was only restored after complete isolation of the medio-basal hypothalamus or after cuts that separated it from dorsal afferents (Maeda et al., 1995). Candidate afferent projections responsible for inhibiting LH release during lactation include GABAergic, dopaminergic, serotonergic, and noradrenergic cell groups that may be activated by the suckling stimulus (Li, Chen, & Smith, 1999a). Much work is needed to determine the specific influences of these cell groups in the regulation of pulsatile LH release during lactation.

The low levels of LH seen in lactation are accompanied by high circulating levels of prolactin (PRL), which is secreted in response to suckling stimulation (Smith & Neill, 1977). This association has led to the suggestion that PRL may play a role in the suppression of reproductive function during lactation (Smith & Neill, 1977). This idea has been reinforced by studies showing that exogenous, systemic PRL administration renders virgin females acyclic (Noel & Woodside, 1993). In lactating rats, however, PRL treatment after the removal of litters early in lactation does not maintain lactational diestrus (Hansen, Sodersten, & Eneroth, 1983). Moreover, while litter removal on day 1 pp is followed by ovulation four days later, pharmacological blockade of PRL with the dopamine D2-like receptor agonist CB154 (bromocryptine) does not restore ovulation until day 11 pp although it has an immediate effect on milk production. (Sodersten & Eneroth, 1984; Woodside, 1991).

Although these data do not support a role for PRL in the suppression of LH early in lactation, there is evidence supporting such a role for PRL in mid-late lactation. For example, suppression of PRL in mid lactation results in a rapid increase in LH and a return to estrus within two days of treatment (Hansen et al., 1983; Lu et al., 1976). There are multiple pathways by which PRL could inhibit the

HPO. PRL can act directly in the ovary to suppress EB synthesis (Dorrington & Gore-Langton, 1981; Dorrington & Gore-Langton, 1982), and it could presumably act on PRL receptors located in hypothalamic nuclei implicated in the control of reproductive function (Grattan, 2001; Pi & Voogt, 2001). A direct effect of PRL on these nuclei, however, seems unlikely given that intracerebral (ic) infusions of PRL into the ventricles, or directly into hypothalamic sites like the PVN, VMH and MPOA, do not result in acyclicity (Sauve & Woodside, 1996; Sauve & Woodside, 2000). It is more likely that these effects are mediated by the stimulatory effects of PRL on the corpora lutea and hence P secretion (Smith & Fox, 1984). Indeed, the effects of PRL suppression on the length of lactational infertility can be reversed by treatment with the P receptor antagonist RU486 without an effect on PRL levels (van der Schoot, Uilenbroek, & Slappendel, 1989).

Given that food restriction prolongs the duration of lactational infertility it is not surprising that it also results in a prolonged suppression of LH levels, which remain suppressed for at least five days following re-feeding (Walker, Mitchell, & Woodside, 1995). Interestingly, no differences were observed in the LH response to an exogenous GnRH challenge between ad lib fed and food restricted lactating rats, suggesting that, as in an ad libitum lactation, in a food restricted lactation the primary deficit is a deficiency in GnRH release (Walker et al., 1995). E2 levels have not been measured in food restricted dams, but one would expect that they would be lower in late lactation than those of ad lib fed rats because of the lack of ovarian stimulation by LH. PRL and FSH levels in food restricted dams are not different from those of ad lib fed dams (Walker et al., 1995; (McGuire et al., 1995). In contrast, P levels are higher in food restricted dams, and remain elevated until at least day 20 pp (Woodside, 1991).

Hypothalamic-pituitary-adrenal axis (HPA) and lactational diestrus. In human populations, conditions of starvation could lead to anovulatory states not only through physiological mechanisms that regulate energy homeostasis, but also through the activation of the neuroendocrine mechanisms that regulate stress (Bongaarts, 1980a; Bongaarts, 1980b). This idea is supported by data showing that the HPO is suppressed by activation of the HPA axis (Rivier, Rivier, & Vale, 1986; Walker, Dallman, Palmer, & Steele, 1993; Rivier, 1995). The inhibitory effects of stress and fasting on LH pulsatility are mediated by activation of brain stem catecholamines, particularly norepinephrine release in hypothalamic centers like the paraventricular nucleus (PVN) (Cagampang et al., 1992; Nagatani et al., 1996b). In cycling rats, it has been suggested that food restriction, like other stressors, increases noradrenergic stimulation of corticotropin releasing hormone (CRH) release from parvocellular neurons in the PVN via A2-noradrenergic receptors. This ultimately suppressess LH pulses (Cagampang, Maeda, Tsukamura, Ohkura, & Ota, 1991; Cagampang et al., 1992; Tsukahara, Tsukamura, Foster, & Maeda, 1999; Tsukamura, Nagatani, Cagampang, Kawakami, & Maeda, 1994). Moreover, EB enhances the inhibitory effects of fasting or glucoprivation on pulsatile LH release, suggesting that this pathway is also important for the negative feedback effects of EB on LH secretion (Cagampang et al., 1997; Cagampang et al., 1992). Finally, CRH producing neurons from the PVN, a hypothalamic center that regulates secretion of adrenal steroid hormones, send direct projections to GnRH neurons and could modulate their secretion directly (Rivest & Rivier, 1993; Rivest & Rivier, 1995). Therefore, the effects of food restriction on the length of lactational infertility could be mediated by increased activity of the HPA.

Indeed, the energetic demands of lactation do affect the hypothalamic-pituitary-adrenal (HPA) axis, and these effects are similar to those that are seen

in animals after chronic stressors (Lightman, 1992; Lightman & Young, 1989; Toufexis et al., 1998; Toufexis & Walker, 1996; Walker et al., 1993). One would assume that these effects are exacerbated by food restriction during lactation because these females are not allowed to compensate for the energy spent in producing milk. The influences of the HPA axis on the reproductive system of lactating rats, however, may be attenuated given that this system is hyporesponsive during lactation (Lightman, 1992; Lightman & Young, 1989; Toufexis et al., 1998; Toufexis & Walker, 1996; Woodside, Robinson, & Amir, 1995; Abizaid & Woodside, 2002). In addition, lesions to the PVN, the main source of CRH, do not restore LH pulses in OVX lactating rats (Tsukamura, Ohkura, Coen, & Maeda, 1993). Finally, the HPA axis of food restricted dams is not markedly different from that of ad lib fed dams. Although adrenocorticotropin hormone (ACTH) is increased in food restricted rats early in lactation, levels of this hormone are not elevated on days 15 or 21 pp in food restricted dams compared to ad lib fed dams and corticosterone levels do not differ between animals in the two diet conditions (Walker et al., 1995).

In sum, these data suggest that the primary deficit in both ad lib fed and food restricted dams is low levels of LH resulting from a reduction in GnRH secretion and that this state is prolonged in under conditions of decreased food availability. The prolonged suppression of GnRH secretion seen in food restricted dams does not result from changes associated from increased suckling intensity or frequency, and does not seem to be associated with changes in metabolic fuels available for oxidation or with hyperactivity of the HPA axis. It seems, however, that nutritional signals like leptin may shorten the lactational diestrus of rats if the main outlet of energy expenditure, milk production, is blocked as done by the dam itself in 48 hour fast, or surgically by the GC manipulation that prevents milk release. Leptin may exert this effect through a

reduction in NPY release from the ARC on to GnRH neurons and/or terminals, but this effect is dependent on the energetic state of the dam.

Nonetheless, low concentrations of LH levels seen in both ad lib fed and food restricted dams lead to a decrease in ovarian stimulation and hence the absence of E2 secretion from the ovary that is necessary to stimulate an LH surge. Furthermore other data suggests that even if the positive feedback signal from E2 were present, the mechanisms through which it would stimulate the LH surge are also inhibited during lactation. This evidence is described in the next section

Lactation and the positive feedback effects of E2

It is possible that, in addition to the inhibitory effects of the suckling stimulus and changes in metabolic signals like leptin, the reproductive axis of lactating rats is quiescent because of a decrease in its response to the hormonal and/or circadian signals that produce ovulation. During lactation, E2 levels are low, and do not reach the levels necessary for the priming of an ovulatory response until about day 15 pp (Smith & Neill, 1977). However, restoring circulating E2 levels may not be sufficient to fully restore the positive feedback effects. Indeed, the positive feedback effects of steroid hormones on ovulation may be altered during lactation and after food restriction. For example, Smith (1978) gave an acute E2 treatment to lactating rats at either day 5, 10 or 15 pp and compared the LH surges elicited to those of virgin females given the same treatment. These studies demonstrated that E2 induced LH surges in all animals, but that these surges were attenuated in lactating rats in comparison to those seen in virgin females on proestrus. Hodson et al (Hodson, Simpkins, & Meites, 1978), also reported that acute E2 treatment followed by P produced attenuated

LH surges on day 10 pp; OVX increased the magnitude of the E2-induced surges. Finally, Coppings and McCann showed that chronic treatment with E2 silastic capsules induced surges of LH in intact lactating rats on the day following the implantation of the capsules, but these failed to reoccur on subsequent days (Coppings & McCann, 1979). These studies show that even if circulating levels of E2 are restored in lactating rat by E2 replacement, ovulation may not occur because the LH surge response to E2 is attenuated.

Hypotheses

Previous research has focused on the suppression of basal levels of LH as the main mechanism through which food restriction prolongs lactational infertility. Ovulation, however, depends both on sufficient FSH and LH secretion to stimulate the production of an ovulatory follicle and sensitivity to the positive feedback effects of the estrogen secreted by that follicle. Given that a reduced response to such positive feedback signals has been reported in ad lib fed lactating rats (Coppings & McCann, 1979; Hodson et al., 1978; Smith, 1978), the following experiments examined whether a prolongation of such reduced sensitivity plays a role in the increased length of lactational infertility produced by food restriction, and the pathways mediating this effect in both ad lib fed and food restricted rats. Chapter 2 examined whether: 1) food restricted dams would show E2-induced LH surges that are attenuated for a longer period of time than those of ad lib fed dams; 2) the removal of the ovaries, the primary source of circulating P, or treatment with P receptor antagonists would restore the ability of E2 to induce LH surges in food restricted dams; and 3) giving exogenous P treatment would extend the period of insensitivity to the effects of E2 on the LH surge of ad lib fed dams.

Exogenous GnRH treatment to food restricted dams produces similar LH responses to those seen in ad lib fed dams (Woodside & Jans, 1995). This suggests that food restriction does not alter pituitary responsiveness to GnRH, and that the prolonged lactational diestrus results from a prolonged inhibition in GnRH release from the hypothalamus. Therefore, any suppression of LH release after E2 treatment to food restricted dams would potentially be due to a reduction of E2 induced GnRH output. It has been shown previously that a 48-hour fast that attenuates the E2-induced LH surge in intact hamsters also reduces the proportion of GnRH neurons that are activated by gonadal steroids for the induction of the LH surge (Berriman, Wade, & Blaustein, 1992). Similarly, aging female rats that show attenuated LH surges similar to those of E2 treated dams also show less preovulatory neuronal activation in both AVPV and GnRH neurons (Krajnak et al., 2001; Le et al., 2001). It is not known, however, how these neural correlates of the LH surge are affected during lactation. Experiments in Chapter 3 assessed the responsiveness ad lib fed and food restricted dams to E2 in areas like the AVPV, and within GnRH neurons in the OVLT using FOS-IR as an index of neuronal activation. It was expected that if food restriction prolonged insensitivity to the positive feedback effects of E2, this would be reflected in patterns of FOS-IR in the AVPV and in the proportion of GnRH neurons co-expressing FOS-IR within the OVLT (see Figure 3).

Finally, it is thought that E2 acts in the AVPV to induce progesterone receptors, and these are necessary for the LH surge (Levine, 1997; Levine et al., 2001). Thus, it is possible that any effect of food restriction on the E2-induced LH surge of lactating rats is the result of a decrease in the number of ER that are targeted by E2, and/or by a decrease in their ability to induce PRs. This last issue was also addressed in Chapter 3 (see Figure 3).

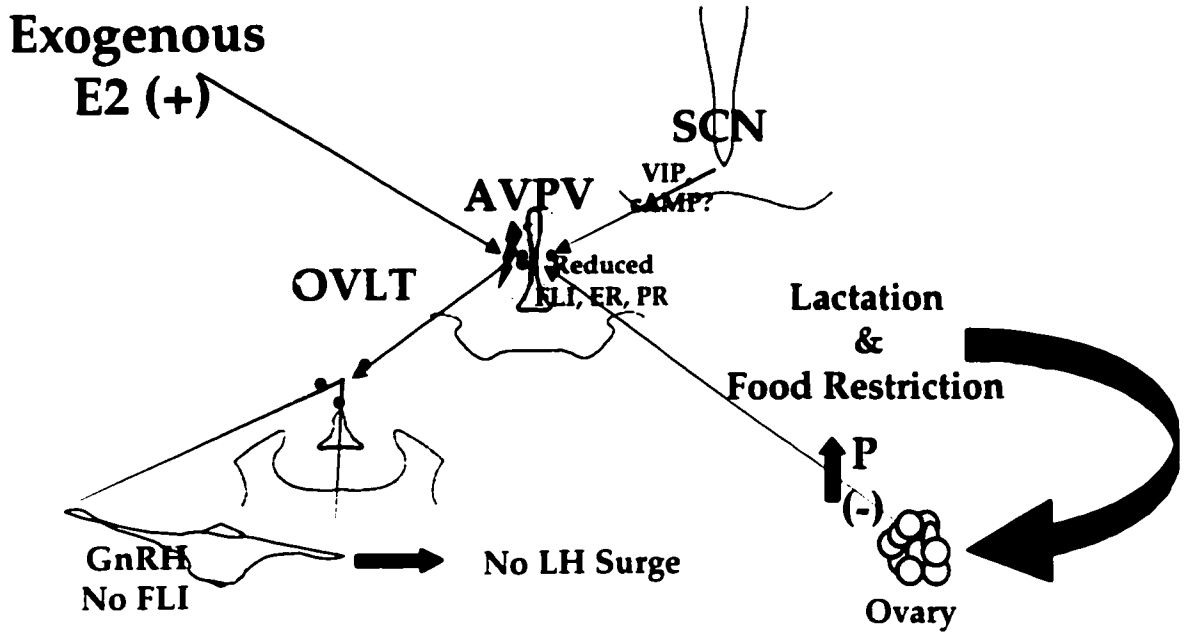

Figure 3.- Hypothetical neuroendocrine mechanism by which food restriction during lactation prolongs lactational diestrus. Elevated P levels that are seen during lactation attenuate LH surges by interfering with the neuroendocrine mechanisms subserving the positive feedback signals of E2, thereby delaying ovulation. This state may be exacerbated and/or prolonged by food restriction because food restricted dams secrete higher levels of P for an extended period of time.

CHAPTER 2

Effects of food restriction on the positive feedback effects of E2 on LH release during lactation

The experiments described in this chapter were designed to address three issues: 1) whether the time course of restoration of sensitivity to the positive feedback effects of estrogen differed between ad lib fed and food restricted rats, 2) the effect of removing ovarian influences during lactation on this positive feedback system and 3) the role of progesterone in the modulation of sensitivity to the positive feedback effects of E2.

Experiment 1

Exogenous administration of E2 induces LH surges in lactating rats, but these surges are attenuated in comparison to those seen in preovulatory cycling rats, or in OVX steroid treated virgin rats (Coppings & McCann, 1979; Hodson et al., 1978; Smith, 1978). Whether food restriction further attenuates the magnitude of the LH surge and/or prolongs the duration of hyporesponsiveness to exogenous E2 administration in lactating rats is not known. Previous data, however, have shown that 48 hours of food deprivation during estrous and metestrous days of the estrous cycle in female hamsters attenuates the E2 induced LH surge (Berriman et al., 1992). Similarly, an acute 72 hour fast results in the suppression of the steroid induced LH surge in steroid treated OVX female virgin rats (Watanobe & Schioth, 2001). Given these data, we hypothesized that a food restriction regimen that prolongs lactational infertility

and the suppression of basal LH release, would also result in either a more profound suppression of the LH surge mechanisms and/or a state of hyporesponsiveness to the positive feedback effects of E2 that is prolonged compared to that seen in ad lib fed dams.

The LH surge response is commonly examined in virgin animals with the use of exogenous administration of E2 combined with an injection of P on the morning of the expected LH surge (i.e. Lee et al., 1990b). Because lactating rats have high concentrations of plasma P, the use of a treatment of E2 alone that has been previously used to elicit LH surges in both cycling and lactating rats was selected (Smith, 1978). To validate this treatment, a pilot study was conducted giving this E2 treatment to OVX virgin female. Plasma LH concentration from these animals are depicted in Figure 4. As shown in this figure, E2 treatment increased peak LH concentrations dramatically from those seen on the morning of the expected surge.

In this experiment the LH responses to this E2 treatment were compared between ad lib fed and food restricted dams at 1) peak lactation when neither group has typically shown a vaginal estrus (day 15 pp); 2) a day in which ad lib fed animals, but not food restricted dams, begin showing vaginal estrus (day 20 pp); and 3) a day at which food restricted dams begin to show estrus smears (day 25 pp).

General Method

Animals. Female Wistar rats weighing between 220-240 gm were obtained from Charles River Breeding Farms (St. Constant, Québec). Upon arrival, animals were housed four rats per cage in stainless steel cages (50 cm X 20 cm X 15cm). One week later, a male rat was introduced to some of the group cages for

the purpose of mating. Female rats were housed individually in shoe-box cages (45 cm X 25 cm X 20 cm) as soon as pregnancy became noticeable, whereas unmated animals were housed individually in similar cages just prior to the onset of the study. All animals were kept under a 12 hour light/dark cycle (lights on at 08:00h), at a constant temperature (20°C ± 1°), and humidity. All procedures used in these experiments were approved by the Concordia University Animal Care committee, and followed the guidelines set by the Canadian Council for Animal Care.

Procedure

The day on which pregnant rats gave birth was assigned as day 0 pp. Litters were culled to eight pups on Day 1 postpartum (pp). Finally, food intake and body weight of all animals (non lactating, lactating and their litters) were recorded throughout the experiments. Lactating rats were assigned to groups according to the day of lactation on which blood samples were collected (day 15, 20, or 25 pp) and diet condition (ad lib or food restricted). Six groups were formed in total: 1) Ad lib day 15 (AL15, n=5); 2) Ad lib day 20 (AL20, n=5); 3) Ad lib day 25 (AL25, n=6); 4) Food restricted day 15 (FR15, n=7); 5) Food restricted day 20 (FR20, n=7); 6) Food restricted day 25 (FR25, n=5).

Food Restriction. Animals in the food restricted groups were given 50% of a previously determined ad libitum intake for each of days 1-14 pp.

Jugular Cannulation. Two days prior to blood sampling animals were anesthetized with a mixture of ketamine and xylazine (6 mg ketamine and .86 mg xylazine/ 100 gm of body weight) and were equipped with a jugular silastic catheter (Dow corning; OD= 0.047 in, ID= 0.025 in) filled with heparinized saline (50 U heparin/ml). The catheter was inserted 25 mm into the right jugular vein

so that it rested on top the atrium of the heart. It was then secured to the vein with surgical thread, and exteriorized between the scapulae.

E2 Treatment. On the day of surgery animals received a subcutaneous (sc) priming injection of E2 (Estradiol Benzoate, ICN) (1μg/.1 ml of peanut oil), followed by a second sc injection of E2 given at 09:00 of the following morning (250 μg/.1 ml of peanut oil). This dose was chosen because it has previously used to induce LH surges in lactating rats (Smith, 1978) and, as shown by our pilot study, is effective in producing robust LH surges (20-25 ng/ml) in virgin OVX animals within our colony (see figure 4). Finally, to ensure the E2 treatment was effective in inducing vaginal cornification, vaginal smears were collected starting on the first day of E2 treatment.

Blood Sampling. Blood sampling took place every hour beginning at 11:00h and ending at 21:00h. Two hours prior to sampling (09:00 hours), the jugular catheter was connected to a 30 cm piece of PE50 tubing (Beckton Dickinson) and exteriorized out of the cage. Animals were then left undisturbed with their litters until sampling began. At each sampling, 400 μl of blood were withdrawn through the extension tubing and were replaced with equal amounts of heparinized saline. All animals appeared undisturbed by this procedure and most slept or nursed their pups throughout the sampling phase.

LH RIA. Blood samples were centrifuged soon after collection, and plasma pipetted and stored at -20°C until assayed. Selected samples were assayed in duplicate using a commercial kit for LH radioimmunoassay (Amersham Pharmacia Biotech Ltd., Montréal, Canada). Sensitivity for the kit ranged from .08-50 ng LH/ml of plasma. Intra-assay variability was determined at 8.4%, inter-assay variability was assessed at 6.5%, and non-specific binding was less than 5%.

Statistics. A 2 X 3 X 2 repeated measures analysis of variance (ANOVA)

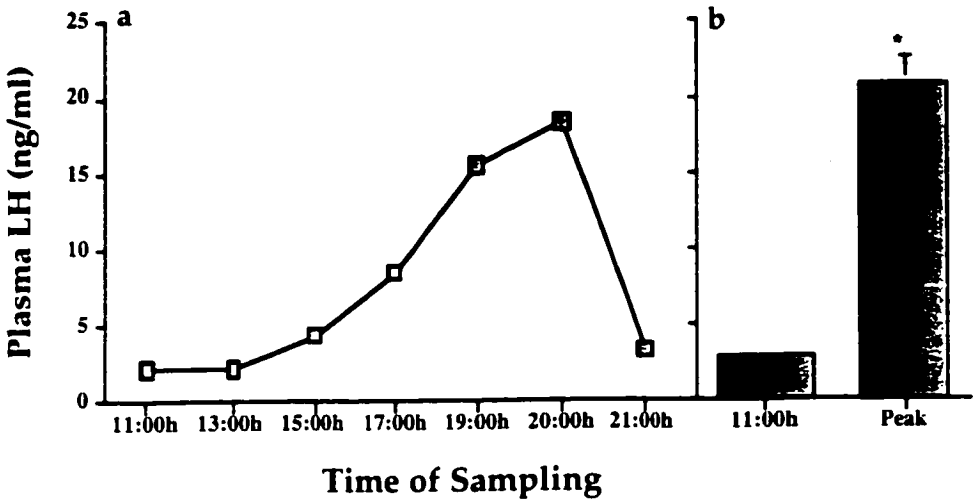

Figure 4.- Plasma LH after E2 treatment in OVX female virgin rats. Panel a) Example of an LH response to E2 treatment across the sampling day. As seen in this panel, E2 treatment produced a surge in LH release between 19:00 and 20:00 hrs (see panel a). Panel b) Group means ± SE of E2-induced baseline and peak LH concentrations. Mean LH concentrations at the time of the peak were significantly different from those at 11:00 on the day of the expected LH surge (see panel b).

with Diet (ad lib fed vs. food restricted) and Day of Lactation (Day 15, 20 and 25pp) as the between subjects variables and Time (11:00 vs. peak LH levels) as a within subjects variable was used to analyze differences in LH levels after E2 treatment between the different groups.

Individual t-tests were conducted to compare overall differences in food intake, dam weight change from day 1 pp, and daily litter weight gain between ad lib fed and food restricted dams on day 15 pp. Similar t-tests were conducted on data from dam weight change and litter weight gain on those animals tested on day 20 pp and on those tested on day 25 pp.

Results

The ability of E2 to generate positive feedback response in ad lib fed and food restricted females on days 15, 20 and 25 pp is shown in Figures 5 and 6. Statistical analyses of these data showed that the overall LH responses to E2 were smaller in food restricted dams compared to ad lib fed dams (significant main effect for Diet ($F(1,29)= 4.88$, $p < .05$). Also the overall LH responses to E2 increased as lactation advanced (significant main effect for Day of Lactation ($F(2,29)= 6.46$, $p < .05$)). Finally, a significant main effect for Time of Sampling ($F(1,29)= 22.53$, $p < .05$) revealed that overall, lactating rats showed significantly higher levels of circulating LH in the afternoon following E2 treatment than in the morning following E2 treatment.

A significant Day of Lactation X Time of Sampling interaction effect ($F(2,29)= 5.64$, $p < .05$) followed by Post hoc one way ANOVAs demonstrated that peak LH responses to E2 were significantly higher in day 25 pp dams compared to those on day 20 or day 15 pp dams. In addition, a significant Diet X Time of sampling interaction ($F(1,29)= 4.49$, $p < .05$) followed by post hoc one

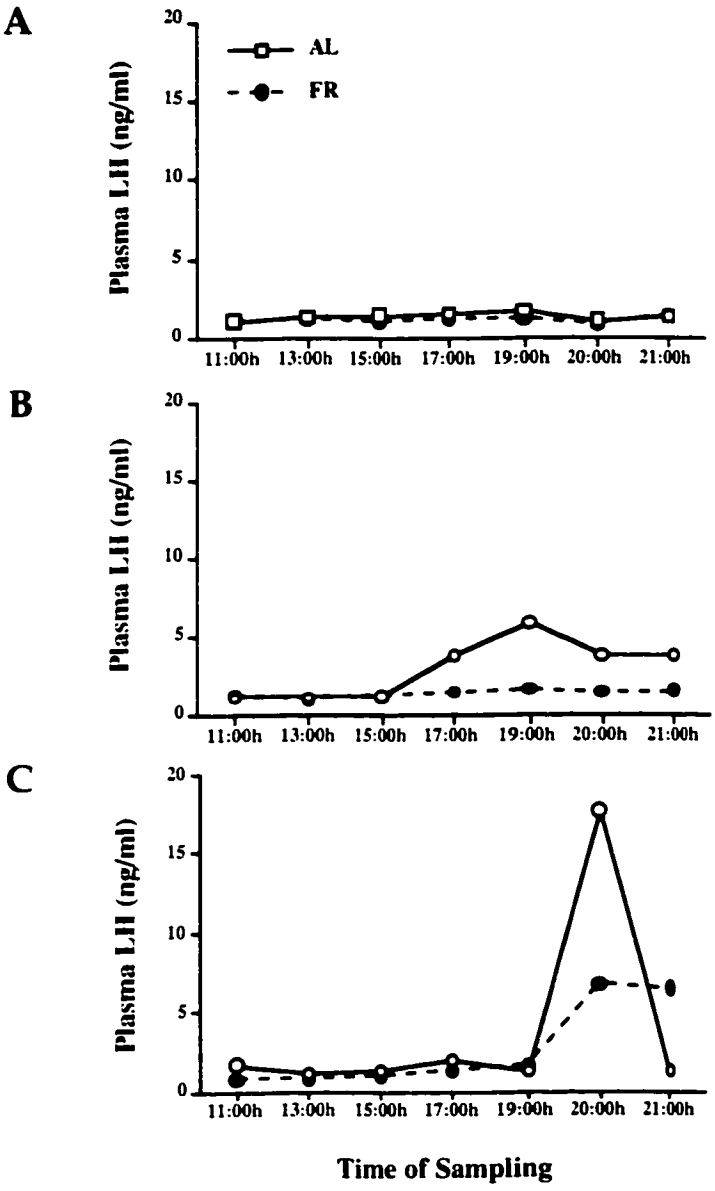

Figure 5.- Examples of LH responses to exogenous E2 administration in ad lib fed (AL) and food restricted (FR) lactating rats. Panel A depicts sample data from day 15 pp dams, panel B shows representative data from dams sampled on day 20 pp, and panel C shows data from representative animals on day 25 pp.

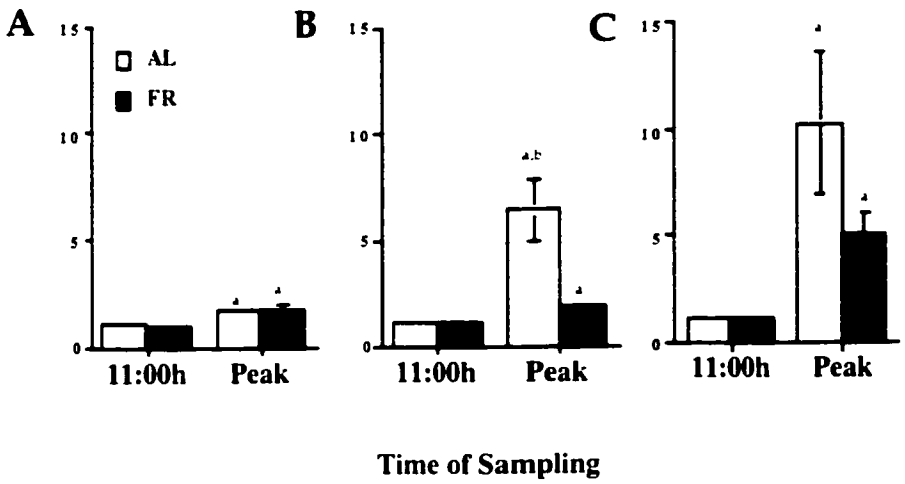

Figure 6.- Mean (± SE) LH concentrations in ad lib fed (AL) and food restricted (FR) dams on the day following E2 treatment. Panel A depicts mean LH levels of day 15 pp dams at 11:00h, and at the time in which their LH concentrations peaked. Panel Bdepicts LH concentrations after the same treatment on day 20 pp, and panel C shows LH concentrations after E2 treatment to day 25 pp dams. As seen in this figure Food restricted dams show a prolonged insensitivity to the positive feedback effects of EB on LH release.

a= significant from 11:00h, p.< .05

b= significant from food restricted, p.< .05

way ANOVAS revealed that peak LH responses to E2 treatment were smaller in food restricted dams compared to peak LH responses to E2 treatment seen in ad lib fed dams.

Posthoc independent samples t-tests revealed that peak LH concentrations after E2 treatment were not different on day 15 pp ($t(10)= -.02$, $p.>. .05$). By day 20 pp, peak LH concentrations increased dramatically in ad lib fed dams and were significantly higher than those of food restricted dams ($t(10)= 3.07$, $p.< .05$). By day 25 pp, peak LH concentrations increased in food restricted dams and were comparable to those seen in ad lib fed dams ($t(9)= .27$, $p.> .05$). Finally, the ANOVA conducted on the overall data yielded no significant Diet X Day of Lactation X Time of Sample interaction ($F(2,29)= 1.23$, $p.> .05$).

Statistical analyses of control data showed that food restricted dams consumed overall less food throughout the food restriction regimen ($t(37)= 21.47$, $p.< .05$) and amounted to an average of 52% of the intake of the ad lib intake from animals in this experiment (see Figure 7).By day 15 pp, food restricted dams had lost 18% and ad lib fed dams gained 15% of their day 1 pp weight ($t(37)= 5.44$, $p.< .05$; see Figure 7). By day 20 pp, food restricted dams had regained the weight lost throughout the food restriction period and weighed about 5% above their day 1 pp weight. Their mean weight change, however, was still below that seen in ad lib fed dams on the same day ($t(25)= 4.03$, $p.<.05$). The t-test conducted on the weight change from day 1 pp data revealed that by day 25 pp food restricted dams had gained comparable amounts to that gained by ad lib fed dams from their respective day 1 pp weights ($t(12)= 1.88$, $p.< .05$).

Litters nursed by food restricted dams grew at a slower rate than those nursed by ad lib fed dams throughout the first two weeks of lactation (56% of ad lib, $t(37)= 13.66$, $p.<.05$; see Figure 7). When comparing the mean daily weight

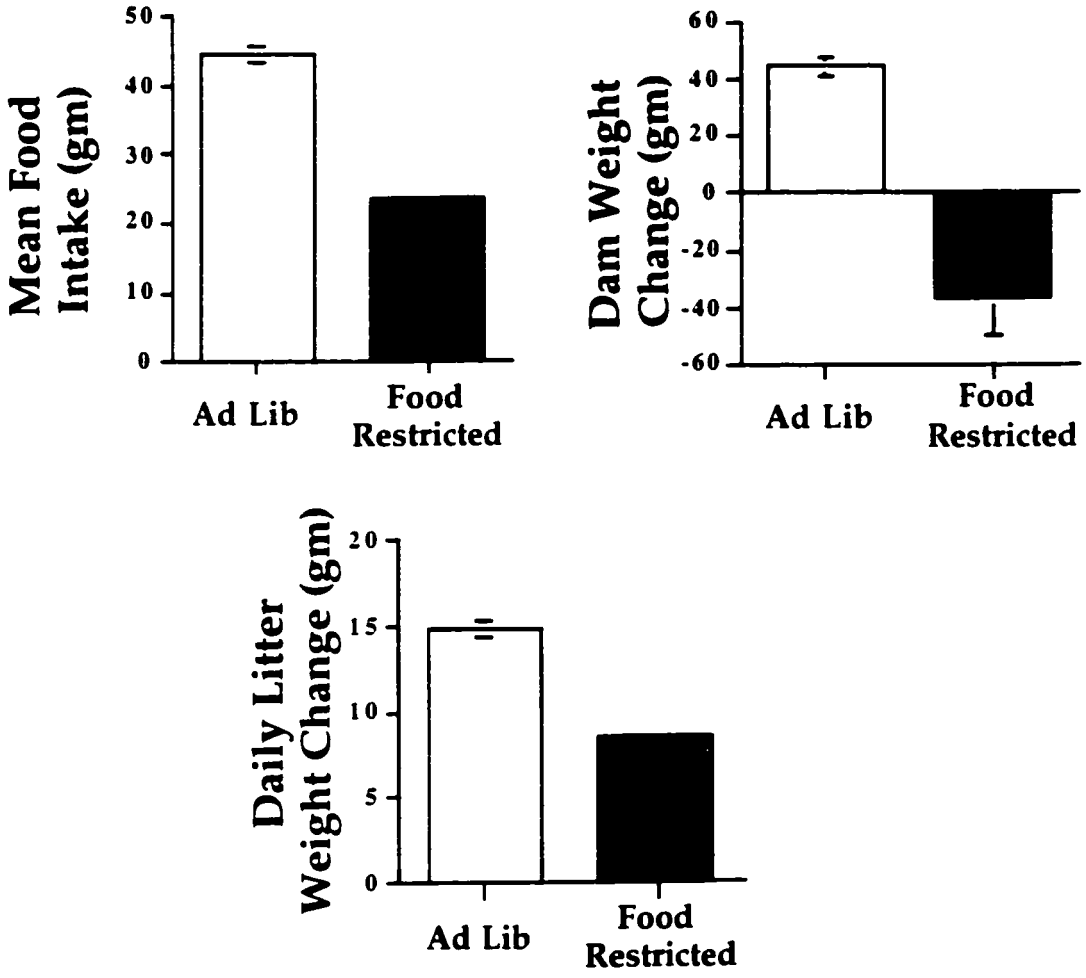

Figure 7.- Mean (± SE) Food intake, dam weight change and daily litter weight change during the first two weeks of lactation for all animals in Experiment 1. As seen in this figure, food restricted animals ate approximately 52% of the ad lib intake in this experiment, resulting in a significant drop in dam weight (17% of day 1 pp weight) and a decrease in milk yield as reflected by daily litter weight change.

gain of litters from food restricted dams and that of litters of ad lib fed dams from day 15 to day 20 pp no significant differences were found ($t(25) = .13$, $p > .05$), suggesting that the rate of weight gained by litters from food restricted dams increased soon after dams were given free access to food. All litters gained similar amounts of weight from day 20 to day 25 pp ($t(12) = .99$, $p > .05$).

Discussion

Results from Experiment 1 reveal three major findings. First, they show that day 15 pp lactating rats nursing eight pups have a small response to exogenous E2 treatment that is not different between ad lib fed and food restricted dams. Second, they show that, although LH release after E2 treatment increases in ad lib fed dams by day 20 pp, it remains small in the food restricted dams. Finally, by day 25 postpartum the LH surges induced by E2 treatment are increased and are comparable in ad lib fed and food restricted dams. These data, therefore, correlate well with the duration of the lactational diestrus of ad lib fed and food restricted dams respectively (Woodside, 1991), and suggest that in addition to maintaining low levels of basal LH secretion, food restriction during lactation also prolongs the reduction in sensitivity to the positive feedback effects of E2.

These results are also consistent with previous research in which lactation was linked to a state of reduced responsiveness to the positive feedback effects of E2 on the reproductive system. For example, Smith reported that exogenous estradiol challenges similar to those used in our study induced only attenuated LH surges in lactating rats at days 5, 10 and 15 pp compared to those induced in virgin animals following the same E2 treatment (Smith, 1978). Attenuated surges have also been reported in lactating animals after E2 followed by progesterone

treatment (Hodson et al., 1978).

It is possible that the prolonged inability to produce substantial LH surges following E2 treatment in food restricted dams is due to lower circulating levels of E2. Lactating rats show very low levels of circulating E2 that gradually rise during the second week pp (Smith & Neill, 1977; Taya & Greenwald, 1982b). This gradual restoration of E2 levels may be important to prime the mechanisms regulating E2-induced LH surges. Thus, the low basal levels of LH seen in food restricted dams could result in low circulating E2, and hence, play a role in the prolonged insensitivity to E2. In support of a priming role of E2 on LH surge induction, Tsukamura and colleagues have shown that OVX lactating rats show daily LH surges and elevated LH pituitary stores 48 hours after being equipped with silastic capsules filled with E2 (Tsukamura, Maeda, & Yokoyama, 1988). This treatment, however, only induced surges of a small magnitude in intact lactating rats, suggesting that ovarian factors produced during lactation such as P or inhibin may also act to inhibit the LH surge system.

Comparison of figures 4 and 6 show that even on day 25 pp, the increases in LH observed in both ad lib fed and food restricted dams after E2 treatment were relatively small compared to those seen in OVX virgin females and resemble those seen in aging female rats. This may reflect the fact that the dams in the current studies were with their litters at all times and that the amount of suckling stimulation they were receiving was sufficient to maintain some suppression of LH release. Indeed, Coopings et al. have shown that the magnitude of E2-induced LH surges in lactating rats at peak lactation is inversely related to the number of pups that the dam is nursing (Coppings & McCann, 1979). The pathways through which such suckling-induced inhibition might be expressed are not known, but it is conceivable that they involve direct effects of suckling stimulation on peptide or neurotransmitter systems (Li et al., 1998a; Li

et al., 1999a). Alternatively, the daily circadian signal, which is a prerequisite for the initiation of the LH surge may be attenuated during lactation as is the case in aging female cycling rats (Wise, 1982).

While the average group LH peaks occurred between 19:00h and 20:00h, the timing of the peak in LH concentration for individual animals was variable particularly in dams that showed a larger surge in LH. It may be that the priming regimen used to stimulate the LH response in this experiment contributed to this variability. Here we used a priming dose of E2 followed by a bolus dose of E2. While E2 is indispensable for the induction of the LH surge, the peak of pre-ovulatory P seems to be an important factor not only in enhancing its magnitude, but also in advancing it, and perhaps tightening the timing of the LH surge. Similar variability in the timing of peak LH secretion has been reported in intact cycling females, however, thus it remains possible that the variation we observed reflected individual variation in rhythmicity (Gans & McClintock, 1993).

In summary, the results obtained in this experiment suggest that the duration of lactational diestrus results not only from inhibition of pulsatile LH release (Smith & Neill, 1978), but also from an attenuated response to the positive feedback effects of E2. Moreover, food restriction seems to prolong both of these effects. The mechanisms underlying these effects are examined in the following experiments

Experiment 2

As seen in Experiment 1, the positive feedback effects of E2 on LH release are suppressed at peak lactation (day 15 pp), and remain suppressed in food restricted dams for at least five days post re-feeding (day 20 pp). By day 25 postpartum there is no longer an effect of diet condition on the E2 induced surge. Previous research has implicated an ovarian factor in the suppression of positive feedback effects during lactation. For example, Hodson et al. showed that E2-induced LH surges are greater in magnitude in OVX than in intact lactating rats (Hodson et al., 1978). This experiment examined the effects of ovariectomy on the ability of exogenous E2 to induce an LH surge in ad lib fed and food restricted females on Days 15 and 20 postpartum.

Method

Animals in this experiment were kept under the same laboratory conditions as those in Experiment 1. Similarly, E2 treatment, jugular cannulation, blood collection and plasma analysis for LH concentrations were as described in Experiment 1. The food restriction ration was modified in this experiment because OVX lactating rats eat more than intact dams.

Procedure

Four groups were formed according to the diet condition they were in (ad lib fed vs. food restricted) and the day of lactation in which their blood was collected (day 15 and 20 pp): 1) OVX Ad lib fed Day 15 (n=6); 2) OVX Ad lib fed Day 20 (n=6); 3) OVX Food Restricted Day 15 (n=6; and 4) OVX Food Restricted

Day 20 (n=6). On day 1 pp, lactating animals were anesthetized with a mixture of ketamine and xylazine (6 mg ketamine and .86 mg xylazine/ 100 gm of body weight) and their ovaries were dissected bilaterally. The incision was then sutured and sprinkled with antibiotic powder (Cicatrin).

Statistics. A 2 X 2 X 2 repeated measures analysis of variance (ANOVA) with Diet (OVX ad lib fed vs. OVX food restricted) and Day of lactation (Day 15, and 20 pp) as the between subjects variables and Time (11:00 vs. peak LH levels) as a within subjects variable was used to analyze differences in LH levels after E2 treatment between the different groups. Repeated measures ANOVAs using either Diet (Ad lib vs. food restricted) or Day of lactation (Day 15,and 20 pp) as the between groups variables and Time (11:00 vs. Peak) as the within subjects variable were used as post hoc tests.

Data for food intake, dam and litter weight were analyzed as in Experiment 1.

Results

The LH responses to E2 treatment in food restricted and ad lib fed OVX dams on days 15 (top panel) and 20 pp (bottom panel) are shown in Figure 8. Analysis of these data revealed that, regardless of diet condition, LH levels were significantly higher at the peak than those seen at 11:00h (main effect for time of sampling, $F(1,20)= 28.43$, $p.< .05$). A non-significant main effect for diet suggested that the overall LH responses were similar in both ad lib fed and food restricted dams ($F(1,20)= 1.66$, $p.> .05$). Similarly, animals did not differ significantly in their LH response on either day 15 or day 20 pp (main effect for day of lactation, $F(1,20)= .097$, $p.> .05$). Interaction effects were not statistically significant ($p. > .05$, see Figure 8).

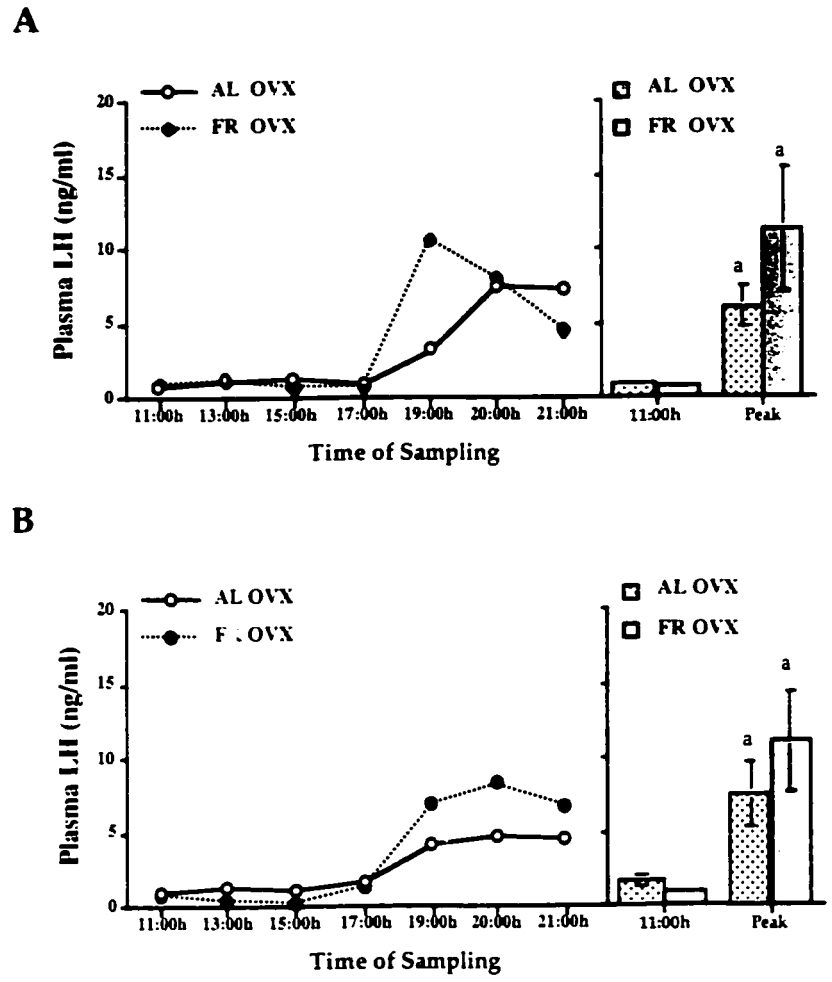

Figure 8.- LH discharges after E2 treatment in OVX ad lib fed and food restricted lactating rats on day 15 (panel A) and day 20 (panel B) pp. As seen in this figure, exogenous E2 treatment elicited comparable LH surges in ad lib fed and food restricted ovx lactating rats at both days examined, suggesting that ovariectomy liberates the suppressive effects of food restriction on E2-induced LH surges. Line graphs depict data from a representative animal from each group and bar graphs show mean (± SE) for each group.

a= significant from 11:00h, $p < .05$.

A between groups t-test revealed that in average, food restricted dams ate significantly less (54.8%) than ad lib fed dams (t (22)= 17.68, p.< .05) during the first two weeks of lactation. During this same period, food restricted dams lost an average of 63 grams whereas ad lib fed dams gained about 40 grams over their day 1 pp weight. This change was shown to be statistically significant (t(22)= , p.< .05)(See Figure 9). Food restricted dams, however, recovered so that by day 20 pp they weighed the same as they did on day 1pp. Ad lib fed dams still weighed about 40 grams above their day 1 pp weight (significant from food restricted, t(10)= 5.37, p.< .05).

The average daily litter weight gain over the first two weeks of lactation was compared using a between groups t-test. These tests revealed that litters nursed by ad lib fed dams gained more weight than those nursed by food restricted dams (t(22)= 9.61, p.< .05; see Figure 9). From days 15 to day 20 pp, however, litters nursed by dams in the food restricted group gained the same amount of weight daily as did litters nursed by ad lib fed dams (t(10)= -.35, p.> .05).

Discussion

These results show that removal of the ovaries, leads to a restoration of the ability of E2 to produce LH surges in lactating rats regardless of diet condition. Interestingly, LH surges are restored even in food restricted animals on day 15 pp, a time of great energetic challenge compounded by the challenge of food restriction. Finally, LH responses to E2 were similar between OVX ad lib fed dams and OVX food restricted dams on day 20 pp and did not differ from those on OVX dams on day 15 pp. These results are in agreement with others who have shown that E2 treatment induces larger LH surges in OVX dams than

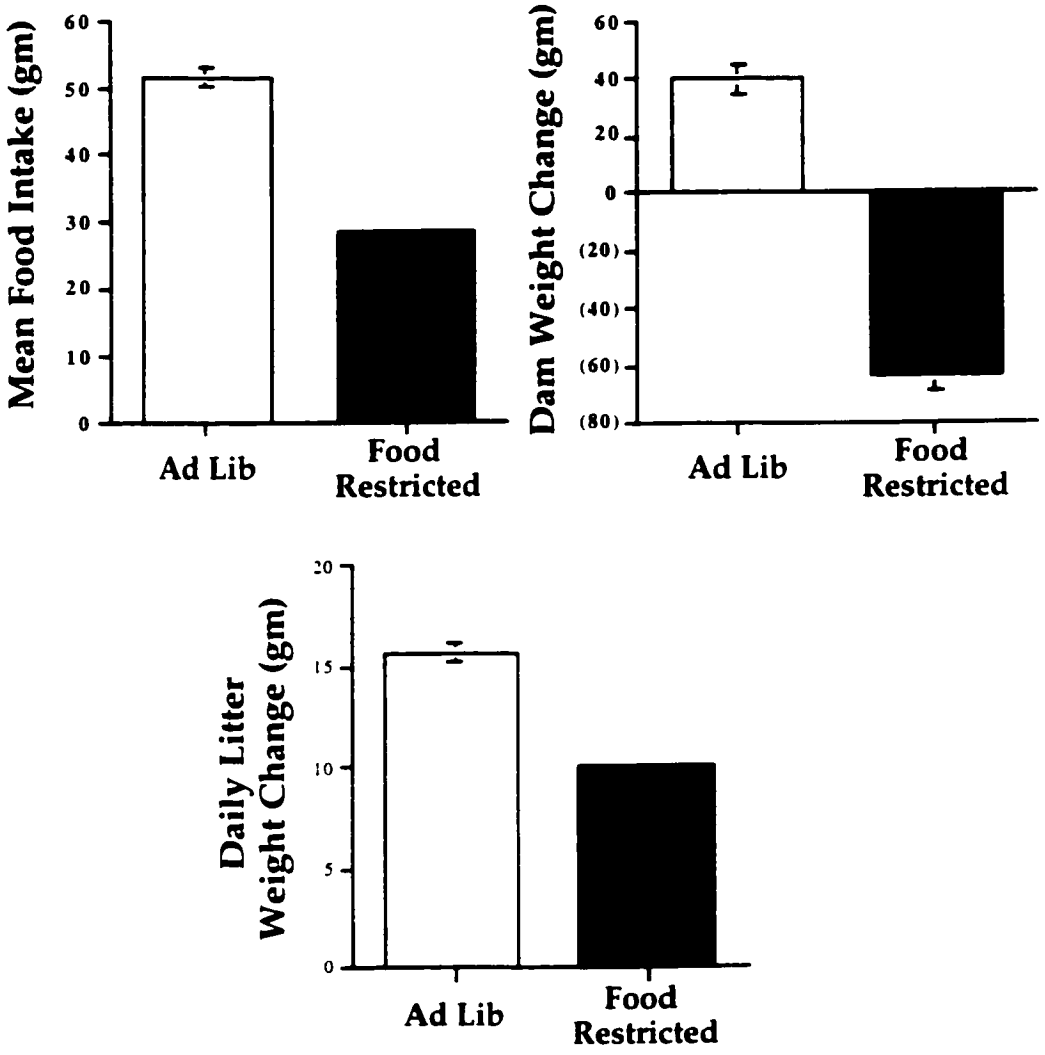

Figure 9.- Mean (±SE) Food intake, dam weight change and daily litter weight change during the first two weeks of lactation for all animals in Experiment 2. As seen in this figure, food restricted animals ate aproximately 54% of the ad lib intake in this experiment, resulting in a significant drop in dam weight (17% of their day 1 pp weight) and a decrease in milk yield as reflected by daily litter weight change.

in intact animals on days 10 and 15 pp (Hodson et al., 1978).

The differences in the LH responses to E2 in OVX animals cannot be accounted for by differences in the dietary regimen that these animals followed compared to that of intact rats in Experiment 1. Although dams in Experiment 1 received less food than the OVX food restricted dams in this experiment, the diet regimen followed in this experiment was tailored to reflect 50 % of the ad libitum intake of OVX lactating rats which is higher than that of intact ad lib fed dams. The proportion of body weight lost by food restricted females in both experiments was similar.

The ovary is a source of a number of steroid and peptide hormones that could influence surge activity (Taya & Greenwald, 1982a). The ovarian factor, inhibin, has been shown to suppress follicular development and ovulation but does so by acting on the release of FSH (Taya & Greenwald, 1982a; Taya & Sasamoto, 1980). Given that FSH levels are only low during the first five days of lactation, and that they rapidly increase thereafter in both ad lib fed and food restricted dams, however, it is unlikely that inhibin produces the attenuated LH responses to E2 (McGuire et al., 1995; Taya & Greenwald, 1982a). It is more likely that P plays the major role in suppressing the E2 induced LH surge in ad lib fed and food restricted dams. It is well established that if P is administered acutely on the morning of the expected surge it potentiates the E2-induced LH surge (DePaolo & Barraclough, 1979; Everett, 1961a). If, however, P is given to OVX females before the E2 treatment, or if P levels are chronically elevated, then the E2-induced LH surge is blocked (DePaolo & Barraclough, 1979; Everett, 1961a).

In lactating rats, circulating levels of P rise in the first few days pp, and peak at about day 12 postpartum, and decrease thereafter (Grota & Eik-Nes, 1967; Smith & Neill, 1977; Woodside, 1991). In food restricted dams, P levels are about 50% higher than in ad lib fed dams on day 12 pp, and they remain elevated

until at least day 20 pp, five days after free access to food has been restored (Woodside, 1991). Given these data it seems reasonable to suppose that the elevated P levels that are seen after food restriction are responsible for the prolonged suppression of the LH response to an E2 challenge. In contrast to this idea, Smith reported that E2 treatment induced LH surges in OVX lactating dams on day 5 pp that were increased by either acute P on the morning of the expected surge, or by chronic P treatment via silastic implants (Smith, 1981). Although these effects tended to disappear by day 15 pp, it is not known if chronic P would decrease or potentiate the LH surge on day 20 pp OVX ad lib fed dams. Thus, in Experiment 3 we further investigated the possibility that elevated P causes the suppression of the LH response to the positive feedback effects of E2.

Experiment 3

The results of Experiment 2 suggest that ovariectomy restores the ability of E2 to produce LH surges in lactating rats regardless of diet condition, implicating an ovarian factor in the suppression of the LH response to exogenous E2 during lactation. P concentrations are high during lactation, and remain elevated in food restricted dams for a period of time which correlates with their inability to show EB-stimulated LH secretion of a significant magnitude (Woodside, 1991; Experiment 1). Previous data has shown that high concentrations of P prevent E2-induced LH surges in OVX animals (Everett, 1961a; Freeman, 1994). Therefore, P seems to be a good candidate as the ovarian factor that suppresses the E2 induced LH surge during lactation, and one that could prolong this state in food restricted dams.

If high levels of circulating P are indeed the critical factor underlying the differential ability to respond to E with an increase in LH secretion in food restricted and ad lib fed lactating rats, then an exogenous treatment that increases P in ad lib fed dams should inhibit the LH surge. This possibility is investigated in Experiment 3a.

Conversely, blocking the action of P on its receptors should restore LH responsiveness to E2 in food restricted rats. RU486 is a competitive progesterone receptor antagonist with a relatively long half-life of up to 12 hours (Baulieu, 1991). When RU486 binds to the PR, it causes conformational changes, as does progesterone, but these differ from those reported when P binds the PR and result in defective binding of the PR onto the segments of DNA at which PR acts to increase transcription (Baulieu, 1991).

The administration of RU486 has been used successfully to enhance basal LH levels in day 15 pp lactating rats nursing eight pups (Lee, Haisenleder,

Marshall, & Smith, 1989a), and when given chronically from day 1 pp to intact lactating rats, shortens lactational diestrus to a duration of day 13 pp (van der Schoot et al., 1989). Its ability to modulate the LH response to E2 during lactation, however, has not been examined. Because P has both an inhibitory and stimulatory effect on the LH surge one would expect that blocking P receptors with RU486 would also have both stimulatory and inhibitory effects. Evidence indicates that this is indeed the case. For example, RU486 given on the morning of the expected preovulatory or steroid-induced LH surge prevents the surge from occurring (Chappell & Levine, 2000). In contrast, the progesterone antagonist RU486 rescued an E2 induced LH surge in ewes exposed chronically to P but only if the RU486 is given concurrently with E2 (Skinner, Bouchard, & Caraty, 1999).

It is important to note that RU486 also acts as a glucocorticoid receptor antagonist. The effects of this drug on the LH surge, however, have been attributed primarily to the blockade of P receptors because RU486 binds to PR with a higher affinity than it does to glucocorticoid receptors, and cortisol levels do not change during the LH surge (Skinner et al., 1999).

To investigate the hypothesis that removal of the inhibitory effects of P in food restricted rats would restore responsivity to the positive feedback effects of E, in Experiment 3b groups of food restricted lactating rats were given vehicle or one of two protocols of RU486 injections. The RU486 protocols differed only in the timing of drug administration and were based on the drug administration regimen used by Caratay et al (Skinner et al., 1999). So that RU486 was administered at 12-hr intervals concurrent with E2 administration. The difference between the two protocols was that the final injection of RU486 in one group occurred 24 h prior to sampling whereas in the second group it was given 12 hrs before sampling began. In both cases, the effects of RU486 should have

dissipated prior to the surge onset.

Method

Animals in these experiments were kept under the same laboratory conditions and guidelines for animal care followed in Experiment 1. Similarly, E2 treatment, jugular cannulation, blood collection and plasma analysis for LH concentrations were as described in Experiment 1. Food intake was not recorded in these experiments.

Procedure

Experiment 3a. Here we compared the ability of E2 to induce LH release in OVX ad lib fed lactating rats implanted with silastic capsules filled with P, with that of ad lib intact or ad lib cholesterol treated OVX animals on day 20 pp. Four groups were formed: 1) Intact (n=6); 2) OVX + Cholesterol (n=6); 3) OVX + P - P (n=6; and OVX + P (n=6). Ovariectomies were performed as described in Experiment 2. On day 1 pp, OVX animals were implanted with three silastic capsules (30 mm length, 1.98 mm i.d., 3.18 mm o.d.) containing either P (Sigma) or cholesterol implanted subcutaneously (sc) between the scapulae. In the OVX + P - P group, the P implants were removed on day 15 pp in an attempt to mimic the patterns of circulating P that are seen in the ad lib fed dam. In the OVX + P group, the progesterone implants were not removed in an attempt to keep P levels elevated to levels that are comparable to those seen in food restricted dams.

Experiment 3b. In this experiment we compared the ability of E2 to induce an increase in LH release in food restricted animals given oil vehicle or receptor

the P receptor antagonist RU486 at peak lactation (day 15 pp). The LH response to E2 challenge was compared among three groups of animals: 1) FR + Oil (n=6); 2) FR + RU486 A(n=6); and 3) FR + RU486 B (n=6). The RU486 1 group received an sc RU486 injection (5 mg/kg in sesame oil; Rousell-Uclaf, a kind gift from Dr. C.-D. Walker) 36, 24, and 12 hrs. prior to the onset of blood collection and in concert with both the priming (1 μg) and the bolus (250μg) administration of E2. Animals in the RU486 2 group received similar injections of RU486 48, 36, and 24 hours prior to blood collection getting P before the bolus dose of E2 but after the priming dose of E2. Surgeries for the jugular cannulation, hormone treatment, blood collection and blood sample analyses were conducted as in the previous experiments.

Results

Experiment 3a. LH responses to E2 treatment in intact, OVX + chol, and P treated groups are shown in Figure 10. A 4X2 (treatment X time of sampling) mixed ANOVA conducted on these data showed that peak LH levels were significantly higher than LH levels at 11:00h (significant main effect for time of sampling, $F(1,19)= 45.25$, $p.< .05$). A significant main effect for group ($F(3,19)= 7.83$, $p.< .05$) followed by post hoc tests revealed that intact and OVX + chol dams had higher LH levels after E2 treatment than OVX dams given progesterone

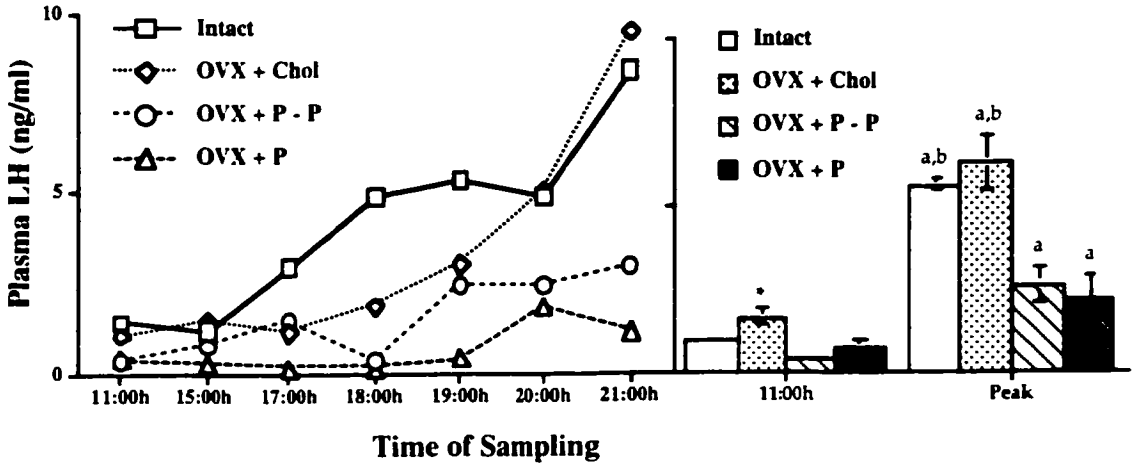

Figure 10.- Plasma concentrations of LH after E2 treatment in intact and OVX ad lib fed dams given implants of cholesterol or P. The left panel shows examples of LH levels during the day of the expected E2-induced surge. The right panel shows mean (± SE) plasma concentrations of LH on the first sample and the peak for each individual animal. As seen in this figure, E2 elicited a significantly higher LH discharge in intact and cholesterol treated dams than in ovx dams given P implants.

a= significant from 11:00h, p.< .05

b= significant from OVX + P and OVX + P - P

*= significant from Intact, OVX + P - P, and OVX + P at 11:00h, p.< .05.

implants (p.< .05, Fisher LSD). A treatment X time of sampling interaction that approached significance suggested that the group differences in LH were seen primarily at the time in which peak LH levels were attained (F(3,19)= 2.91, p.= .06). A posthoc between groups one way ANOVA showed that animals in the OVX + Chol group had higher concentrations of LH at 11:00h than animals in the other groups (F(3,19)= 6.96, p.< .05). Peak LH levels were lower in P-treated dams than those seen in the intact and OVX + Chol groups (F(3,19)= 5.18, p.< .05).

As expected, OVX dams gained more weight than intact dams regardless of whether they were given cholesterol or P as revealed by a between groups ANOVA (F(3,19)= 4.88, p.< .05). This effect was not reflected in lactational performance given that a between groups ANOVA conducted on the mean daily weight gain of pups nursed by dams in this experiment failed to find any significant differences between the groups (F(3,19)= .99, p.> .05; See Figure 11).

Experiment 3b. Figure 12 depicts the LH responses to EB treatment in food restricted dams given oil or RU486 at different time points. A 3X2 (treatment X time of sampling) mixed ANOVA revealed that LH levels were higher at the time of the peak response than at 11:00h when the first sample was collected (significant main effect for time of sampling, F(1,14)= 17.46, p.< .05). A significant treatment X time of sampling interaction (F(2,14)= 3.88, p.< .05) revealed that RU486 treated dams differed from oil treated dams in the magnitude of their LH response to EB treatment. A posthoc one way ANOVA showed no significant differences between the groups at 11:00h (F(2,14)= .97, p.> .05). A second one way ANOVA followed by posthoc tests (Fisher's LSD) showed that animals in the RU486 A group showed a nearly significantly larger peak LH responses after EB than oil treated dams (F(2,14)= 3.24, p.= .07). This effect was not seen in animals given the RU486 B protocol.

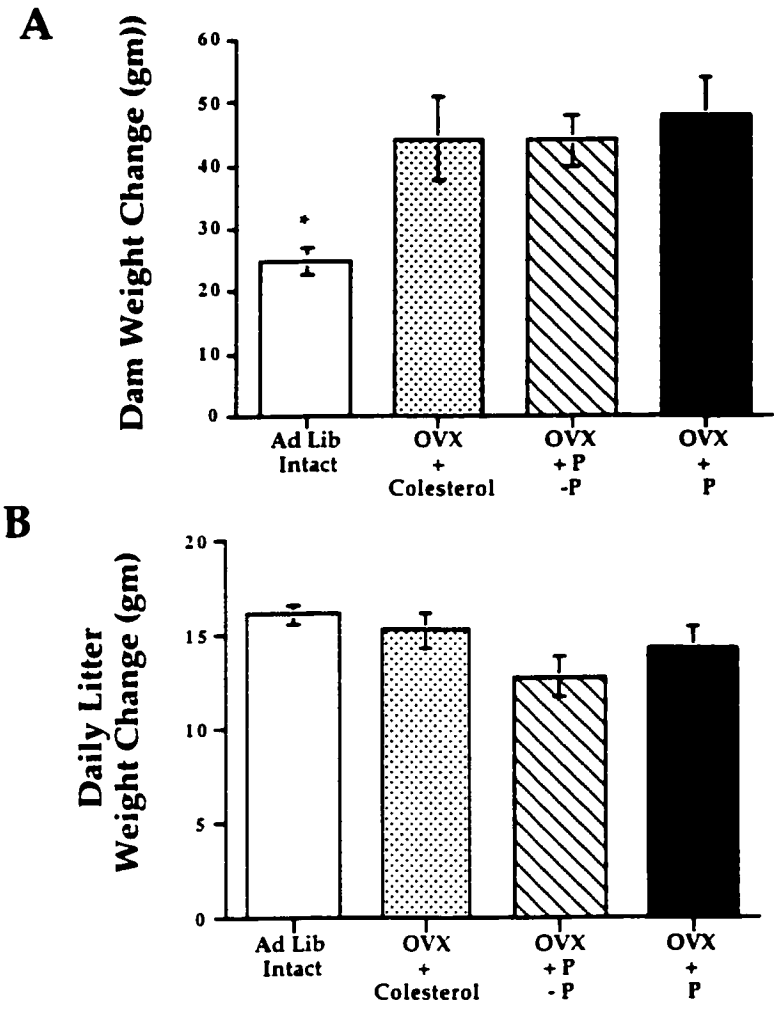

Figure 11.- Mean (±SE) dam weight and daily litter weight change in intact dams and in dams ovariectomized on day 1 pp and given cholesterol (OVX + Chol), P for two weeks (OVX + P - P), or P for three weeks (OVX + P). A seen in panel A, intact dams gained less weight than ovx dams given cholesterol or P. This effect, however did not affect the their ability to feed their youg given that there were no group differences in the daily litter weight gain. All animals were ad lib fed throughout the study.

* = significantly different from the rest of the groups, p. < .05

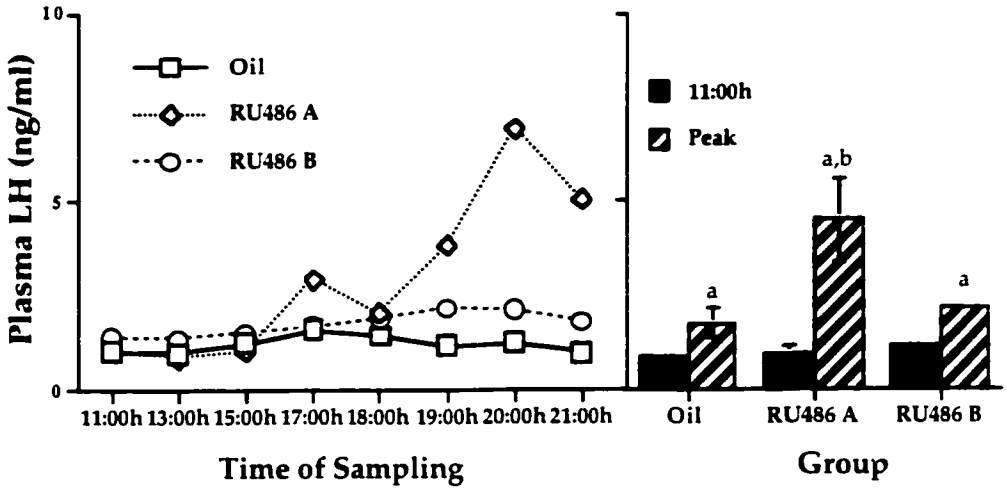

Figure 12.- Plasma concentrations of LH after E2 treatement in food restricted dams given sesame oil or RU486 36, 24, and 12 hrs. (RU486 A), or RU486 given 48, 36, and 24 hrs. before blood collection began (RU486 B). The left panel depicts data from a representative animal in each group across the day of blood sampling. The right panel shows mean (± SE) plasma concentrations of LH on the first sample (11:00h) and the peak for each group.

a= significant from 11:00h, p. < .05.

b= significant from control, p. < .05.

Analyses on dam weight change showed that dams lost comparable amounts of weight across the experiment regardless of the drug treatment they received, and regardless of when the drug was administered ($F(2,14)= .14$, $p.> .05$). Similarly, RU486 did not significantly affect overall litter weight gain ($F(2,14)= .60$, $p.> .05$; see Figure 13)

Discussion

Results from Experiment 3a and 3b directly implicate progesterone in the suppression of E2-induced LH surges. OVX animals given progesterone implants for either 2 or 3 weeks failed to show LH surges on day 20 pp after E2 treatment, whereas intact or OVX dams given cholesterol filled implants showed LH responses to E2 on the same day. In Experiment 3b we document that the LH surge may be rescued in intact food restricted dams at peak lactation when the P receptor antagonist RU486 is given at the appropriate time.

Chronic progesterone treatment has been reported to enhance the E2-induced LH surge in OVX lactating rats on day 5 pp and this effect tends to decrease as lactation advances so that LH surges on day 15 pp OVX dams given P are similar to those of intact dams (Smith, 1981). That day 5 pp OVX dams show a enhanced E2-induced LH surge when given P implants may seem contradictory to our results in Experiment 3a, and to the hypothesis that P suppresses the surge. One way to explain this discrepancy in results is that it is the duration of P exposure that is critical. Thus, when P is given for five days, it enhances the LH surge. When given for a two-week period, however, it may become inhibitory.

In addition, the mechanisms that suppress ovulation early in lactation may be different from those that suppress it later in lactation. In fact, there is a

considerable amount of evidence to suggest that this is so. The post-castration rise in LH can be inhibited by suckling but not by PRL in early lactation but can be inhibited by PRL in late lactation (Smith, 1981). In addition when P secretion is indirectly suppressed by daily bromocriptine treatment there is a longer latency for the return to estrous in dams when the drug is given in early lactation than when drug administration starts in mid lactation (Hansen et al., 1983). One would expect that the ability of P to inhibit reproductive function during lactation would correlate with high circulating levels of this hormone. P levels do not attain their peak concentrations until the middle of the second week pp coinciding with the time points at which the effects of inhibiting P's actions are strongest (Grota & Eik-Nes, 1967; Smith & Neill, 1977).

Unexpectedly, OVX dams that received P implants for only two weeks failed to show an E2-induced LH surge. This particular group was designed to mimic the patterns of P secretion seen in intact ad lib fed dams across lactation. Thus, it was expected that on day 20 pp, five days after the removal of their P implants, they would show an LH surge that was comparable to that seen in intact dams. This result could be explained by simply taking into account that intact animals have gradually increasing titers of E2 that may actually begin priming the system so that it responds to the exogenous E2 treatment that was used to elicit LH surges. Tsukamura and colleagues have shown that chronic E2 treatment given to OVX lactating rats via silastic implants produce LH surges that gradually increase in magnitude, presumably because they increase GnRH responsiveness in the pituitary (Tsukamura et al., 1988). In contrast, P-treated dams in Experiment 3a are OVX and thus, have little circulating E2 concentrations throughout the study. This lack of naturally

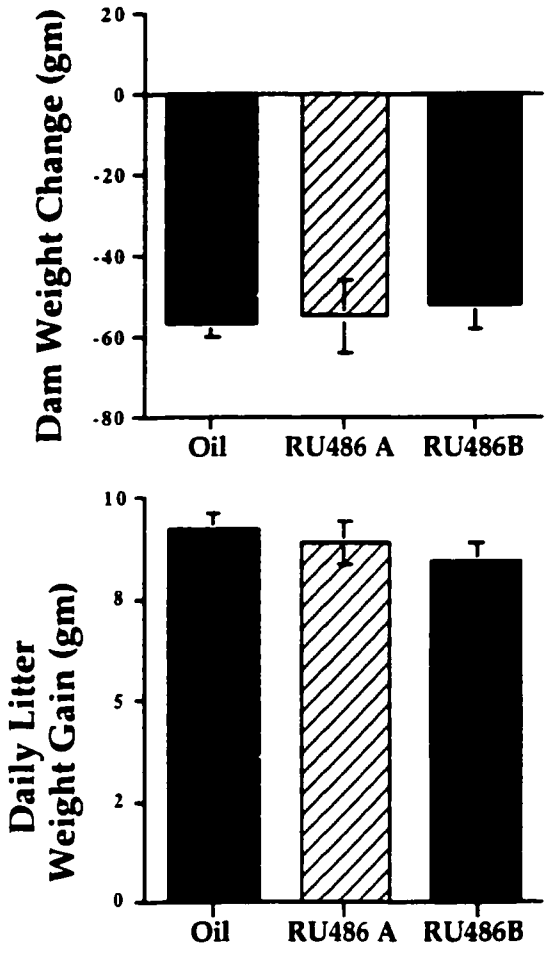

Figure 13.- Mean (± SE) dam weight change and daily litter weight gain in food restricted day 15 pp dams given either oil or one of two RU486 injection protocols. There were no significant effects of the RU486 on neither of these measures (p.> .05).

occurring E2 priming, along with the sustained two week chronic progesterone treatment may have resulted in reduced responsiveness to E2.

In Experiment 3b, RU486 was used to produce pharmacological withdrawal of progesterone, and in doing so, to restore the ability of E2 to induce LH surges in intact food restricted dams at peak lactation. We found that food restricted dams given RU486 36, 24 and 12 hours prior to the onset of blood collection had LH responses that tended to be larger in magnitude than those of oil treated dams, and those of animals whose RU486 treatment had ended 24 hours prior to blood collection. These results suggest that this specific RU486 treatment rescues, at least temporarily, the E2-induced LH surge from the profound inhibition produced by the food restriction-induced increase in P concentrations.

Together the results of experiments 3a and 3b suggest that high circulating levels of P are able to suppress an E2-induced LH surge and that differences in circulating P levels contribute to differences in the ability of ad lib fed and food restricted dams to respond to E2 administration with an increase in LH secretion. The mechanism through which P modulates these effects is most likely through its effects on its own receptor.

According to Levine, the availability of PR in the AVPV and pituitary is crucial to the generation of an LH surge (Levine, 1997; Levine et al., 2001). Activation of the PR by P provokes a conformational transformation in the PR that enables it to bind to its DNA binding domain in the nucleus resulting in further transcription (Turgeon & Waring, 2000). At the same time, this process also increases the activity of enzymes that degrade and ultimately downregulate the number of receptors available for binding (Lange, Shen, & Horwitz, 2000). Thus when P levels are high PR is down regulated. Downregulation of PR would not only reduce the effects of P itself but would also prevent the ligand-

independent transactivation of these receptors, by cAMP for example.

As noted earlier, the ability of E2 to induce an LH surge depends in part on its ability to upregulate PR (Herbison, 1998). Transcription of the P receptor (PR) is enhanced by E2 acting on its nuclear receptors in most tissues, including brain and pituitary (MacLusky & McEwen, 1978; Roy, MacLusky, & McEwen, 1979). Thus after E2 treatment, there is a marked increase in the presence of the PR protein that is available for binding (MacLusky & McEwen, 1978; Roy et al., 1979). *In vitro* studies show that high levels of progesterone prevent the upregulation of PR usually observed following E2 stimulation (Turgeon & Waring, 2000). Thus the high circulating levels of P seen in food restricted rats may not only serve to downregulate the PR but also to interfere with the ability of E2 to increase PR levels. Blockade of PR with RU486, on the other hand, can rescue gonadotrope PRs from progesterone induced proteosome degradation (Turgeon & Waring, 2000). Moreover, this effect is mediated by the presence of E2 (Turgeon & Waring, 2000). Thus RU486 administration in the current experiment was expected both to rescue PR number and the ability of E2 to increase PR.

In summary, the LH surges seen in animals in the RU486A group may have been the product of a combined increased transcription of PRs concurrent with the withdrawal of the inhibitory actions of P, and decreased PR degradation with the ultimate result of having enough PRs available for activation on the morning of the expected LH surge.

The differential efficacy of the two RU486 administration protocols probably reflects the critical importance of timing of PR receptor availability for the induction of an LH surge. According to the scenario described above, RU486 should be present at a time that allows E2 to have its stimulatory effect on the PR. This may indeed be the case with animals in the RU486 A group which was

given the drug 36, 24, and 12 hours prior to the morning of the expected surge.

In contrast, the timing of RU486 administration may have failed to rescue the surge in RU486 B dams simply because the effects of this antiprogestin may have disappeared by the time the E2 treatment would be expected to increase the synthesis of new PRs, enabling P to continue its inhibitory effects on LH release and to degrade newly synthesized PRs before a surge could occur.

To conclude, the results from the experiments described in this chapter strongly suggest that lactating rats are hyposensitive to the positive feedback effects of E2 and that food restriction prolongs the duration of this hyposensitivity. They also suggest that ovarian P may modulate this effect. Because the effects of food restriction are thought to be exerted on the central mechanisms that regulate GnRH release, in the next series of experiments we have attempted to determine how and when food restriction affects the hypothalamic events associated with the E2-induced LH surge.

CHAPTER 3

Effects of food restriction on the positive feedback signals of E2 In hypothalamic regions associated with the LH surge

In the previous Chapter we documented that the lactational diestrus of food restricted dams is in part associated with a prolonged suppression of the positive feedback effects of E2 on LH release that are necessary for ovulation to occur (Experiment 1). These effects are mediated by elevated P levels because ovariectomy or an appropriately timed RU486 treatment restores E2 responsiveness in food restricted dams, while chronic P treatment inhibits E2 responses in ad lib fed dams at a time at which they are normally present (Day 20 pp; Experiments 2 and 3). The next series of studies were conducted in an attempt to elucidate how lactation affects some of the events within the anterior hypothalamus that are associated with the induction of the E2-induced LH surge, and how food restriction may further modulate these events. We also hoped to reveal the role that P may play in regulating any changes we observed.

The hypothalamus plays deterministic role in the mechanisms that regulate both the pulsatile and surge release of LH that is necessary for ovulation. GnRH neurons scattered across the anterior basal forebrain release pulses of GnRH into the portal blood system, and this peptide acts on godatrophs in the pituitary to activate the release of LH (Silveman, 1988). The release of GnRH from the anterior hypothalamus into the pituitary is, therefore, a critical factor regulating LH release and stimulating the HPO (Levine, 1997).

GnRH release is the result of a harmonic interaction between inhibitory and excitatory neural and endocrine signals that allow sufficient release of this peptide to generate the pulsatile and surge release of LH (Freeman, 1994; Levine,

1997). The physiological changes that occur during lactation, however, produce a state in which inhibitory signals are enhanced, and excitatory signals are attenuated to produce a state in which hypothalamic release of GnRH is dramatically inhibited. This inhibition of GnRH secretion produces two major alterations to the pituitary: 1) a decrease in LH pulsatility; and 2) a decrease in GnRH receptors that reduces the ability of the pituitary to respond to GnRH (Smith, 1984; Smith & Neill, 1977; Taya & Greenwald, 1982a).

Pituitary responsiveness to exogenous GnRH stimulation is not different between ad lib fed and food restricted dams (Walker et al., 1995). Because of this, we have attributed the prolonged basal levels of LH release and the prolonged lactational diestrus of food restricted dams to a prolonged inhibition of basal GnRH secretion from the hypothalamus (Abizaid et al., 1997; Walker et al., 1995). The suppression of basal GnRH release from the hypothalamus that results in low basal LH concentrations in food restricted dams, may be related to increased inhibitory signals acting on GnRH neurons. For example, increased chronic NPY secretion from the ARC of lactating rats may act directly on GnRH neurons or on their terminals to inhibit GnRH secretion into the pituitary gland (Abizaid et al., 1997; Malabu et al., 1994; Smith, 1993). This effect may be prolonged in food restricted dams in which ARC NPY protein content and synthesis is further enhanced (Abizaid et al., 1997).

Interestingly, increased levels of P may play a role in the mechanisms that maintain basal LH secretion low in ad lib fed dams and possibly in food restricted dams. Restoration of LH pulses in ad lib fed lactating rats can be attained within 72 hours after an exogenous GnRH treatment that mimics the physiological patterns of GnRH secretion from the hypothalamus (Lee et al., 1989b). This effect can be accelerated if dams are given daily injections of the progesterone receptor antagonist RU486 (Lee et al., 1989a). These data suggest that elevated levels of P

can act to restrain GnRH secretion from the hypothalamus and, by doing so, suppress LH secretion from the pituitary.

In contrast to the basal release of GnRH seen during lactation, little work has been conducted on how lactation, with or without food restriction, affects the hypothalamic mechanisms by which E2 stimulates gonadotropin secretion. Again, because the LH responses to exogenous GnRH treatment are not affected by food restriction during lactation, it is likely that the deficit in E2-induced LH release observed in food restricted dams or in those given P implants are related to effects of these manipulations on the hypothalamus (Walker et al., 1995). Knowing that the release of basal GnRH is suppressed during lactation, it seems reasonable to propose that the response of GnRH neurons to E2 stimulation is also affected so that less hypothalamic GnRH would be released into the pituitary to produce an LH surge. Furthermore, it is also possible that food restriction prolongs this state and that P plays a key role in the mechanisms underlying these effects. The following experiments were conducted to determine if food restriction attenuates the response to E2 in hypothalamic sites that control the release of GnRH.

Experiment 4

The idea that ovulation was regulated by the action of the brain was first postulated in the 16th century by Haighton (1797; cited in Sawyer & Kawakami, 1961). This was later confirmed by experiments in which brain lesions rendered female animals acyclic, as well as by experiments demonstrating that electrical stimulation of different brain regions led to ovulation at times during the estrous cycle at which ovulation does not naturally occur (Everett, 1961b). Of these regions, the preoptic area proved to be the site at which electrical stimulation almost always led to an ovulatory response similar to that obtained on the evening of proestrus (Everett, 1961b).

The anatomical location for a central control center of ovulation was confirmed later by the discovery of GnRH, and by experiments using more localized lesions. These studies showed that the location of GnRH producing neurons was in fact within areas of the brain that, when destroyed, resulted in anestrus (Silveman, 1988). The majority of lesions in the anterior hypothalamic region including the SCN result in a state of constant vaginal estrous, and hyporesponsiveness to the positive feedback effects of E2 (Wiegand et al., 1978). Discrete lesions to the OVLT, however, resulted in a state of prolonged diestrus (Terasawa & Kawakami, 1974; Wiegand & Terasawa, 1982; Wiegand et al., 1980), a fact that could be attributed to an alteration of gonadotropin secretion.

The OVLT is a region anterior to the optic chiasm that surrounds the rostral most portion of the third ventricle. Interestingly, that the largest proportion of GnRH secreting neurons are located just caudal to the OVLT, and these neurons seem to play an important role in the regulation of LH release (Silverman, Jhamandas, & Renaud, 1987). For example, about 50 % of these neurons are activated, as determined by an increase in FOS-IR and JUN-IR within

these neurons, at around the time of the LH surge (Lee et al., 1990a). A significant number of GnRH /FOS-IR neurons are seen in OVX animals that receive E2 treatment alone (about 20% of GnRH neurons caudal to the OVLT), and after E2 + P treatment (about 40-50% of GnRH neurons caudal to the OVLT) (Lee et al., 1990b). Thus, if P treatment is given following E2 administration, it enhances both the induction of FOS-IR within OVLT GnRH neurons and the LH surge. In contrast, P given prior to E2 treatment both decreases the proportion of GnRH cells that also show FOS-IR, and inhibits the LH surge (Le, Attardi, Berghorn, Blaustein, & Hoffman, 1997a; Petersen, McCrone, Keller, & Shores, 1995).

Another region closely associated with the generation of the LH surge is the AVPV. Selective lesions of the AVPV, a discrete region surrounding the anterior third ventricle and just caudal to the OVLT, result not only in a state of persistent vaginal estrous (Wiegand & Terasawa, 1982), but also in a decrease in hormonally-induced FOS-IR within GnRH neurons in the OVLT (Le et al., 2001). Neuronal activation of the AVPV correlates positively with the preovulatory increase in FOS-IR within GnRH perikarya in the OVLT (Le et al., 1999). Furthermore, treatment with E2 alone (Insel, 1990), or with P (Auger & Blaustein, 1997) increases FOS-IR in the AVPV of OVX virgin rats at the time of an expected LH surge.

These data suggest that the AVPV is important for the integration of hormonal and neuronal signals that act to produce LH surges. Other data that support this contention include the fact that the AVPV is connected to the SCN where the neuronal daily signal that activates the surge is thought to originate, and to the ARC where metabolic and hormonal signals may be integrated to regulate energy homeostasis (Gu & Simerly, 1997). The AVPV also contains a dense concentration of E2 receptors (Shughrue, Lane, & Merchenthaler, 1997;

Shughrue et al., 1998b). According to Levine, E2 acts on these receptors to increase the transcription of progesterone receptors that, as previously reviewed, are particularly important for the induction of an LH surge (Levine, 1997; Levine et al., 2001). Interestingly, FOS-IR in the AVPV occurs within neurons that express progesterone receptors, and it is therefore possible that the degree of FOS-IR in these neurons reflects ER induced and/or PR induced transcription (Greco et al., 2001).

In this Experiment we hypothesized that if lactational diestrus is in part the result of hyposensitivity to the positive feedback effects of E2 on the hypothalamus, and food restriction prolongs this state, these changes would be reflected in a reduced ability of E2 to trigger the neuronal events that surround LH surges. One would predict, therefore, that FOS-IR within GnRH neurons in the OVLT and in the AVPV would be attenuated during lactation, and that this hyporesponsive state would be prolonged in food restricted dams. Specifically, we expected that when given E2 treatment, both ad lib fed and food restricted dams would show comparable degree of neuronal activation as measured by FOS-IR in the AVPV, and within GnRH neurons on day 15 pp., a time at which ad lib fed and food restricted dams do not differ in their LH response to E2. We also expected that as lactation progressed, E2-induced neuronal activation in these regions would increase faster in ad lib fed dams than in food restricted dams.

Method

Animals in these experiments were kept under the same laboratory conditions as in the previous studies. Similarly, food restriction was as described in Chapter 2. Food intake, dam weight and litter weight were recorded daily in

these experiments.

Procedure

Experiment 4a. This experiment was conducted to compare the ability of E2 to induce FOS-IR in the AVPV of ad lib fed and food restricted dams at different stages of lactation. Similar to Experiment 1, Ad lib fed and food restricted lactating rats were assigned to groups according to the day of lactation on which they were sacrificed after E2 treatment (days 15, 20 and 25 pp). Six groups were formed in total: 1) Ad lib day 15 (AL15, n=7); 2) Ad lib day 20 (AL20, n=6); 3) Ad lib day 25 (AL25, n=6); 4) Food restricted day 15 (FR15, n=7); 5) Food restricted day 20 (FR20, n=6); 6) Food restricted day 25 (FR25, n=6). All animals were given the same E2 treatment used in the experiments discussed in Chapter 2. A pilot study showed that, as with the LH responses, the E2 treatment was effective in inducing significant increases in FOS-IR within the AVPV and in GnRH neurons in OVX virgin rats (See Figures 14 and 15).

On the day of sacrifice, animals were given an overdose of sodium pentobarbital between 19:00 and 20:00h, and were transcardially perfused with ice cold saline followed by 4% paraformaldehyde in .1M phosphate buffer (ph 7.3-7.4). Brains were collected, post fixed for 36-48 hours in a 30% sucrose in 4% paraformaldehyde solution, and stored at -80° C until the tissue was sectioned. All brains were cut on a cryostat in order to obtain four sets of 25 μ coronal sections per animals that were stored in cryoprotectant (Watson, Wiegand, Clough, & Hoffman, 1986).

Single Labeling Immunocytochemistry for Fos. One in four sections from each animal were processed for FOS-IR using a standard protocol. Briefly, sections were washed 4-5 times in Tris buffered saline (TBS), and quenched in a .3%

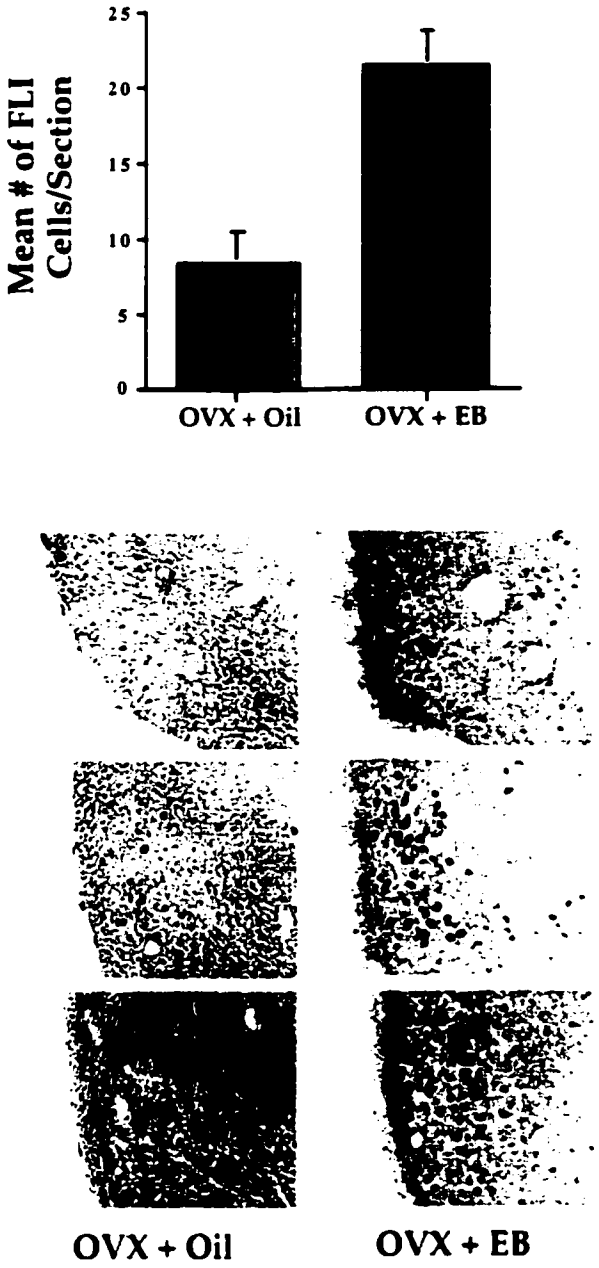

Figure 14.- Changes in the expression of FLI across the AVPV of OVX virgin female rats treated with oil or E2. A seen in this figure, E2 treatment increased the number of cells expressing FLI across the AVPV.

*= significant, p.< .05.

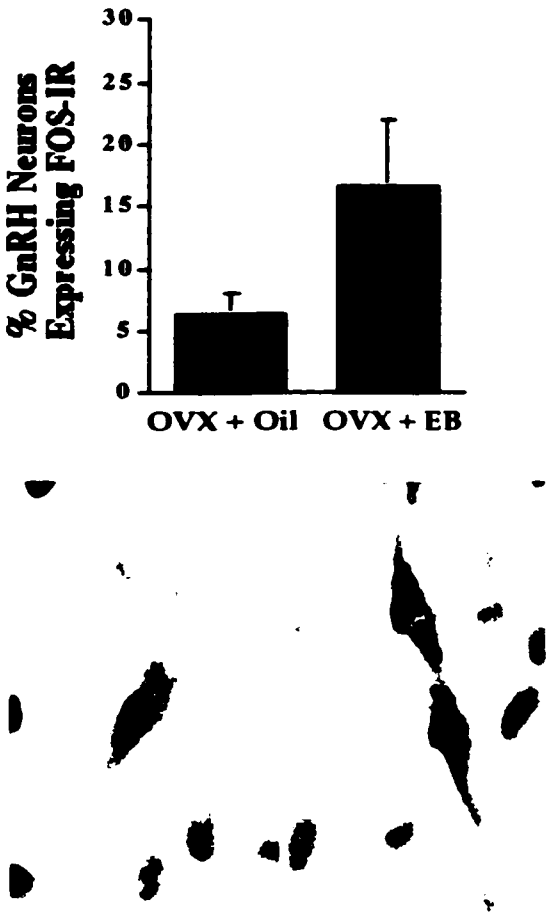

Figure 15.- Proportion of GnRH neurons co-expressing FLI after E2 treatment in OVX virgin rats. As seen in this figure E2 increased the number of GnRH cells that were also immunopositive for the Fos protein (top panel). The bottom panel shows digital images of double (left) and single (right) labelled GnRH cells around the OVLT.

*= significant, p.< .05.

solution of H2O2 for 30 minutes. The sections were washed again 3-4 times, incubated in pre-blocking solution (3% normal goat serum (NGS), .3% Triton X (TTX) in TBS) for 1h and incubated in the primary IgG solution (1% NGS, .3% TTX, and rabbit anti-Fos (aFos ab-5, 1:180,000, Oncogene) in TBS) for 48h at -4°C. After several washes tissue sections were incubated in biotinylated goat anti-rabbit IgG and avidin-biotin complex (ABC complex) as directed by the Vector elite kit. The tissue was then washed 3-4 times and developed using the Vector DAB and NiSO4 kit to produce a dark blue stain on the tissue.

Stained sections were mounted on gel coated slides, dehydrated, and coverslipped. Images of sections containing the AVPV as shown in plates 17, 18 & 19 of the Swanson atlas of the rat brain (Swanson, 1998), were captured using a digital camera mounted on a microscope and connected to a Macintosh G4 power computer. Cells expressing FOS-IR within the AVPV were counted using the NIH imaging software (version 1.62). The region of the AVPV counted consisted of an area of 75μ x 415 μ next to the third ventricle, an area recently reported to be the most sensitive to E2 treatment (Le et al., 2001; see Figure 16). The cell counts from all sections of the AVPV were averaged for each animal to obtain a mean number of FOS-IR cells per section for the selected brain region.

Experiment 4b. This experiment was designed to compare the proportion of GnRH neurons that shows FOS-IR after E2 treatment using a second set of sections from the same animals used in Experiment 4a. Two sets of sections from the previous studies, one from an ad lib day 15 dam and one from a food restricted day 15 dam, were not included in the study due to tissue attrition.

Double Labeling Immunocytochemistry for GnRH and Fos. All sections were processed for FOS-IR as shown above. Following exposure to the DAB /NiSO4 chromagen, sections were rinsed and incubated in anti-GnRH primary antibody solution (LR-1, 1:50,000; Dr. Benoit: a kind gift from Dr. J. Pfaus) for 48 hrs. After

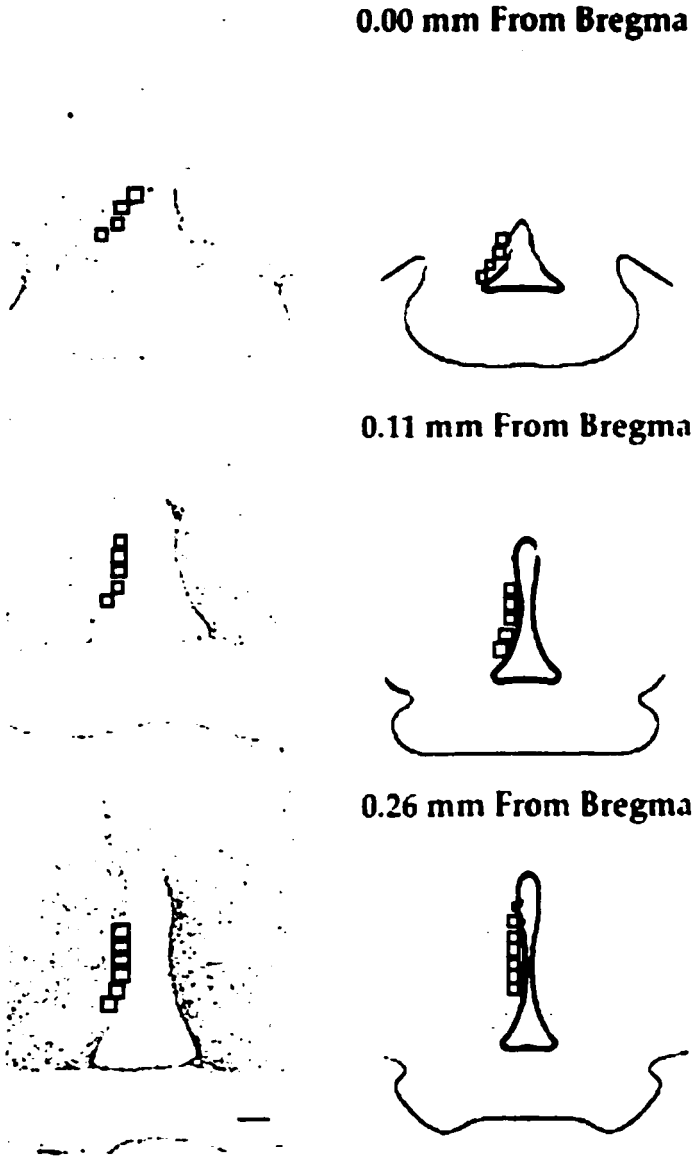

Figure 16.- Digital images and diagrams of the different levels of the AVPV organized rostrocaudally. The squares represent $70\mu^2$ grid boxes as drawn with the NIH image analysis after images were captured with a digital camera. Only cells expressing FOS-IR within these borders were used for our analysis. Bar represents 100μ.

incubation with the biotinylated antibody and the ABC reagent, staining was developed using DAB without NiSO4. Sections were then mounted on gel coated slides and coverslipped. Images of sections containing the OVLT, as seen in plates 16 and 17 of the Swanson atlas of the rat brain (Swanson, 1998), were captured to determine the percentage of GnRH neurons activated after EB treatment. GnRH-positive neurons were counted across each section, and the number of these neurons that were also FOS-IR positive was also recorded. Data from these sections was expressed as a percentage of GnRH neurons expressing FOS-IR.

Statistics. The cell counts from both Experiments 4a and 4b were analyzed with a 2X3 (Diet X Day of lactation) between groups ANOVAs, using Fisher PLSD as a Post Hoc test when required. In addition we also conducted a 2 X 3 (Diet X Day of Lactation) between groups ANOVA on the total number of GnRH neurons observed regardless of whether they were activated or not. Food intake, body weight, and litter weights were evaluated as in Experiment 1. Because the degree of activation of GnRH neurons has been correlated with the expression of FOS-IR in the AVPV, we also conducted a Pearson correlation between the number of FOS-IR positive neurons within the AVPV and the proportion of GnRH neurons expressing FOS-IR within each animal.

Results.

Experiment 4a. Figure 17 shows the mean number of FOS-IR cells in the AVPV of ad lib fed and food restricted dams on days 15, 20, and 25 pp. The ANOVA conducted on these data showed that overall, E2 treatment resulted in significantly less neuronal activation in the AVPV of food restricted dams compared to ad lib fed dams (main effect for Diet, $F(1,32)= 4.44$, $p.< .05$). A significant main effect for Days showed that overall AVPV FOS-IR in response to E2 increased as lactation progressed ($F(2,32)= 3.67$, $p.< .05$). Post Hoc tests

revealed that FOS-IR was significantly higher on day 25 pp dams, compared to that seen in day 15 or day 20 pp dams ($p.< .05$). Although there was no significant diet X day interaction effect ($F(2, 32)= 1.23$, $p.> .05$), closer inspection of the data suggested that ad lib fed dams showed more FOS-IR in the AVPV on days 20 and 25 pp. Given that our *a priori* prediction was that FOS-IR in the AVPV of ad lib fed dams would be higher earlier in lactation than FOS-IR in the AVPV of food restricted dams, we conducted individual t-tests on the data from day 20 and day 25 pp. Results from these tests showed that day 20 pp ad lib fed dams had more FOS-IR neurons in the AVPV than food restricted dams after E2 treatment ($t(10)= 2.02$, $p.< .05$, one tailed test). Furthermore, a second t-test revealed that this difference was still detectable by day 25 pp although it was not statistically significant ($t(10)= 1.41$, $p.=.09$; one tailed test; see Figure 17).

Experiment 4b. Figure 18 shows the mean proportion of GnRH neurons that co-express FOS-IR at the time of an expected LH surge in ad lib fed and food restricted dams on day 15, 20, and 25 pp. The ANOVA conducted on these data revealed that overall, there were no significant differences between ad lib fed and food restricted dams in the percentage of GnRH neurons expressing FOS-IR ($F(1,30)= 1.46$, $p.> .05$). A significant main effect for Days ($F(2,30)= 5.58$, $p.< .05$) revealed that the proportion of GnRH neurons expressing FOS-IR in response to E2 increased as lactation progressed. Post Hoc tests showed that the proportion of GnRH neurons expressing FOS-IR was greater in day 20 and day 25 pp dams compared to day 15 pp dams. There was no significant interaction effect ($F(2,30)= .73$, $p.< .05$) (see Figure 18).

Finally, a Pearson correlation conducted on all the data from both experiments 4a and 4b revealed a significant association between the mean number of FOS-IR expressing neurons within the AVPV and the proportion of GnRH neurons expressing FOS-IR after E2 treatment ($r(36)= .46$, $p.< .05$). We

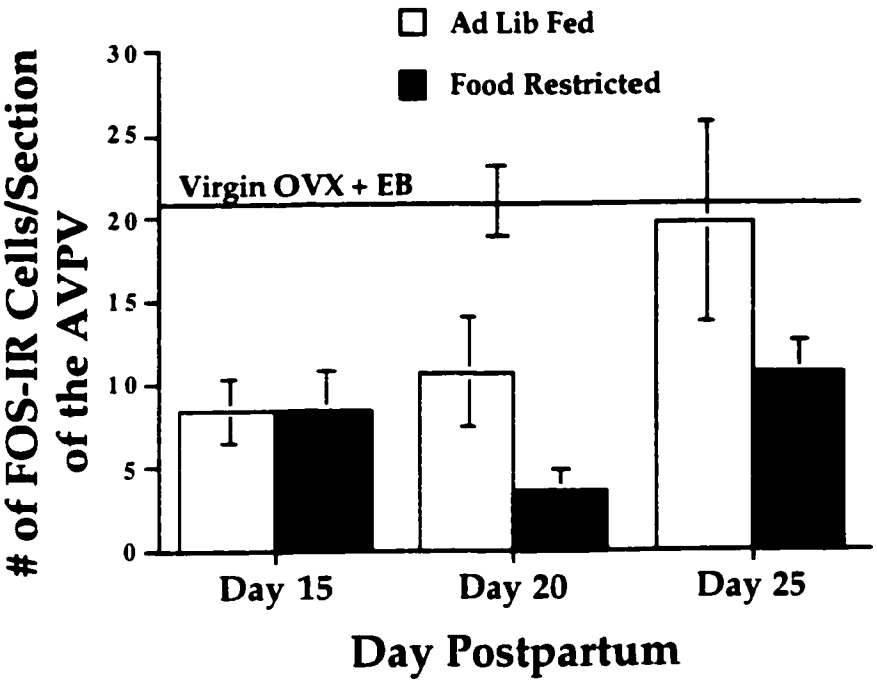

Figure 17.- Neuronal activation within the AVPV of ad lib fed and food restricted dams on days 15, 20, and 25 pp given E2 treatment as reflected in the mean number of FOS-IR cells per section. FOS-IR in the AVPV of virgin OVX females given EB in a pilot study is also included. As seen in this figure, overall FOS-IR was reduced in the AVPV of food retsricted dams ($p.< .05$). In addition, overall E2-induced FOS-IR immunoreactivity within the AVPV was attenuated on days 15 and 20 pp, and had increased by day 25 pp ($p. < .05$).

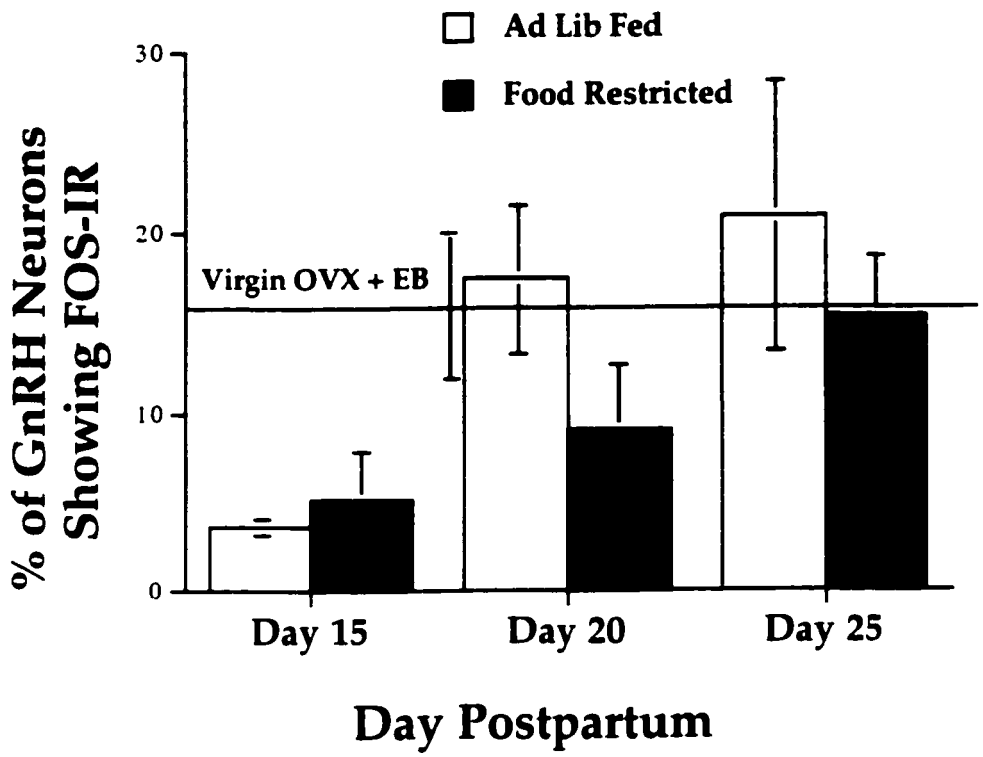

Figure 18.- Proportion of GnRH cells co-expressing FOS-IR after E2-treatment to ad lib fed and food restricted dams on either day 15, 20 or 25 pp. In addition, the proportion of GnRH neuron that showed FOS-IR after E2 treatment to virgin OVX females in a pilot study was also plotted. As seen in this figure, E2 treatment induced an overall increase in the proportion of GnRH neurons showing FOS-IR in day 20 and 25 pp dams, but not in dams at peak lactation (day 15 pp; $p.< .05$). Food restriction during lactation did not further affect the proportion of GnRH neurons showing FOS-IR.

then decided to conduct a Post Hoc correlation analysis using the data from both immunocytochemical studies and that from Experiment 1. We found near significant correlations between E2-induced peak LH levels and E2-induced FOS-IR in the AVPV ($r(34)= .316$, $p.= .07$), and between E2-induced peak LH levels and the proportion of E2-induced FOS-IR in GnRH neurons across lactation ($r(34)= .305$, $p.= .08$).

There were no significant changes in the number of GnRH stained neurons observed in the forebrain of the dams in this study (see Appendix X)

As in Experiment 1, food restricted dams ate about 50% of the ad lib intake (significant from ad lib, $t(33)= 17.71$, $p.< .05$), resulting in a significant decrease in body weight compared to their day 1 pp weight (Food restricted $\underline{M}=$ -62.35 ± 3.94 gm versus ad lib $\underline{M}= 48.6 \pm 4.85$ gm; $t(34)= 17.75$, $p.<.05$). By day 20 pp, food restricted dams had recovered the weight lost over the food restriction period ($\underline{M}= 17.96 \pm 2.90$ gm above their day 1 pp weight), but their weight change was still significantly different from that of ad lib fed dams (Ad lib fed $\underline{M}=$ 51.86 ± 3.83 gm above their day 1 pp weight; $t(22)= 7.06$, $p.< .05$). These differences were still evident by day 25 pp(Ad lib fed $\underline{M}= 33.21 \pm 3.77$ gm above their day 1 pp weight, versus food restricted $\underline{M}= 18.90 \pm 4.87$ gm above their day 1 pp weight; $t(10)= 2.32$, $p.< .05$).

Also as in experiment 1, the food restriction protocol produced a reduction in daily weight gained by the litters (ad lib $\underline{M}= 14.89 \pm .56$ gm versus Food restricted $\underline{M}= 9.35 \pm .22$ gm; $t(34)= 9.21$, $p.< .05$). After refeeding, litters growing with food restricted dams gained as much weight daily than those nursed by ad lib fed moms from days 15 to 20 pp ($t(22)= .95$, $p.> .05$), and from day 20 to day 25pp ($t(10)= 3.10$, $p.< .05$; See Appendix 2).

Discussion

The results from experiments 4a and 4b suggest that there are some differences between ad lib fed and food restricted dams in the neuronal events associated with the E2-induced LH surge during lactation. In Experiment 4a results show that FOS-IR in the AVPV in response to E2 treatment varied across lactation, being minimal on day 15 pp, and increasing as lactation progressed. The expression of FOS-IR was also overall significantly lower in food restricted dams, an effect that could be attributed to differences on days 20 and 25 pp, where the mean number of FOS-IR expressing tended to be lower in food restricted dams than in ad lib fed dams. In Experiment 4b, it was found that the proportion of GnRH neurons co-expressing FOS-IR increased between days 15 and day 20 pp but there were no significant differences on the activation of these neurons in response to diet.

Previous studies have shown that E2-induced LH surges correlate with the activation of E2 sensitive neurons within the AVPV and activation of a subgroup of GnRH neurons in the OVLT of female rats (Le et al., 1999; Le et al., 2001). To our knowledge, these are the first reports describing both FOS-IR in the AVPV and within GnRH neurons in response to E2 in lactating rats. Furthermore, these data are also the first showing changes in neuronal activation at these sites in correlation with the time of weaning. Moreover, these data correlate significantly with the patterns of LH responsiveness to E2 treatment during lactation seen in Experiment 1.

Results from this experiment show a diet effect on the activation of AVPV neurons but not in GnRH neurons after E2 treatment. It is also important to note that, although FOS-IR in GnRH neurons increased from day 15 pp to day 20 pp,

FOS-IR in the AVPV remained low until day 25 pp. These results suggest first that the effects of diet on the neural events that regulate the LH surge may occur primarily within the AVPV. Second, they point to a dissociation between the factors that restrain neuronal activation in the AVPV and those that do so in GnRH neurons during lactation. Moreover, these data suggest that a higher proportion of GnRH neurons showing FOS-IR is not always associated with a surge in LH release.

There is evidence supporting the contention that the expression of FOS-IR within GnRH neurons is not always consistent with GnRH and LH release. For example, it has been reported that α1 adrenergic receptor blockers suppress the LH surge but only have a small effect in reducing the proportion of GnRH neurons expressing FOS-IR at the time of an expected surge (Le, Berghorn, Smith, & Hoffman, 1997b). These data suggest that FOS-IR within GnRH neurons may be related to changes in the transcriptional activity of these cells and not to GnRH release *per se*. Wang and colleagues have provided evidence that GnRH mRNA is increased in those neurons that are also FOS-IR-positive, suggesting that FOS-IR in these neurons reflects activation of transcription that leads to the synthesis of new GnRH peptide (Petersen et al., 1995; Wang, Hoffman, & Smith, 1995). One could therefore propose that during lactation the ability of E2 to increase transcription within GnRH is reduced at peak lactation, but is restored by day 20 pp regardless of diet. Furthermore, because the pattern of neuronal activation in GnRH neurons is restored by day 20 pp, but that in the AVPV is still reduced at this time, suggests that during lactation the reduced responsiveness of GnRH neurons to E2 priming may be mediated through mechanisms other than those affecting the response to E2 in the AVPV. Therefore, the inability of E2 to induce LH surges in food restricted dams by day 20 pp is related to insensitivity to E2 in the AVPV and not in GnRH neurons themselves.

In spite of the differences in the patterns of activation within GnRH neurons and in the AVPV there was a significant correlation between FOS-IR in the AVPV and the proportion of GnRH neurons co-expressing FOS-IR. This supports previous data demonstrating a strong association in the neuronal activation patterns of these cells (Le et al., 1999; Le et al., 2001). Le et al. have shown that lesions to the AVPV result in a lack of activation of GnRH neurons in steroid treated OVX rats (2001). In these studies the degree of AVPV lesion matched not only the degree of GnRH neuron activation but also the magnitude of LH surges. It is therefore likely that the AVPV plays a key role in the mechanisms that produce LH surges in both cycling and lactating rats.

The levels of FOS-IR in both the AVPV and within GnRH neurons were low compared to those reported in other studies (Lee et al., 1990a; Lee et al., 1990b). This could be explained by differences in the hormonal treatment that was given. In the present studies we chose to give an E2 treatment that induced reliable LH responses in virgin females, and one used previously in lactating rats (Smith, 1978) because E2 is the critical factor to induce the LH surge and its neuronal correlates (Herbison, 1998). Treatment with E2 followed by P on the morning of the expected surge, however, enhances both LH surges and neuronal activation within GnRH neurons and in the AVPV (Auger & Blaustein, 1997; Lee et al., 1990b). It is therefore possible that the overall levels of FOS-IR were low because we used E2 alone as a treatment.

In general, results from these studies suggest that there is a general pattern of neuronal activation in the AVPV that correlates with the E2-induced LH surges in ad lib fed and food restricted dams. AVPV increases in FOS-IR seen across late lactation in ad lib fed dams are retarded in food restricted dams, in which E2-induced LH surges are attenuated for a longer period of time. The mechanisms by which steroid hormones lead to a preovulatory increase in FOS-

IR in the AVPV at around the time of the LH surge are still under investigation. It is thought that E2 acts on its classical nuclear receptors (ERα and ERβ), which, upon binding to E2, act as transcription factors to induce the synthesis of P receptors (Herbison, 1998). As proposed by Levine (Levine, 1997; Levine et al., 2001), activation of these receptors by cAMP and/or P is the key event that leads to the dramatic increase in the release of GnRH and LH. Given the timing of the presence of FOS-IR in the AVPV, and the fact that it is enhanced using an E2 + P protocol, it is likely that this is an index of PR receptor activation via ligand independent and/or dependent mechanisms. It is therefore possible that lactation reduces the ability of E2 to induce P receptors, with the end result being that lactating rats having less P receptors. If E2-induced transcription of P receptors in the AVPV is reduced, activation by the circadian signal or by preovulatory P would be reduced resulting in low levels of FOS-IR in the AVPV and a suppressed LH surge. The data from Chapter 2, would also suggest that food restriction prolongs the inability of E2 to induce P receptors, and that this effect would be mediated by the elevated levels of P seen in these animals. The possibilities that food restriction affects the ability of E2 to transcribe P receptors, and that P plays an important role in mediating this mechanism are evaluated in Experiments 5 and 6.

Experiment 5

Results from the last experiment show that events in the hypothalamus that occur in concert with the E2-induced LH surge and which are presumed to play a role in the central mechanisms mediating the surge are changed during lactation. Given that the results of Experiments 2 and 3 implicated P in the suppression of the LH surge one could propose that P might be playing an important role in suppressing the hypothalamic response to E2 administration in both ad lib fed and food restricted dams.

Both LH surges, and the presence of FOS-IR within the AVPV and in GnRH neurons, are events that occur on proestrus afternoon in intact animals, and proestrus evening in E2-treated OVX animals (Le et al., 1999; Le et al., 2001; Lee et al., 1990a; Lee et al., 1990b). LH surges, and presumably their neural correlates, are delayed for 24 hours if the animal is anesthetized early on the afternoon of the expected surge (Everett, 1961a). Because of these data, it has been proposed that E2 prepares the hypothalamus to be responsive to a circadian daily signal that early in the afternoon of proestrus, provides an acute stimulus that rapidly increases the release of GnRH. Therefore, the role of E2 is to provide sufficient stimulation so that the surge system is ready to be activated by the time the circadian signal is produced (Levine, 1997; Levine et al., 2001). In Levine's model, the role of E2 would be to stimulate the production of sufficient number of PR that can be transactivated by the circadian signal. The effects of high levels of P seen during lactation on the E2-induced LH surge could be attributed to an effect upon the ability of E2 to provide an optimal number of PRs for the surge to occur (Levine, 1997; Levine et al., 2001).

Figure 19 depicts two ways by which P could suppress the positive feedback effects of E2 resulting in reduced neuronal activation in the AVPV and

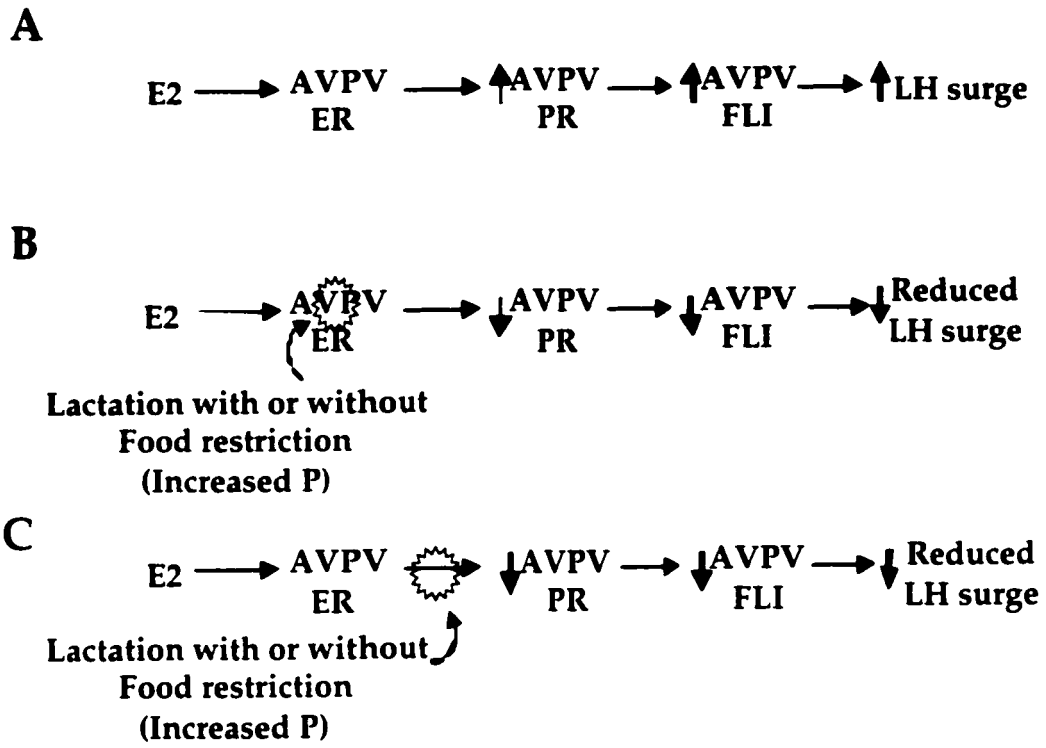

Figure 19.- Hypothetical mechanisms through which lactation with or without food restriction could act at the AVPV to attenuate the E2-induced LH surge. Panel A depicts how E2 binds to E2 receptors in the AVPV to induce transcription of P receptors, key elements for the E2 induction of the LH surge. In panel B describes the hypothesis that increased P as a result of lactation with or without food restriction could decrease the number ofE2 receptors that are available for binding resulting in reduced P receptor transcription. Panel C describes the scenario in which increased P has an effect in the events that occur after E2 has bound its receptors, thereby decreasing PR transcription. Both B and C would ultimately result in the attenuation of the E2-induced LH surge.

in LH release. The first mechanism suggests that the high levels of P seen in food restricted dams reduces sensitivity to E2 by reducing the number of estrogen receptors (ERs) in brain regions associated with the control of reproductive function. Indeed, elevated P has been linked to a decrease in ERs in the uterus and pituitary (Attardi, 1984). Evidence for the ability of P to reduce ERs in the hypothalamus remains controversial, with some reports claiming a reduction in ERs (Blaustein & Brown, 1984; Brown & MacLusky, 1994), while others report no effects (Attardi, 1984). These studies have only looked at the effects of acute treatments of P. It remains possible that the chronically elevated levels of P during lactation, and those seen after food restriction, enhance this putatively inhibitory effect of P on ERs, ultimately reducing sensitivity to E2.

If chronically elevated P levels decrease the number of ERs, one would expect that on day 20 pp intact food restricted dams should have a lower number of ERs in the AVPV than ad lib fed dams. In addition, one would also expect that OVX food restricted dams would have higher number of ERs in the AVPV than ad lib OVX dams given P implants. In this experiment these hypotheses were tested by comparing the basal number of E2 receptor α (ERα) immunopositive neurons in the AVPV of animals within the above mentioned groups of lactating rats.

Method

The general methodology in this study was similar to that used in the previous experiments. Ovariectomies, food restriction protocols, and standard laboratory conditions were as described in Chapter 2. In this study, however, no EB treatment was given.

Procedure

On day 1 pp, lactating rats were assigned to one of four groups: 1) Ad lib fed intact (AL intact, n=7); 2) Food restricted intact (FR Intact, n=5); 3) Ad lib fed OVX + progesterone (AL Ovx + P, n=6); 4) Food restricted OVX + cholesterol (FR OVX + Chol, n=4). On day 20 pp, animals were perfused and serial brain sections were processed for ERα immunocytochemistry.

Immunocytochemistry for ERα. Animals were sacrificed between 10:00 and 11:00h. At the time of sacrifice, animals were given an overdose of sodium pentobarbital, and were transcardially perfused with ice -cold saline followed by 4% paraformaldehyde. Brains were collected, post fixed for 36-48 hours in a 30% sucrose solution mixed in 4% paraformaldehyde, and then stored at -80° C until the tissue was sectioned. All brains were cut on a cryostat so as to obtain 25 μ coronal sections. One in four sections from each animal were processed for ERα immunoreactivity using a protocol that was similar to that used for FOS-IR. Thus, sections were washed 4-5 times in Tris buffered saline (TBS), and quenched in a .3% solution of H2O2 for 30 minutes. The sections were washed again 3-4 times, incubated in pre-blocking solution (3% normal horse serum (NHS), .3% Triton X (TTX), and 1% bovine serum albumin (BSA) in TBS) for 1h and incubated in the primary IgG solution (1% NGS, .3% TTX, 1% BSA, and mouse anti-ERα (monoclonal anti ERα ID-5, 1:500, Dako) in TBS) for 48h at -4°C. After several washes tissue sections were incubated in biotinylated horse anti-mouse IgG (1:200) and avidin-biotin complex (ABC complex) as directed by the Vector elite kit. The tissue was then washed 3-4 times and developed using the Vector DAB and NiSO4 kit to produce a dark blue stain on the tissue.

ERα- stained sections were mounted on gel coated slides, dehydrated, and coverslipped. Sections containing the AVPV were captured using a digital camera mounted on a microscope and connected to a Macintosh G3 power computer.

The number of cells expressing ERα within the same region of the AVPV that was examined in Experiment 4a, were counted using the NIH imaging software. The counts were averaged for each animal to obtain a mean number of ERα-positive cells per section of the AVPV.

Statistics. One way between groups ANOVAs followed by post hoc tests (Fisher's LSD) were conducted to compare group differences in the mean number of ERα within the AVPV.

Repeated measures 4X2 ANOVAs with group (ad lib intact, food restricted intact, ad lib fed OVX + P, and food restricted OVX + chol) as the between factor and time (day 15 and day 20 pp) as the within factor were used to compare dam weight change from day 1 and average daily litter weight gain.

Results

Figure 20 depicts sample pictures of ERα immunoreactivity within the AVPV of lactating rats in the different groups. As seen in Figure 21, there were no significant differences in the mean number of ERα-expressing neurons in the AVPV between the groups ($F(3,19)= .97$, $p.> .05$).

Statistical analyses of the control data revealed that lactating rats underwent significant body weight changes throughout the duration of the study (significant main effect for group, $F(3,19)= 6.26$, $p.< .05$). A significant main effect for time suggested that overall, dam weight change was more pronounced just after the end of the food restriction period (day 15 pp) than by the end of the experiment (day 20 pp; $F(1,19)= 273.5$, $p.< .05$). A significant group X time interaction effect showed that the patterns of weight change in the groups of dams differed across the two points of lactation examined ($F(3,19)= 77.57$, $p.< .05$). A posthoc one way ANOVA followed by posthoc tests revealed that on day

Figure 20.- Sample digitized images of AVPV sections immunostained for ERα with a monoclonal antibody (ID5 clone, Dako, 1:500 dilution). As seen in this figure, lactating rats showed a large concentration of ERα immunopositive cells across the rostro-caudal extent of the AVPV.

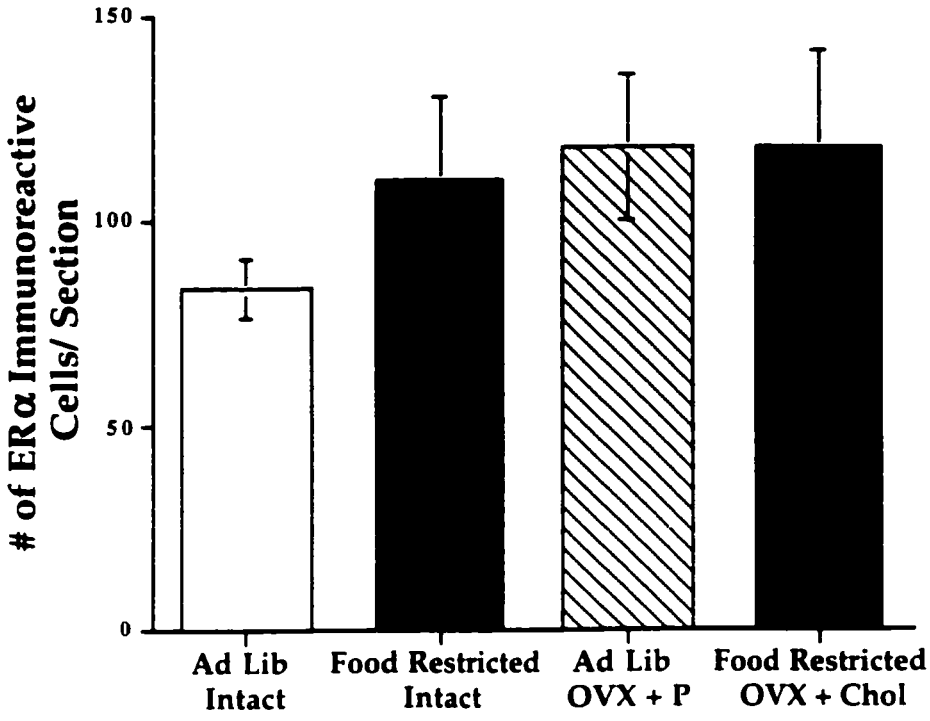

Figure 21.- Mean (±SE) number of cells expressing ERα immunoreactivity per section of the AVPV of intact and OVX lactating rats either food restricted or ad lib fed and sacrificed on day 20 pp. As seen in this figure, neither food restriction nor P treatment to ovx lactating rats affected the number of ERα immunoreactive cells in the AVPV.

15 pp dams in the food restricted groups had lost a significant amount of weight, whereas dams in both ad lib fed groups had gained weight ($F(3,19)= 55.62$, $p.< .05$). Although food restricted dams lost comparable amounts of weight during this period regardless of whether they were OVX or not, ad lib fed intact dams gained more weight than ad lib fed OVX dams given P ($p.< .05$, Fisher LSD). By day 20 pp dams in the food restricted groups had recovered the weight lost during the food restriction period, but were still below the weight change observed in ad lib fed dams ($F(3,19)= 5.18$, $p.< .05$). By this time, however, ad lib fed OVX dams given P showed similar patterns of weight change as those in the intact ad lib fed group.

As expected, all litters gained weight during the experiment (main effect for time, $F(1, 19)= 117.56$, $p.< .05$). A group main effect showed that litters nursed by food restricted dams gained less weight than those nursed by ad lib fed dams ($F(3,19)= 5.33$, $p.< .05$). As revealed by a significant group X time interaction effect, the rate of weight gain of the litters varied during the first two weeks of lactation, and between day 15 and day 20 pp ($F(3,19)= 9.29$, $p.< .05$). A significant post hoc one way ANOVA showed that pups in the food restricted litters gained in average less weight daily than those in the ad lib fed litters during the first 15 days pp ($F(3,19)= 33.64$, p. .05). The average daily weight gain between days 15 and day 20 pp, however, was not different amongst the different groups ($F(3,19)= .62$, $P.< .05$; see Appendix X).

Discussion

Results from this experiment show that neither food restriction during the first two weeks of lactation, nor chronic P treatment affect basal levels of ERα immunoreactivity in the AVPV of lactating rats. These results suggest that food

restriction-induced increments in P do not inhibit the E2-induced LH surge in lactating rats via a reduction of ERα number. This supports other data demonstrating that P treatment has little if any effects on hypothalamic ER number and expression (Attardi, 1984) even when P is chronically increased as it was in our food restricted dams or in OVX ad lib fed dams given P implants. That food restricted dams showed no significant changes in ERα expression within the AVPV is also consistent with previous data showing that virgin female hamsters show no changes in AVPV ERα immunoreactivity after a 48 hour fast (Li, Wade, & Blaustein, 1994).

That ERα immunoreactivity was not changed by either the diet or hormonal manipulations does not necessarily mean that P does not affect ER sensitivity to its ligand. One way by which food restriction or P could affect ER sensitivity to E2 without decreasing the number of ERα is by decreasing its binding capability. In support of this, Wade and colleagues have demonstrated that there is lower binding affinity to ER in the MPOA of diabetic animals although the concentration of ER number in this area is actually increased in these animals (Li et al., 1994; Siegel & Wade, 1979). It is not known, however, if the ERα present in the dams from our study differ in their capacity to bind E2, and further studies using receptor binding assays need to be conducted to address this issue.

Although sensitivity to E2 is often associated with the presence of ERα, it is possible that during lactation, sensitivity to the stimulatory effects of E2 is mediated by ERβ receptors. Indeed, it has been shown that genetically engineered mice lacking the ability to produce ERα are still capable of ovulating, and producing LH surges (Couse & Korach, 1999), but these surges are attenuated. In addition, distribution of ERβ is different between males and females, with females having a larger concentration of ERβ - synthesizing cells in

the AVPV, particularly in an area similar to that analyzed in our study (Orikasa, Kondo, Hayashi, McEwen, & Sakuma, 2002). Moreover, antisense oligonucleotides for the ERβ message disrupt the cycle in adult females in a manner that was similar to discrete lesions of the AVPV (Le et al., 2001; Orikasa et al., 2002), suggesting that ERβ could play a role in the regulation of the LH surge. Given that both ERs can form heterodimers to induce transcription (Cowley, Hoare, Mosselman, & Parker, 1997; Pettersson, Grandien, Kuiper, & Gustafsson, 1997), it is possible that an optimal number of ERβ receptors are necessary for E2 to have its full effect on the transcription of factors that ultimately lead to a preovulatory surge of LH release.

Whether the ability of E2 to induce an LH surge is decreased by the inability of E2 bind to its receptors, or by alterations in cellular mechanisms that occur after E2 has bound to its receptor, one would expect that both of this putative events would be reflected in an inability of E2 to induce PR. Using this rationale, the next experiment was designed to evaluate the ability of exogenous E2 to induce PR in the AVPV of lactating rats with different dietary histories and/or hormonal status.

Experiment 6

In the previous experiment it was shown that neither food restriction nor chronic P treatment altered the number of ERα available for transcription within the AVPV of lactating rats. As illustrated in Figure 19, a second mechanism through which food restriction and the chronic high levels of P that result from this manipulation could inhibit the LH surge would be through a reduction in the ability of the activated ER to increase PR levels.

There is considerable evidence suggesting that reproductive function is highly dependent on the ability of ERs to increase the number of PRs (MacLusky & McEwen, 1978), and that this may be compromised in situations where there is a negative energy balance state. For example, food deprived female hamsters show less E2-stimulated PR immunoreactivity within the MPOA and medial amygdala than ad lib fed females, although no differences were found in the AVPV (Du, Wade, & Blaustein, 1996). Work on the Zucker rat has shown that this genetically diabetic strain of animals has altered physiological and behavioral reproductive responses to the positive feedback effects of E2 (Olster & Auerbach, 2000). For example, OVX female Zucker rats given E2 + P treatment show attenuated LH surges and lower lordosis quotients than control animals (Olster & Auerbach, 2000). These hormonal and behavioral patterns are reflected in the inability of the steroid treatment to induce the expression of PR within the AVPV and VMH of these animals (Olster & Auerbach, 2000).

If the same mechanism is responsible for attenuating the LH surge during lactation, than E2 should be less effective in inducing PR within the AVPV at a time at which these are needed for the induction of the LH surge. It would therefore be expected that on day 20 pp, intact ad lib fed dams would show more AVPV PR expression than food restricted dams after E2 treatment. Also, given

that P plays an important role in inhibiting the E2-induced LH surge, ovariectomizing food restricted dams would increase, and chronic P treatment to ad lib fed OVX lactating rats would decrease the number of PRs that are expressed in the AVPV on day 20 pp after E2 treatment. These hypotheses were tested in the present experiment.

Method

In this study one compared the ability of E2 to induce PR expression in the AVPV of lactating rats assigned to groups similar to those formed in Experiment 5. Therefore, ad lib fed intact (n= 13), food restricted intact (n= 11), ad lib fed OVX + P (n= 12), and food restricted OVX + chol (n= 11) were given the same E2 treatment that has been used throughout this thesis, and were sacrificed between 10:00h and 12:00h on the day of the expected LH surge. Their tissue was then processed for PR immunocytochemistry along with a set of sections obtained from the untreated dams used in the previous Experiment. In addition, sections of OVX virgin females treated with E2 (n= 5) or with oil (n= 6) were added to the immunocytochemical assay to compare the effectiveness of E2 in inducing PR expression between virgins and lactating rats. All animal care, surgical procedures, laboratory conditions, tissue collection and processing, and image analysis were conducted as described in Experiment 5.

Immunocytochemistry for PRs. Sections collected were processed for PR-immunoreactivity using a protocol similar to that for ERα immunoreactivity. Following incubation in the blocking solution, sections were rinsed and incubated in primary antibody solution (mouse anti PR MA1-410, 1:500, Affinity Bioreagents, mixed in TBS with 1% BSA, 1% NHS and .3% TTX) for 72 hrs. After incubation with the horse anti-mouse biotinylated antibody and the ABC

reagent, staining was developed using nickel intensified DAB. Sections were then mounted on gel coated slides and coverslipped.

Statistics. A 5 X 2 (group X treatment) between subjects ANOVA was conducted to analyze the ability of E2 to induce the expression of PR in the AVPV. Control organized as in Experiment 5. Statistical analyses on these data were designed to compare these measures with those of dams in Experiment 5, to ensure that females from both experiments were not significantly different in any of these parameters. Therefore, 4 X 2 X 2 (group X treatment X time) within subjects ANOVAs were conducted to evaluate dam weight change from day 1, and daily litter weight change respectively. Virgin females were not included in these analyses.

Results

Figures 22 and 23 show sample sections of the AVPV from animals in the different groups and the mean number of PR induced by E2 in the AVPV of all animals within these groups respectively. As expected E2 induced a significant overall increase in PR immunoreactivity within the AVPV (significant main effect for treatment, $F(1,48)= 23.57$, $p.< .05$)). PR immunoreactivity was not different overall across the groups tested (main effect for group, $F(4,48)= 1.05$, $p.> .05$). The ability of E2 to induce PR immunoreactivity differentially across the groups was suggested by a nearly significant interaction effect ($F(4,48)= 2.26$, $p.= .08$). Post hoc t-tests revealed that, E2-treated virgin OVX females showed more PRs in the AVPV than oil treated OVX virgin females ($t(9)= -2.85$, $p.< .05$). Similarly, E2 treated ad lib intact and food restricted OVX + cholesterol dams showed a significantly higher number of PRs in the AVPV than untreated females in the same conditions (Al intact $t(11)= -2.38$, $p.< .05$; food restricted OVX + chol $t(9)= -$

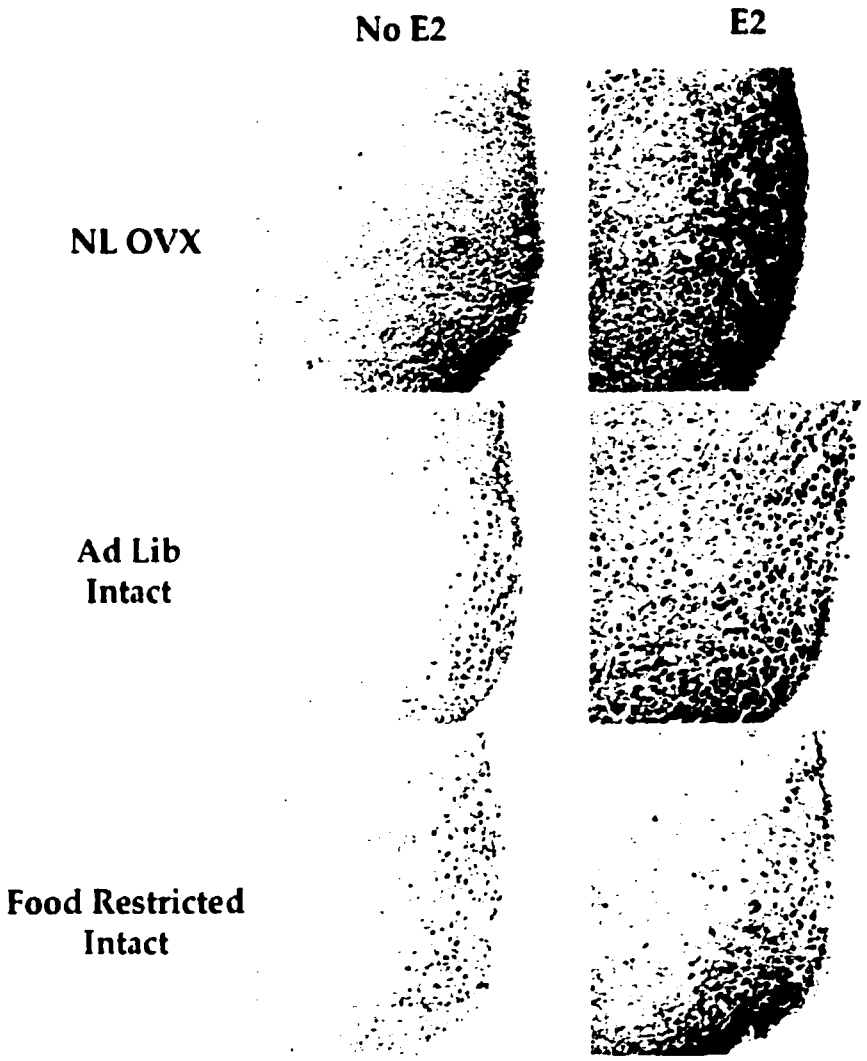

Figure 22.- Sample digitized pictures of the AVPV of virgin and lactating rats showing PR immunoreactivity with or without E2 treatment and sacrificed on day 20 pp. Sections were stained using a monoclonal antibody recognizing occupied and unoccupied PRs of both subtypes (PR-A and PR-B; mouse anti-PR Ig 1:500, Affinity Bioreagents).

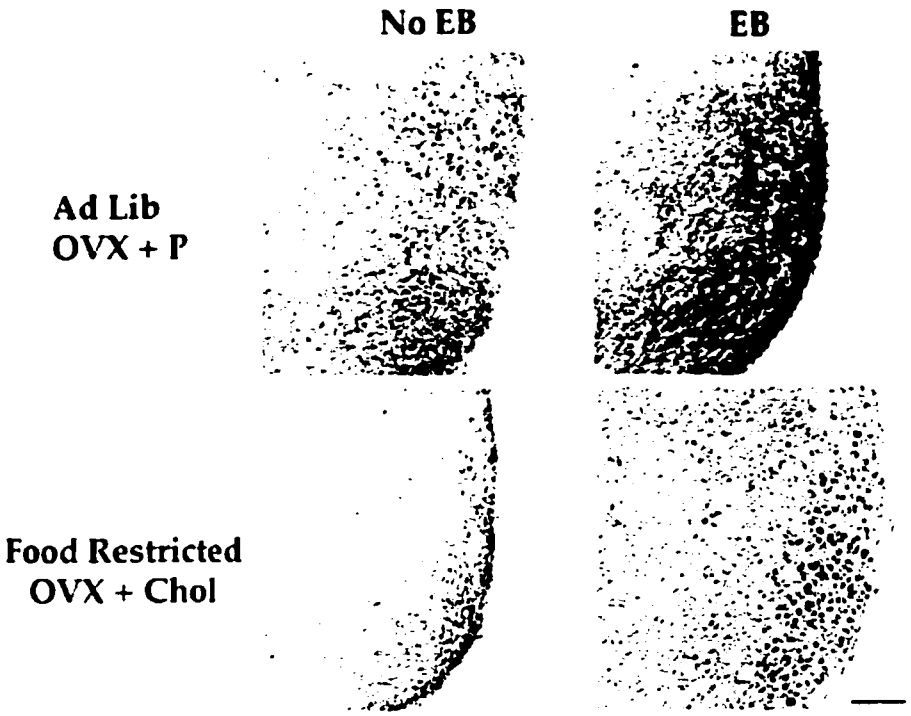

Figure 22 (cont.).- Sample digitized images of PR-immunostained sections of the AVPV of OVX lactating rats treated with or without E2. Ad lib fed dams were given P implants, whereas food restricted dams were given cholesterol implants on the day of ovariectomy (day pp) and all were sacrificed on day 20 pp. Measurement bar= 50μm.

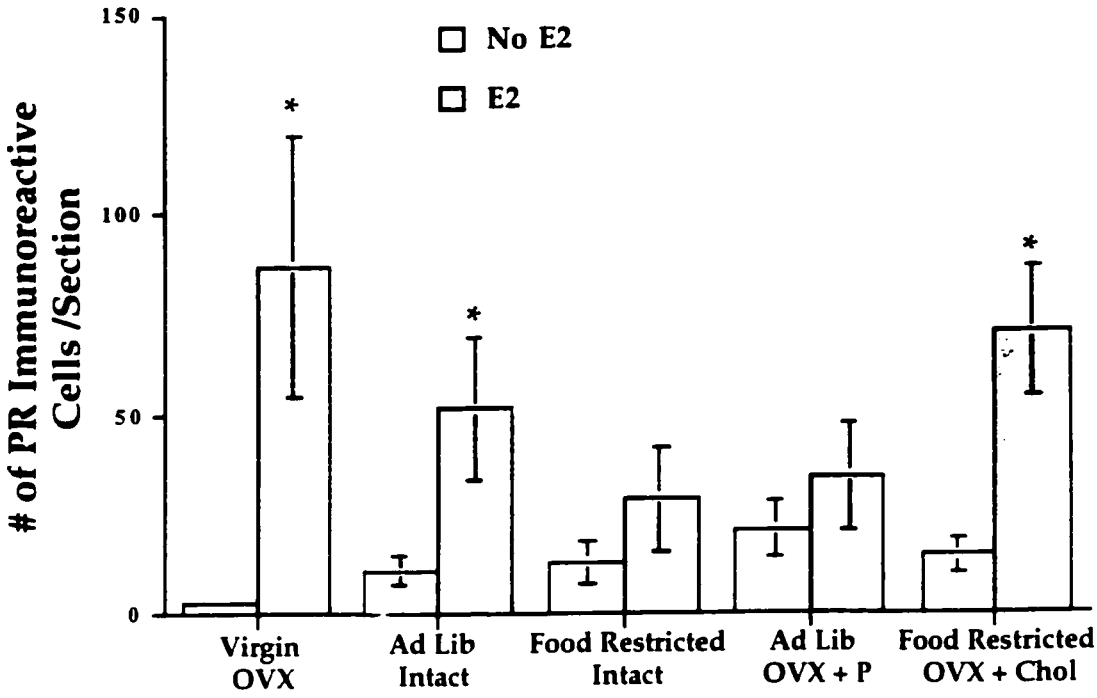

Figure 23.- Mean (±SE) number of PR detected in animals with or without E2 treatment. As seen in this figure, E2 treatment significantly increased the number of PRs in the AVPV of virgin OVX, ad lib intact and food restricted OVX lactating dams but had no such effect in the AVPV of food restricted intact and ad lib OVX + P dams.

*= significant from untreated animals, p.< .05.

3.05, p.< .05). In contrast, no differences were found in the number of PR detected in the AVPV of between treated and untreated animals in food restricted intact or ad lib fed OVX + P dams (food restricted t(9)= -1.03, p.> .05; ad lib OVX + P t(10)= -.98, p.> .05).

Statistical analysis of the dam weight change data revealed that dams changed in weight across lactation (main effect for time, F(1,39)= 275.94, p.< .05). As expected, this weight change was different across the groups within the experiment (main effect for group, F(3,39)= 60.45, p.< .05). Weight change, however, did no vary in response to treatment (F(1,39)= .16, p.> .05). A significant time X group interaction effect demonstrated that the change in weight from day 1 was different in some groups compared to others across at days 15 and 20 pp (F(3,39)= 94.6, p. < .05). Post hoc one way between groups ANOVAs on the data from each day showed that on day 15 food restricted groups had, as expected, lost a significant amount of weight in contrast to ad lib fed dams who had gained weight over the same time period (F(1,43)= 109.31, p.< .05). By day 20 pp, dams in the food restricted groups had recovered some of the weight lost during the food restriction period, but their weight change was still below that seen in the groups of ad lib fed dams (F(3,43)= 6.55, p.< .05; See Appendix X). A significant time X treatment interaction effect showed that overall, dams lost a significant amount of weight during the first two weeks of lactation, but had gained weight by day 20 pp (F(1,39)=6.37, p.< .05). Finally, treatment did not differentially affect dams in any of the groups (group X treatment interaction effect, F(3,39)= .19, p.< .05), regardless of the time point of lactation that was analyzed (time X group X treatment interaction effect, F(3,39)= .72, p.< .05).

A second 4 X 2 X 2 repeated measures ANOVA conducted on daily litter weight gain at either day 15 or day 20 pp showed that litters continued to gain

weight across the study (main effect for time, $F(1,39)= 246.2$, $p.< .05$). A significant main effect for group demonstrated that some litters gained less weight daily than others throughout this time ($F(3,39)= 15.25$, $p. < .05$). A significant main effect for treatment also revealed that overall, litters nursed by animals treated with E2 gained less weight daily than those in the untreated groups ($F(1,39)= 4.94$, $p.< .05$). Analyses of interaction effects showed that the litters within the different groups gained weight differentially at either on days 15 and 20 pp (significant time X group interaction, $F(3,39)= 23.71$, $p.< .05$). Post hoc one way between groups ANOVAs showed that by day 15 pp litters nursed by ad lib intact dams gained more weight on daily basis than those nursed by ad lib fed OVX + P dams or by food restricted dams ($F(3,43)= 42.35$, $p.< .05$). Litters in the food restricted groups gained less weight every day in comparison with litters in the ad lib groups ($p. < .05$). Significant results from a second one way ANOVA showed that on day 20 pp litters growing with ad lib fed OVX + P dams gained less weight from days 15 to day 20 pp. ($F(3,43)= 4.23$, $p.< .05$). There were no significant time X treatment ($F(1,39)= .37$, $p.> .05$), group X treatment ($F(3,39)= 1.26$, $p.> .05$), or time X group X treatment interaction effects ($F(3,39)= .39$, $p.> .05$).

Discussion

In this study it is shown that a dose of E2 that is capable of inducing a significant amount of PR expression in the AVPV of virgin OVX females also induces PR expression in the AVPV of lactating rats, but does so to a lesser extent. Furthermore, the ability of E2 to induce PR during lactation may be compromised if the animal is food restricted, or if the dam is treated with P chronically. Conversely, ovariectomy on day 1 pp restores the ability of E2 to

induce PR in food restricted dams.

These results suggest that the inability of E2 to induce LH surges in lactating rats is in part related to an alteration in the mechanisms through which E2 induces the transcription of PR in the AVPV. These results support previous data suggesting that the presence of sufficient amounts of PRs in the AVPV is necessary for the E2 induced release of GnRH and LH surges in virgin OVX rats (Chappell & Levine, 2000). In these studies, treatment with PR receptor antagonists or with PR antisense oligonucleotides on the morning of an expected E2-induced surge completely blocked GnRH and LH surges (Chappell & Levine, 2000). These results also suggest that P is critical for food restriction to inhibit on the transcription of PRs given E2 induced was able to induce more PR in the AVPV of food restricted dams OVX on day 1 pp and given cholesterol implants, than that of ad lib fed dams OVX on day 1 pp and given P implants. These data, along with data from Experiment 3b where LH surge was restored in food restricted dams with RU486 treatment concurrent with E2 priming, propose that insensitivity to the positive feedback effects of E2 are primarily mediated through the action of P upon its nuclear receptors.

Although the results from the present study provide clear evidence that food restricted intact rats, and ad lib fed OVX + P rats show a suppressed response to E2-induced PR receptor protein expression in the AVPV when compared to virgin females, differences within the lactating groups are more subtle. For example, no statistical differences were obtained in the number of E2-induced PRs in the AVPV of ad lib intact versus that of food restricted intact dams. The lack of a difference between these two groups may reflect a general suppression of the ability of E2 to induce PR during lactation. Nevertheless the comparison with the virgin OVX females suggests that the degree of suppression seen in the food restricted intact is more profound.

The mechanisms through which chronic P during lactation and after food restriction inhibit the ability of E2 to induce PRs are not known, but they may include interference with a variety of intracellular events that lead from the binding of E2 to its receptors to the degradation of the PR protein. One of such events involves the recruitment of cofactor proteins by the E2 receptor to enhance transcription. Steroid hormone receptors act as transcription factors that are normally activated upon binding of their specific ligand. Once binding occurs, conformational changes allow the receptor to recruit a host of protein cofactors, known as steroid hormone coactivators, which then improve the capability of the receptor protein to dock to its response element within the nucleus (McKenna, Lanz, & O'Malley, 1999). Interestingly, different steroid receptors compete for these same coactivator proteins suggesting that they may be a limiting factor for the transcriptional effects of different hormones within the same cell (McKenna et al., 1999). Coactivators like the steroid receptor coactivator-1 (SRC-1) protein, and the cAMP response element-binding protein (CBP) have been both found to be regulated by changes in steroid hormones, and are also located in regions of the brain that are involved in the regulation of the LH surge and sexual behavior (Molenda, Griffin, Auger, McCarthy, & Tetel, 2002). In addition, blocking the synthesis of these proteins inhibits the transcriptional effects of E2 reducing not only PR immunoreactivity, but also disrupting sexual behavior in female rats (Molenda et al., 2002). It is then possible that in the lactating rat, the high levels of P binding to the basal number of PRs in the AVPV, decrease the level of coactivator proteins like SRC-1 and CBP that receptors for E2 can use for their full effect on the transcription of new PR protein. This effect would be prolonged in the food restricted dam as a consequence of further elevated P plasma concentrations.

The antibody used in the present study was manufactured to detect both

the PR A and B subtypes. Recent data, however, suggests that E2, at least 3 hours following treatment, induces primarily the transcription of the PR B subtype at both the hypothalamus and pituitary (Scott, Wu-Peng, & Pfaff, 2002). Furthermore, the PR A subtype seems to act as a corepressor for the transcriptional capabilities of the PR B subtype, and that of other steroid hormone receptors including ER (Giangrande, A. Kimbrel, Edwards, & McDonnell, 2000). These data hint to the possibility that ratios in the concentrations of the PR A and PR B receptor subtypes within the AVPV may determine the transcriptional events that lead to the release of LH surges, and increased P during lactation and food restriction could alter this ratio to produce infertility.

Finally, the presence of P prior to and during E2 priming may also reduce the number of PRs via a mechanism of enhanced proteosomic degradation. Upon binding to its receptors, P activates the production of enzymes that cause a downregulation of the numbers of receptors detectable by immunoreactivity in gonadotropes even in the presence of E2 (Turgeon & Waring, 2000). A similar mechanism may also take place at the AVPV and other hypothalamic sites associated with reproductive function, where chronic presence of P could bind to E2-induced PRs and reduce their number.

A caveat to the above mentioned hypothesis is that in our study, basal PR numbers in the AVPV were similar across the groups of lactating rats examined regardless of their diet history or whether they were given P implants or not. In contrast, virgin OVX females that did not receive E2 stimulation had few detectable PR-positive neurons in the AVPV. This suggests that the basal transcription of the PR within the AVPV is regulated by factors other than ovarian steroid hormones during lactation. One can speculate that there is either a basal level of PR transcription within AVPV cells, or that this basal number of

PRs is a sequelae to those PRs that were synthesized during pregnancy (Numan et al., 1999). Alternatively, it is possible that immunocytochemical quantification is not precise enough to detect subtle differences in the basal number of PR within the AVPV of the different groups.

In conclusion, food restriction attenuates the number of PR detectable by immunocytochemistry after E2 treatment. It is likely that P mediates this effect, because ovariectomy restores E2-induced PR immunoreactivity in the AVPV of food restricted dams, and chronic P treatment reduced E2-induced PR immunoreactivity in the AVPV of OVX ad lib fed dams. These results suggest that P attenuates the E2 induce LH surge during lactation by interfering with the induction of PR by E2 acting in the AVPV. Food restriction may enhance this effect by maintaining elevated P levels.

Chapter 4

General Discussion

The positive feedback effects of E2 are necessary for the induction of a preovulatory LH surge that ultimately leads to ovulation (Everett, 1961a; Freeman, 1994; Herbison, 1998; Levine et al., 2001). During lactation, ovulation is arrested, an effect that is due in part to a state of hyporesponsiveness to the positive feedback effects of E2. This is reflected in attenuated LH surges after exogenous E2 treatment (Coppings & McCann, 1979; Hodson et al., 1978; Smith, 1978). These two facts led to the hypothesis that in food restricted dams, in which lactational diestrus is prolonged by about a week, sensitivity to the positive feedback signals of E2 is either further reduced or extended for a longer period of time. It was also hypothesized that P would play an important role in the mechanisms that suppress the positive feedback effects of E2 on LH release, and finally, that P acted on the hypothalamus, specifically the AVPV, to reduce LH responsiveness to E2. The results from the experiments reported in this thesis support these hypotheses and open up some new avenues for future studies.

Hormonal responses to E2 in food restricted dams

Food restriction extends the period of insensitivity to the positive feedback effects of E2.- In Experiment 1, it was found that food restriction did indeed prolong the state of hyporesponsiveness to the positive feedback effects of E2. Although no differences between the E2-induced LH surges in food restricted and ad lib fed dams on day 15 pp were observed, the LH response to E2 remained minimal in food restricted dams on day 20 pp, whereas it increased

in ad lib fed dams at this time. The LH response to exogenous E2 treatment of food restricted dams was not restored to ad lid fed levels until about day 25 pp.

It is also clear from this experiment that LH surges of lactating rats on day 15 pp were smaller than those shown by OVX virgin females in the pilot study. In the case of the day 15 pp lactating rats, peak LH concentrations were only of the magnitude of about 2 ng/ml, whereas peak LH concentrations in OVX virgin females rats were about 20 ng/ml. These data are consistent with those of others showing that the magnitude of E2-induced LH surges was significantly reduced in lactating rats after E2 treatment in comparison with that of non-lactating rats (Smith, 1978; Hodson et al., 1978; Coppings & McCann, 1979)).

The effects of diet on the LH response to E2 were evident on day 20 pp when food restricted females still showed a reduced response to E2 stimulation compared to that seen in ad lib fed. These results confirm that dietary manipulations not only inhibit the basal release of LH, but they also prolong the inability of E2 to produce LH surges in lactating rats. In addition, they show that, as with the basal release of LH, the effects of food restriction are long lasting, extending for a period of at least five days after refeeding begins.

The effects of chronic food restriction appear to be dependent on the reproductive state of the female inasmuch as chronic food restriction does not blunt the positive feedback effects of E2 on the LH release of virgin females, even if they are rendered acyclic by this dietary regimen (Sprangers & Piacsek, 1997). Nevertheless, complete food deprivation does inhibit preovulatory or steroid-induced LH surges in female rats, suggesting that extreme changes in food availability can affect the LH surge mechanism (Watanobe & Schioth, 2001). In the case of the acute fasting, a situation in which the life of the animals is at peril, the surge mechanism is affected so as to prevent the rupture of ova that are already formed. In the chronic food restriction, the primary effect is to delay

follicular maturation (Wade & Schneider, 1992; Wade et al., 1996). In the food restricted lactating rat, it seems that both mechanisms are at work to prolong infertility (Taya & Greenwald, 1982a; Walker et al., 1995). Moreover, the effects of food restriction on reproductive function in lactating rats extends beyond the food restriction period, whereas in cycling females these effects are reversed soon after the females are given full access to food (Wade & Schneider, 1992; Wade et al., 1996).

Results from Experiment 2 suggest that an ovarian factor is primarily responsible for the suppression of the positive feedback effects of E2 during lactation. We found that lactating females OVX on day 1 pp showed large LH surges on both days 15 and 20 pp. Furthermore, food restriction did not affect the ability of E2 to induce surges in these animals. These data support previous studies reporting that ovariectomy restores the ability of E2 to induce LH surges in ad lib fed lactating rats (Hodson et al., 1978). In addition it provides evidence suggesting that food restriction during lactation increases the production of an ovarian factor that then alters the LH response to E2.

Progesterone (P) is the ovarian factor mediating the effects of food restriction on E2-induced LH surges.- In Experiment 3, the possibility that P secretion from the ovaries is the critical factor regulating the LH responses to E2 during lactation was explored for two reasons. First, plasma concentrations of P are markedly increased during lactation to concentrations that are about 2-4 times higher than those seen during the estrous cycle. P concentrations peak at around mid lactation (days 10-13 pp), and gradually decrease to the levels of cycling females by about day 20 pp. coinciding with the times at which lactating females begin showing estrous cyclicity (Grota & Eik-Nes, 1967; Smith & Neill, 1977). Second, food restricted dams have higher levels of P that peak later in lactation (days 15-17 pp) and that are still elevated by day 20 pp, coinciding with

the low levels of basal LH concentrations reported in these animals (Woodside, 1991).

Results from this experiment clearly implicate P in the attenuation the E2-induced LH surge in food restricted lactating rats. In Experiment 3a it was found that chronic P treatment with silastic implants for either 15 or 20 days following parturition, markedly attenuated the E2-induced LH surge on day 20 pp of ad lib fed OVX dams compared to that seen in cholesterol treated OVX , or intact ad lib fed dams. The importance of P in regulating sensitivity to the positive feedback effects of E2 was also underlined by results in Experiment 3b. Here, treatment with the P receptor antagonist RU486 restored the ability of food restricted dams to produce LH surges on day 15 pp. RU486 treatment, however, is only effective when given concurrently with the E2 treatment. Like P, RU486 may have a dual effect on LH release so that it facilitates E2 induced LH surges when P is inhibitory. In contrast, RU486 may attenuate E2-induced LH surges when P stimulates LH release.

Overall, the results of Experiments 1, 2, and 3 clearly show that food restriction can prolong lactational infertility in part by an attenuation of the positive feedback effects of E2 on LH release. Furthermore, they suggest that P mediates this effect.

In the next series of studies were focused on describing the ability of food restriction and P to reduce the positive feedback effects of E2 in the hypothalamus of lactating rats. This was done because the primary alteration underlying lactational infertility is thought to involve a suppression of the release of GnRH. While it is established that pituitary responsiveness to GnRH stimulation is decreased during lactation (Smith, 1984), this is due in part to a lack GnRH stimulation on the pituitary. Furthermore, pituitary responsiveness does not differ between ad lib fed and food restricted dams (Walker et al., 1995).

Neuronal Responses to E2 in food restricted dams

The pattern of FOS-IR in the AVPV correlates with the pattern of LH release after E2 treatment in ad lib fed and food restricted dams.- There are several pieces of evidence suggesting that the AVPV and the OVLT are key sites in the integration of the hormonal and neuronal signals that result in the surge release of GnRH and LH. Lesions to these regions result in an anovulatory state and in a lack of LH surges after steroid hormone treatment (Le et al., 2001; Wiegand & Terasawa, 1982; Wiegand et al., 1978; Wiegand et al., 1980). Other data have shown that a steroid hormone treatment that produces a surge in LH release also induces the expression of FOS-IR in both the AVPV (Auger & Blaustein, 1995; Insel, 1990), and in a subgroup of GnRH neurons in the OVLT in correlation with the timing of the LH surge (Lee et al., 1992; Lee et al., 1990a; Lee et al., 1990b). In Experiment 4 the ability of E2 to induce these two neuronal events in ad lib fed and food restricted dams on days 15, 20, and 25 pp was examined.

Results from Experiment 4a showed that, as with LH release, FOS-IR in the AVPV after E2 treatment varied as a function of the day of lactation in which the animals were tested. Thus, regardless of diet, lactating animals showed lower FOS-IR in the AVPV at peak lactation (day 15 pp), and on day 20 pp than on day 25 pp in response to E2 treatment.

More important, within the context of the present thesis, was the fact that overall, E2 was less effective in inducing FOS-IR in food restricted dams than in ad lib fed dams. These results are also the first to provide evidence that E2-induced FOS-IR within the AVPV is reduced by changes in food availability. This reduction was particularly apparent on day 20 pp, a time at which ad lib fed

dams showed more FOS-IR cells than food restricted dams in response to E2. As with the effects on E2-induced LH secretion, the effects of food restriction on FOS-IR in the AVPV were long lasting, remaining evident after five days of free access to food.

As shown in Experiment 4b, the patterns of neuronal activation after E2 treatment within the AVPV differed from those seen within GnRH neurons of the same lactating rats. Here we reported that the proportion of GnRH neurons also showing FOS-IR at the time of an expected E2-induced LH surge increased from the levels seen at peak lactation, to a significantly higher proportion by day 20 pp. No further increase in the proportion of GnRH/FOS-IR cells was seen on day 25 pp. These data show that the stimulatory effects of E2 on the activation of a subgroup of GnRH neurons are reduced at peak lactation compared to those later in lactation. In contrast to the results in Experiment 4a, food restriction did not significantly affect the proportion of GnRH neurons that were activated by E2 treatment. These results suggest that the response of GnRH neurons to E2 stimulation is altered during lactation but that food restriction does not prolong this effect.

Results from experiments 4a and 4b suggest that food restriction attenuates E2-induced LH surges, in part, by altering the effects of E2 on neuronal activation within the AVPV. According to Levine, the stimulatory effects of E2 are related to the ability of this hormone to stimulate the synthesis of PRs within the AVPV, serving as an integrator of neuroendocrine signals necessary for the induction of the LH surge (Levine, 1997; Levine et al., 2001). Using this model, it was proposed that the changes in the ability of E2 to induce LH surges and neuronal activation in the AVPV might be related to an inability of this hormone to bind to its receptors at this site, or to a deficit in the transcriptional ability of these receptors when occupied. More specifically, we

hypothesized that, if food restriction or P attenuated E2 responses, they would do so by decreasing the levels of E2 receptors in the AVPV. It was also hypothesized that the ability of E2 binding to its receptor or a decrease in the ability of this receptor to induce transcription would be reflected in the synthesis of PRs within the AVPV.

Food restriction does not alter ERα immunoreactivity in the AVPV of lactating rats.- The possibility that food restriction or chronically elevated P would decrease the number of ERα available for binding in lactating rats on day 20 pp was investigated in Experiment 5. Results showed that neither food restriction nor chronic P treatment altered the number of ERα expressing neurons in the AVPV of lactating rats suggesting that these manipulations do not attenuate the LH surge or FOS-IR in the AVPV by reducing the number of ERα available on day 20 pp.

Previous work has shown that changes in food availability produce changes in the number of ERs in the hypothalamus. These changes, however, appear to be site specific. For example, food deprivation, or treatment with metabolic fuel inhibitors, decreases the number of ERα in the VMH but increases the number of these receptors in the MPOA (Li et al., 1994). In the Zucker rat, there is a reduction in the number of ERα positive cells within the AVPV that has been associated with the reduced ability of E2 to induce LH surges in these animals (Olster & Auerbach, 2000). The present results suggest that in the lactating rat the number of ERα positive cells are not affected by food restriction or by P on day 20 pp. The number of ERα available, however, may be affected earlier in lactation when metabolic demands are higher.

The numbers of ERα immunoreactive cells in the AVPV of lactating rats may not reflect the binding capacity of these nuclei. This argument is supported by data in which diabetic animals show an increase in ER in the MPOA, but the

binding capability of these receptors appears compromised as shown by receptor binding assays (Li et al., 1994; Siegel & Wade, 1979). Given the presence of a large number of ERα positive cells in these animals, the lack of binding suggest that either the ERα receptors are not functional, or that other types of E2 receptors are affected by this metabolic challenge.

Food restriction disrupts the ability of E2 to induce PR immunoreactivity in the AVPV of lactating rats and this effect is mediated by P.- As described above, sensitivity to E2 could be evaluated by comparing the ability of this hormone to increase the number of PR immunoreactive cells in the AVPV (Du et al., 1996). Results from Experiment 6, show that food restriction from days 1-14 pp or chronic P treatment to ad lib OVX dams, disrupts the ability of E2 to induce PR immunoreactivity in the AVPV on day 20 pp. In contrast, ad lib fed dams, or OVX food restricted dams given cholesterol implants showed increases in PR immunoreactivity within the AVPV. These data clearly support the notion that the endogenous increases in P secretion in food restricted lactating dams interferes with the ability of E2 to induce the PRs that are necessary for an LH surge. A similar effect has been found in Zucker rats, in which LH surges are attenuated by a similar deficit in E2 signaling (Olster & Auerbach, 2000).

In summary, the present findings suggest that food restriction, in part, prolongs lactational infertility by an attenuation of the positive feedback effects of E2. We also conclude that P mediates this effect primarily by affecting critical neuronal events occurring in the AVPV. In addition to these results, data from these experiments raise questions from which new avenues of research can emerge. These questions are outlined in the subsections that follow.

How does food restriction increase secretion of P?

It is known that food restriction increases plasma P concentrations in the lactating rat, and that this increase in P may plays a role in prolonging the inhibition of tonic LH release seen in late lactation (Woodside, 1991). In addition, as revealed by the present experiments, the effects of food restriction on P secretion during lactation may also mediate the sensitivity to the stimulatory effects of E2 on LH release.

The mechanisms through which food restriction increases P secretion during this reproductive state, however, remain undetermined. It is likely that the source of P secretion lies within the ovaries because circulating P levels in OVX lactating rats drop dramatically regardless of the diet regimen they followed (Walker et al., 1995). P can also be secreted by the adrenal cortex, and food restricted OVX dams have significantly higher levels of P than ad lib fed OVX dams, but the difference in P secretion amongst these groups is far too small to account for the concentrations of P seen in food restricted dams (Walker et al., 1995; Woodside, 1991). Furthermore, given the results from Experiment 2, the inhibitory effects of P on the E2-induced LH surge are governed by P secreted from the ovaries and not to P secreted by the adrenal cortex.

PRL is the primary factor regulating P secretion and the positive feedback effects of E2 in ad lib fed lactating rats. It is well established that PRL acts in the ovary to increase P secretion and it is responsible for the increases of P secretion seen during pregnancy and lactation (Smith, 1984). During lactation, PRL secretion is increased by the suckling stimulus, and these increases are concomitant to increases in P secretion (Smith, 1984).

Interestingly, the E2-induced LH surge can vary in response to the number of young a dam nurses, and in correlation with both PRL and P levels (Coppings & McCann, 1979). These results also suggest that different degrees of suckling stimulation may increase P levels via an increase in PRL secretion, and these may in turn suppress both tonic and E2-induced surge release of LH. This would explain why ad lib fed dams show a prolonged lactational diestrus when given hungry pups to nurse (Woodside & Jans, 1995). This would also explain why daily treatment from day 1pp with bromocriptine or RU486 restores ovulation by day 12-13 pp, so that once the postpartum corpora lutea undergoes atresia, PRL-induced P secretion is not there to reduce basal LH release or attenuate an E2 induced ovulatory responses (Hansen et al., 1983; van der Schoot et al., 1989; Woodside, 1991).

P secretion in food restricted dams is not related to PRL.- In food restricted dams, the suckling stimulus may not play an important role in the secretion of P given that their PRL levels are not any higher than those seen in ad lib fed dams (Walker et al., 1995). It is more likely that in food restricted dams, the increased energetic demands of producing milk for undernourished pups could in turn increase P secretion via PRL independent mechanisms to suppress the E2-induced LH surge. The mechanism for this PRL independent hypersecretion of P remains a mystery.

Low leptin levels during lactation may allow for icreased secretion of P from the ovaries.- Perhaps the answer to this enigma lies in the biosynthesis of steroid hormones. Within steroid hormone secreting glands, cholesterol is internalized into the mitochondria where a family of enzymes produced by the cytochrome P-450 gene transform cholesterol first into P, and subsequently into E2 (Richards & Hedin, 1988). One of the most important enzymes for the regulation of the rate of steroid biosynthsis is the cholesterol side-chain cleavage

cytochrome P-450 (P-450scc), which transforms cholesterol into pregnenalone (Richards & Hedin, 1988). Synthesis of the P-450scc enzyme is regulated by LH, and its levels of expression are increased soon after the LH surge presumably to augment P secretion (Richards & Hedin, 1988). Although LH activates the synthesis of this enzyme, leptin acts in ovarian cells *In Vitro* to limit it (Barkan et al., 1999). It is possible that the low levels of leptin seen in lactating rats liberate the production of P-450scc, therefore resulting in high levels of P. In food restricted dams, P concentrations would be exaggerated given that their leptin levels remain suppressed for an extended period of time (Woodside et al., 2001).

An alternative route through which leptin could regulate P is by limiting the production of the steroidogenic acute regulatory protein (STaR). This protein regulates the transport of cholesterol into the mitochondria for the transformation into steroids (Cherradi, Capponi, Gaillard, & Pralong, 2001). Recent findings show that leptin reduces STaR in adrenal cortex cell cultures, suggesting that in its absence the adrenal cortex and perhaps ovarian cells, overproduce this protein leading ultimately to an increase in P secretion (Cherradi et al., 2001). It should be noted, however, that the effects of leptin on ovarian steroidogenesis have been studied *in vitro* on cell cultures, which leads to questions about the applicability of these results *in vivo*. An approach that might prove more useful in determining whether the lack of leptin promotes oversecretion of P in food restricted dams would be to study the effects of such manipulations *in vivo* on the expressions of proteins and/or enzymes regulating P secretion. One could, for example, compare differences in the synthesis or expression of P-450scc and STaR in ovaries obtained from ad lib fed and food restricted dams at different times during lactation. Furthermore, one could also investigate whether the decrease in steroidogenic responses seen after leptin treatment is also changed during lactation and after food restriction. Indeed,

much work is needed to uncover the factors that generate the high levels of P seen in food restricted lactating rats.

How long do P concentrations need to be elevated to affect the positive feedback effects of E2?

One surprising result from the present studies was that, as shown in Experiment 3a, ad lib fed OVX dams given P for only two weeks remained unresponsive to the stimulatory effects of E2, even though they were free of P for three days before the E2 treatment began. This group was originally designed to mimic the pattern of P secretion seen in intact ad lib fed dams, and thus, it was expected that they would show LH surges comparable to those of intact dams. One possible explanation for this finding is that the presence of P for a two-week period was sufficient to prolong hyposensitivity to the effects of E2 on LH release. This possibility could be evaluated by comparing the E2-induced LH responses in day 15 pp ad lib fed dams OVX on day 1 pp and given P implants for either 5, 10, 15, or 20 days.

Why are the LH surges of lactating rats small in comparison with those of virgin OVX females?

The LH surges elicited by E2 in lactating rats were generally small in comparison with those seen in OVX virgin females. In fact, the surges observed in lactating rats were comparable to those seen in aging females after an LH-inducing steroid treatment (Wise, 1982). Nevertheless, these LH surges may be sufficient for ovulation, as demonstrated by Barraclough et al. and by Wise et al. (Barraclough, Turgeon, & Cramer, 1975; Wise, 1982), who showed that LH

surges of less than half the magnitude of a robust preovulatory LH response can still produce enough LH to induce ovulation. In contrast, the small LH discharges observed in the food restricted dams on day 15 and 20 pp, or in ad lib fed dams given P implants, may not result in follicular rupture (Barraclough et al., 1975). Future studies should be conducted to determine if indeed the LH surges observed in ad lib fed and food restricted dams are sufficient to induce follicular rupture, and to compare the number of mature ova released from these follicles.

The similarities between the LH surges of lactating rats and those of aging female rats in their hormonal responses to E2 may be also reflected in the neuroendocrine events that precede and occur during an LH surge. For example, aging female rats show reduced proportion of FOS-IR in GnRH neurons at the time of an LH surge (Lloyd, Hoffman, & Wise, 1994). Some authors have attributed these changes to an alteration in the circadian signal that provides for the timing of the surge (Wise, 1982).

There is currently much debate on the identity of this circadian signal, but one likely candidate is VIP, which is produced by cells in the SCN and is known to regulate LH release (Scarbrough, Harney, Rosewell, & Wise, 1996). Infusions of antisense oligonucleotides that block the synthesis of this peptide markedly attenuate steroid-induced LH surges (Harney, Scarbrough, Rosewell, & Wise, 1996). Also, VIP cells send projections to both the AVPV and the OVLT where their terminals are in close apposition with GnRH neurons that express FOS-IR at the time of the LH surge (van der Beek et al., 1994). Interestingly, GnRH cell bodies in the OVLT have VIP receptors, suggesting that VIP can act directly upon these GnRH neurons (Smith, Jiennes, & Wise, 2000). In aging females, in which preovulatory LH surges are attenuated to the same extent as those seen in our studies, there is a marked decrease in the proportion of GnRH cells that that are in close contact with VIP terminals and co-express FOS-IR (Krajnak et al., 2001).

This suggests that in aging female rats, GnRH neurons are less sensitive to VIP stimulation, and effect that could also occur in lactating rats. Interestingly, VIP synthesis is increased during lactation (Gozes, Avidor, Biegon, & Baldino, 1989), but it is not known if VIP secretion is altered, or if the number of VIP receptors within GnRH neurons changes during this reproductive state.

Alternatively, the reduced magnitude of the LH surge that occurs during lactation may be related to a reduced pituitary response to GnRH stimulation. As shown by Smith et al, the LH responses to exogenous GnRH treatment are attenuated during lactation, and a specific pattern of GnRH priming is necessary to restore full responsiveness even after litters have been taken away (Lee et al., 1989b). Thus, GnRH priming coincident with E2 treatment might be sufficient to restore the full magnitude of LH surges in lactating rats.

Why does RU486 have a stimulatory effect on E2-induced LH surges in Food restricted dams?

The releasing effect of RU486 on the E2-induced LH surge of food restricted dams appears to contradict several pieces of evidence showing that RU486 blocks E2-induced LH surges in virgin steroid treated females. These contradictory effects can be explained if one considers that P has both an inhibitory and stimulatory effect on LH release. If RU486 is given at a time in which P enhances the magnitude of the LH surge, the surge is dramatically decreased (Chappell & Levine, 2000; Lee et al., 1990b). In contrast, if RU486 is given at a time in which P inhibits the E2-induced LH surge, it has a stimulatory effect on LH release (Skinner et al., 1999).

In the present studies, RU486 was given in two separate treatments so as to block the inhibitory effects of P. Of these treatments, only the one in which

RU486 was given during and after the 250μg E2 injection (RU486 A) was successful in removing the inhibitory effects of food restriction, and presumably high levels of P, on the LH surge of the dams. On the other hand, in the RU486 B treatment the last injection of RU486 was given at the same time as the E2 bolus dose and this was not sufficient to elevate the LH response to E2 in food restricted dams.

This particular effect has been demonstrated previously in the sheep. During the ovulatory cycle of the ewe, there is a long luteal phase that which is characterized by elevated plasma concentrations of P, and basal concentrations of E2. In the follicular phase, P concentrations drop, and E2 levels rise to stimulate the LH surge necessary for ovulation (Feder, 1981). The endocrine changes in the cycle of the ewe are, therefore, very similar to those that seen during lactation in the rat. Interestingly, in studies where the E2-induced LH surge is blocked in OVX ewes by hormone treatments in which P is chronically high, RU486 treatment that is concurrent with E2 treatment restores the LH surge (Skinner et al., 1999).

The degree to which RU486 removed the inhibitory effects of P at the hypothalamus versus the pituitary remains to be determined. In the ewe, RU486 is thought to interfere with the inhibitory effects of P at hypothalamic sites that control the LH surge (Skinner et al., 1999; Skinner et al., 1998). It is therefore possible that the stimulatory effects of RU486 on LH release in food restricted dams seen in Experiment 3b, reflect a liberation of PRs in the AVPV of these animals. In this mechanism, RU486 competes with P to occupy newly formed E2-induced PRs in the AVPV, but unlike P, RU486 does not mark these receptors for degradation (Turgeon & Waring, 2000). Once the activity of RU486 on these PRs ends, they may become available for transactivation by cAMP to induce an LH surge (Turgeon & Waring, 2000). If this is indeed the case, one should see more

PR receptor immunoreactivity in the AVPV of food restricted dams receiving the RU486 A treatment, than in those receiving the RU486 B or vehicle treatment.

Is neuronal activation attenuated in lactating rats following E2 treatment?

Experiment 4a showed that FOS-IR in the AVPV, and within GnRH neurons in response to E2 treatment was low at peak lactation. In average, the levels of neuronal activation at this time were similar to those seen in OVX virgin female rats treated with oil, and lower than those observed in OVX virgin females that were given E2 treatment in a pilot study. These results suggest that both neuronal activation in the AVPV, and that within GnRH neurons, is attenuated during lactation. This assertion, however, cannot be made because our design did not include lactating rats treated with oil as a comparison group. Inclusion of these groups would permit us to determine the basal levels of activation in the AVPV and in GnRH neurons within the AVPV to make a better comparison of how FOS-IR changes in response to E2.

Why are the effects of food restriction reflected in neuronal activation of the AVPV but not in neuronal activation of GnRH neurons?

Overall, E2-induced neuronal activation in the AVPV of lactating animals remained low on day 20 pp, whereas the proportion of GnRH neurons in the OVLT of the same animals had increased by this time. Similarly, while there was an effect of food restriction on the number of FOS-IR cells in the AVPV, no such effect was recorded in the proportion of FOS-IR GnRH neurons. This was surprising considering that these two events are highly correlated with each other, and with the preovulatory or steroid induced LH surge in virgin females

(Le et al., 1999; Le et al., 2001).

There is some recent evidence suggesting that the AVPV and the OVLT respond differently to an E2 treatment that induces LH surges. In these studies, authors found that the message for the rate-limiting enzyme for GABA synthesis, glutamic acid decarboxylase (GAD) 67, is decreased in the AVPV on the afternoon of an E2-LH surge, presumably to allow the LH surge to occur. In contrast, no such changes were observed in the OVLT (Curran-Rauhut & Petersen, 2002). This divergent pattern of gene expression in response to E2 suggests that these two regions may, in spite of their functional association, respond differently to steroid hormone treatment. Furthermore, these differences highlight the importance of future research for a complete understanding of the heterogeneous nature of these responses.

It is also important to note that, while the expression of FOS-IR in GnRH neurons is closely associated with the release of LH, there are instances in which this neuronal correlate is seen in the absence of LH release (Le et al., 1997b). In fact, it is still debated whether FOS-IR in these neurons reflects events that are necessary for a concurrent surge, or if it reflects transcription of genes that increase GnRH synthesis to replenish intracellular stores of this peptide (Hoffman & Lyo, 2002; Rivest & Rivier, 1995). In addition, it is still undetermined if the Fos protein acts to induce transcription of GnRH or to stop it (Bruder, Spaulding, & Wierman, 1996; Eraly & Mellon, 1995). Therefore, the significance of the fact that food restriction does not differentially affect neuronal activation in GnRH neurons cannot be fully evaluated until more is known about the function of the Fos protein in regulating GnRH synthesis and release.

What is the importance of the AVPV in regulating the E2-induced LH surge during lactation?

In general, the present data support the contention that the AVPV is a crucial site in the integration process of both endocrine and neuronal signals that produce the LH surge. These data also support the notion that E2-induced PRs are a critical factor in this process. Little is known, however, about the events that follow the activation of PR-containing neurons, or whether other central pathways implicated in the E2 induction of the LH surge are also affected during lactation.

The AVPV contains cells that produce a variety of peptides and neurotransmitters including enkephalin, dynorphin, neurotensin, GABA, dopamine, cholecistokinin, and CRF (Simerly, McCall, & Watson, 1988; Simerly & Swanson, 1987). It appears that the large majority of neurons within the region of the AVPV that is most sensitive to E2 stimulation synthesize enkephalin and dynorphin, and of these, only dynorphin neurons respond to steroid hormone stimulation (Gu & Simerly, 1994; Simerly et al., 1988; Simerly & Swanson, 1987; Simerly, Young, & Carr, 1996b). Both of these opioid peptides, however, are associated with inhibitory effects on the release of LH so if they participate in the E2-induced LH surge they do so by indirect pathways that remain to be elucidated (Kalra, 1993).

There is a large body of evidence suggesting that excitatory amino acids (EEAs) are the critical stimuli for the surge release of GnRH (Brann & Mahesh, 1995). Within the AVPV, E2 induces increases in EEA receptors that correlate with the LH surge (Gu, Varoqueaux, & Simerly, 1999). These changes are restricted to changes in AMPA receptor subunits, but not to changes in NMDA receptors (Diano, Naftolin, & Horvath, 1997; Ping, Mahesh, Bhat, & Brann, 1997).

The dynamics of kainate receptors within the AVPV have not been investigated. These EEA receptor subtypes, however, are found in GnRH neurons, particularly those that are activated at the time of the LH surge (Eyigor & Jennes, 2000). Interestingly, EEA stimulation of LH release is absent in lactating rats, and this effect is dependent on both suckling stimulation and high levels of P (Pohl, Lee, & Smith, 1989). Therefore, it is possible that steroid hormones interact to induce changes in specific EEA receptors that are unique for each site and that might contribute the an optimal response to glutaminergic stimulation for the release of GnRH. Moreover, the endocrine changes seen during lactation may disrupt the ability of this system to respond to EEA by interfering with the synthesis of the different EEA receptors that are optimal for a surge to occur.

Although the present experiments focused on the AVPV, the positive feedback effects of E2 on the neuronal control of the LH surge have been also reported in other areas of the brain. For example, changes in the plasma concentrations of E2 across the estrous cycle are correlated with changes in the patterns of neuronal activation in adrenergic cell groups within the brain stem that project to the forebrain, particularly in cells that contain ERs and PRs (Haywood, Simonian, van der Beek, Bicknell, & Herbison, 1999; Jennes, Jennes, Purvis, & Nees, 1992; Lee, Moore, Hosny, Centers, & Jennes, 2000). Interestingly, responses mediated by the adrenergic system are attenuated during lactation (Abizaid et al., 2001; Abizaid & Woodside, 2002; Toufexis et al., 1998; Toufexis & Walker, 1996). It is therefore possible that reduced sensitivity to the stimulatory effects of E2 on LH release during lactation and after food restriction may also be reflected in reduced neuronal activation in these brain stem sites.

Similarly, because pituitary responses to exogenous GnRH stimulation are similar in ad lib fed and food restricted dams, we have assumed that the effects of food restriction or increased P take place primarily in the brain (Walker et al.,

1995). Nevertheless, the fact that E2-induced LH surges seen in lactating rats were never of the same magnitude as those seen in OVX virgin female rats suggests that the pituitary could also be less sensitive to E2 stimulation. Here, cAMP resulting from the binding of GnRH or NPY to their respective receptors transacivates P receptors to induce the release of LH (Leupen & Levine, 1999). In lactating rats, high P levels may reduce P receptors in the pituitary, resulting in lower levels of transactivation and ultimately, in low levels of E2-induced LH release. This effect would likely be exacerbated in food restricted dams given their higher P levels. In contrast, this effect would be blocked in lactating rats given the RU486 A treatment.

Finally, in addition to reducing the number of PRs that are available for transcription in the AVPV, the chronically elevated levels of P that are seen in food restricted lactating rats could also inhibit GnRH neurons directly. P can be metabolized centrally and peripherally to produce a series of biologically active molecules that have lower affinity to the PR, but that bind to GABA receptors and exert effects upon this receptors that are similar to those of GABA itself (Majewska, Demirgoren, and London, 1990). While PRs are scarce in GnRH neurons, GnRH neurons contain GABAa receptors that could presumably be activated by P metabolites (Herbison, 1997; Herbison, 1998; Jung, Shannon, Fritschy, & Ojeda, 1998). One of such metabolites, allopregnenalone, has been reported to bind to GABAa receptors and to reduce the release of LH (Schumacher, Coirini, Robert, Guennoun, & El-Etr, 1999). It is not known, however, if allopregnenalone or other metabolites are present in larger concentrations in lactating rats, or if these changes are more prominent in food restricted dams.

Can P act at other sites to alter neuronal responses to E2 in the AVPV of ad lib fed and food restricted dams?

The present studies have shown that nutritional deficits during lactation affect the positive feedback effects of E2 on the AVPV via the inhibitory effects of P. It is possible however, that P also acts at other hypothalamic sites connected to the AVPV to reduce neuronal activation after E2 treatment. The AVPV is connected to several regions of the brain that also contain PRs and ERs, and that are implicated in the regulation of LH release. For example, there is a significant number of reciprocal connections between the ARC and the AVPV suggesting that these regions are functionally related (Gu & Simerly, 1997). It is possible that nutritional information is conveyed from neurons in the ARC to alter neuronal activity in the AVPV and viceversa, and that these signals are also affected by changes in circulating levels of E2 and P (Shughrue et al., 1997; Shughrue, Scrimo, & Merchenthaler, 1998a; Shughrue et al., 1998b). Little is known, however, about how these regions interact to regulate LH release aside from their anatomical connections. Given that the ARC has been suggested as an important area controlling LH pulsatility (Maeda et al., 1995), and the AVPV is important for the LH surge (Levine, 1997), understanding these connections may solve how these two putatively independent systems interact.

How are E2 signals altered within cells in the AVPV of ad lib fed and food restricted dams?

Results from Experiment 6 show that E2-induced PR immunoreactivity within the AVPV is reduced in day 20 pp food restricted dams, or in ad lib fed

OVX dams given P implants. These results showed that chronically increased plasma levels of P reduced the ability of E2 to produce the unbound PRs that may be necessary to elicit a surge in LH. The cellular mechanisms that underlie these effects are not presently known but could include alterations in the processes that enable E2 binding and transcriptional function that ultimately lead to a surge in LH release.

In an attempt to answer this question, it was proposed that food restriction or high concentrations of P during lactation would result in reduced expression of the classical E2 receptor ERα. In Experiment 5, however, it was shown that the number of these receptors in the AVPV was not changed. Recent data suggest that while ERα is important for the induction of a full surge in LH release, the presence of ERβ may be critical for the induction of the surge. A recent study demonstrated that blocking synthesis of the ERβ E2 receptor subtype using antisense oligonucleotides results in the blockade of the LH surge in adult female rats (Orikasa et al., 2002). Therefore, the effects of chronic P during lactation and food restriction may be mediated by changes in the number of ERβ receptors in the AVPV available for binding.

In addition to changes in ERβ receptor number, food restriction or chronic P could alter the effects of E2 once this hormone has bound to its receptor. Upon binding, ERs actively recruit coactivator proteins that enhance the transcriptional capability of these receptors (Mani, Blaustein, & O'Malley, 1997; McKenna et al., 1999). Because all steroid receptors use these coactivator proteins to enhance their transcription, activation of different steroid hormone receptors within one cell may decrease the transcription of their target sequences because they compete for coactivator proteins (McKenna et al., 1999). Therefore the reduced number of PRs that is seen in food restricted or in P treated ad lib OVX dams after E2 treatment could be the result of chronically activated PRs that decrease

the number of coactivator proteins such as SRC-1, or CBP available for the transcription of ERs. Given that this particular interaction between receptors has been detected *in vivo*, and has been linked to the control of sexual behavior (Molenda et al., 2002), a next logical step in our research should be to study the effects of food restriction or P treatment on the expression of these coactivator proteins in the AVPV of lactating rats after E2 treatment.

Finally, signals related to energy balance may also alter the positive feedback effects of E2 directly at the intracellular level. For example, ERα containing neurons in periventricular regions of the anterior hypothalamus also contain leptin receptors (Diano, Kalra, & Horvath, 1998a; Diano, Kalra, Sakamoto, & Horvath, 1998b). In addition, there is increasing evidence for the interactions between the signaling cascades that follow activation of steroid hormone receptors and leptin receptors (Heinlein & Chang, 2002). These pieces of evidence lead to the speculation that bound leptin receptors cause changes in intracellular signals that enhance the transcriptional activity of E2. If true, this would explain why in the Zucker rat, an animal with a genetic mutation that affects the synthesis of the long form of the leptin receptor, the induction of PRs by E2 is disrupted (Olster & Auerbach, 2000). It would also explain why females given leptin antiserum would show attenuated preovulatory LH surges (Watanobe, Suda, Wikberg, & Schioth, 1999). Because leptin levels are low during lactation, and remain low for a longer period of time in food restricted dams, these putative enhancers of transcription may not be present.

Why are the positive feedback effects of E2 attenuated during lactation?

Lactational infertility in rats seems to be regulated by a combination of inhibitory signals that maintain tonic LH concentrations low, and a state of

hyporesponsiveness to the stimulatory effects of E2 that lead to the generation of a preovulatory LH surge. Together, these lead to a delay in follicular maturation, and impose a brake in the release of ova at a time in which the dam may no be ready to invest in a second set young. The present studies provide evidence that food restriction prolongs the presence of this brake with the ultimate result of a delay in ovulation.

The effects of food restriction both on the basal and surge of LH release provide for a mechanism that promotes the survival and production of an optimal number of young in response to changes in food availability. When food is freely available to the rat, lactational infertility only lasts for the period of time in which the young are dependent on their mother for nourishment. If food is only available in restricted amounts the dam postpones future reproductive efforts in order to devote sufficient nourishment to the present litter. A similar effect is seen in rats that become impregnated on the postpartum estrous. These dams also show a prolonged lactational diestrus. All of these examples support the contention that female rats, and possibly all female mammals, have been selected for their ability to manage resource allocation to present and future litters with the ultimate goal of increased reproductive success. The data from these experiments provides for a physiological mechanism underlying this adaptation.

In contrast to rats, lactating females of several other mammal species including humans, do not show increased levels of P across lactation, although they do show an attenuated response to the positive feedback effects of E2 (McNeilly, 1994; McNeilly, 2001; McNeilly et al., 1994). In these females, the suckling stimulus remains the primary inhibitor of basal LH secretion, but it is not known what attenuates the LH response to E2. In addition to this it is not known if food restriction causes an increase in the secretion of P in females from

these species, or if it prolongs reduced sensitivity to the positive feedback effects of E2. It is interesting, however, that leptin secretion is reduced in lactating females of many species (Aoki, Kawamura, & Matsuda, 1999; Block et al., 2001; Johnstone & Higuchi, 2001; Kadokawa, Blache, Yamada, & Martin, 2000; Kunz et al., 1999; Sorensen et al., 2002; Widmaier, Long, Cadigan, Gurgel, & Kunz, 1997). Therefore, as we did with the rat, we can speculate that the positive feedback effects of E2 may necessitate the presence of leptin for the induction of a surge.

Conclusion

Taken together, the experiments conducted in this thesis demonstrate that the lactational diestrus of rats is extended by food restriction in part through an attenuation of the positive feedback effects of E2. These studies also have generated a substantial amount of questions providing for exciting new avenues of research that may provide useful information in the mechanisms that regulate of lactational infertility in the rat and other species of mammals. In addition, answers to these questions may lead to a better understanding of the interaction between metabolic and endocrine signals regulating reproductive function not only during lactation, but also across the reproductive life span of a female mammal.

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APPENDIX A

Source tables for statistical analyses conducted in the experiments contained in Chapter 2

Table 1

Source table for the AVOVA conducted on baseline and peak LH levels data from ad lib fed and food restricted dams on days 15, 20 and 25 pp.

Source	SS	df	MS	F	p
Diet	38.68	1	38.68	4.88	.035
Day	102.46	2	51.23	6.46	.005
Diet X Day	18.73	2	9.36	1.18	.321
Time	179.53	1	179.53	22.53	.001
Time X Diet	35.81	1	35.81	4.49	.043
Time X Day	89.98	2	44.99	5.64	.008
Time X Day X Diet	19.6	2	9.8	1.23	.31
Error	231.12	29	7.97		

Table 2

Source table for the AVOVA conducted on baseline and peak LH levels data from OVX ad lib fed and food restricted dams on days 15, and 20 pp in Experiment 2.

Source	SS	df	MS	F	p
Diet	44.465	1	44.465	1.66	.212
Day	2.59	1	2.59	1.66	.212
Diet X Day	3.35	1	3.35	.12	.729
Time	758.56	1	758.56	28.43	.001
Time X Diet	72.45	1	72.45	2.72	.115
Time X Day	-4.65	1	-4.65	.002	.967
Time X Day X Diet	.9	1	.9	.03	.856
Error	533.56	20	26.68		

Table 3

Source table for the AVOVA conducted on baseline and peak LH levels data from intact, and OVX ad lib fed dams given P or cholesterol implants respectively. All animals were given E2 treatment and sampled on day 20 pp In Experiment 3a.

Source	SS	df	MS	F	p
Group	55.09	3	18.36	7.83	.001
Time	115.5	1	115.5	45.25	.001
Group X Time	22.3	3	7.43	2.91	.061
Error	44.56	19	2.35		

Table 4

Source table for the AVOVA conducted on baseline and peak LH levels data from intact food restricted dams given either oil or one of two RU486 treatment protocols. All animals were given E2 treatment and sampled on day 15 pp in Experiment 3b.

Source	SS	df	MS	F	p
Group	10.1	2	5.05	2.6	.11
Time	24.51	1	24.51	17.46	.001
Group X Time	10.89	2	5.45	3.88	.046
Error	27.15	14	1.4		

Table 5

Source table for t-tests conducted on Dam weight changes data from ad lib fed and food restricted dams in Experiment 1.

Day 15 pp

Group	N	Mean	Std. Error	df	t	p
Ad lib	18	44.17	3.61	37	5.44	.001
Food Restricted	21	-36.39	13.32			

Day 20 pp

Group	N	Mean	Std. Error	df	t	p
Ad lib	13	47.86	5.64	25	4.03	.001
Food Restricted	14	14.92	5.86			

Day 25

Group	N	Mean	Std. Error	df	t	p
Ad lib	7	31.37	4.33	12	.08	.084
Food Restricted	7	19.71	4.41			

Table 6

Source table for t-tests conducted on daily litter weight gain in animals nursed by either ad lib fed or food restricted dams in Experiment 1.

Day 15 pp

Group	N	Mean	Std. Error	df	t	p
Ad lib	18	14.85	1.93	37	13.66	.001
Food Restricted	21	8.43	.89			

Day 20 pp

Group	N	Mean	Std. Error	df	t	p
Ad lib	13	16.54	.81	25	1.56	.131
Food Restricted	14	14.61	.92			

Day 25

Group	N	Mean	Std. Error	df	t	p
Ad lib	7	35.33	2.19	12	.99	.34
Food Restricted	7	32.5	1.81			

Table 7

Source table for t-tests conducted on overall food intake data from ad lib fed and food restricted dams in Experiment 1.

Group	N	Mean	Std. Error	df	t	p
Ad lib	18	44.73	1.07	37	21.47	.001
Food Restricted	21	23.39				

Table 8

Source table for t-tests conducted on Dam weight changes data from ad lib fed and food restricted OVX dams in Experiment 2.

Day 15 pp

Group	N	Mean	Std. Error	df	t	p
Ad lib	12	39.89	5.1	22	14.29	.001
Food Restricted	12	-63.56	5.16			

Day 20 pp

Group	N	Mean	Std. Error	df	t	p
Ad lib	6	42.53	2.68	10	5.375	.001
Food Restricted	6	3.25	6.79			

Table 9

Source table for t-tests conducted on daily litter weight gain in animals nursed by either ad lib fed or food restricted OVX dams in Experiment 2.

Day 15 pp

Group	N	Mean	Std. Error	df	t	p
Ad lib	12	15.73	.51	22	9.61	.001
Food Restricted	12	10.19	.27			

Day 20 pp

Group	N	Mean	Std. Error	df	t	p
Ad lib	6	14.71	1.59	10	-.35	.73
Food Restricted	6	15.39	1.05			

Table 10

Source table for t-tests conducted on overall food intake data from ad lib fed and food restricted OVX dams in Experiment 2.

Group	N	Mean	Std. Error	df	t	p
Ad lib	12	51.75	4.65	22	17.68	.001
Food Restricted	12	28				

Table 11

Source table for one way ANOVA conducted on Dam weight change data from ad lib fed intact and ad lib fed OVX dams given cholesterol, P for two weeks or P for three weeks in Experiment 3.

Descriptive statistics

Group	N	Mean	Std. Error
Intact	6	3.83	7.95
OVX + Cholesterol	6	24.02	6.72
OVX + P – P	6	38.65	4.39
OVX + P	5	26.25	7.14

ANOVA table

Group	df	SS	MS	F	p
Between	3	3732.71	1244.24	4.88	.011
Within	19	4848.01	255.16		
Total	22	8580.72			

Table 12

Source table for one way ANOVA conducted on litter weight gain data from ad lib fed intact and ad lib fed OVX dams given cholesterol, P for two weeks or P for three weeks in Experiment 3.

Descriptive statistics

Group	N	Mean	Std. Error
Intact	6	15.86	.48
OVX + Cholesterol	6	14.72	1.16
OVX + P – P	6	13.8	.87
OVX + P	5	15.39	.99

ANOVA table

Group	df	SS	MS	F	p
Between	3	14.15	4.72	.99	.418
Within	19	90.36	4.76		
Total	22	104.51			

Table 13

Source table for one way ANOVA conducted on Dam weight change data from intact food restricted dams given oil or RU486 in two different injection protocols in Experiment 3.

Descriptive statistics

Group	N	Mean	Std. Error
Oil	5	-59.62	4.06
RU4861	6	-58.35	9.7
RU4862	6	-54.33	6.4

ANOVA table

Group	df	SS	MS	F	p
Between	2	1.84	.92	.61	.56
Within	14	21.24	1.52		
Total	16	23.08			

Table 14

Source table for one way ANOVA conducted on Litter weight gain data from intact food restricted dams given oil or RU486 in two different injection protocols in Experiment 3.

Descriptive statistics

Group	N	Mean	Std. Error
Oil	5	9.2	.47
RU4861	6	8.88	.54
RU4862	6	8.39	.52

ANOVA table

Group	df	SS	MS	F	p
Between	2	1.84	.92	.61	.56
Within	14	21.24	1.52		
Total	16	23.08			

APPENDIX B

Source tables for statistical analyses conducted in the experiments contained in Chapter 3.

Table 15

Source table for the AVOVA conducted on the FLI in the AVPV data from ad lib fed and food restricted dams on days 15, 20 and 25 pp in Experiment 4.

Source	SS	df	MS	F	p
Diet	275.64	1	275.64	4.43	.043
Day	455.94	2	227.97	3.67	.037
Diet X Day	152.53	2	1.23	1.23	.307
Error	1988.64	32			

Table 16

Source table for the AVOVA conducted on the proportion of GnRH neurons expressing FLI from ad lib fed and food restricted dams on days 15, 20 and 25 pp. in Experiment 4.

Source	SS	df	MS	F	p
Diet	152.93	1	152.93	1.46	.236
Day	1173.2	2	586.6	5.6	.009
Diet X Day	152.68	2	76.34	.73	.491
Error	3143.91	30	104.78		

Table 17

Source table for one way ANOVA conducted on the number of ERα immunoreactive cells in the AVPV from intact ad lib and food restricted dams, and OVX ad lib fed and food restricted dams given P and cholesterol respectively in Experiment 5.

Descriptive statistics

Group	N	Mean	Std. Error
Ad lib intact	6	83.33	7.07
Food Restricted intact	6	109.94	16.36
Ad lib OVX + P	6	117.72	18.04
Food Restricted OVX + Chol	5	117.73	23.7

ANOVA table

Group	df	SS	MS	F	p
Between	3	4676.76	1558.92	.97	.42
Within	19	30532.72	1606.98		
Total	22	35209.48			

Table 18

Source table for one way ANOVA conducted on the mean dam weight of intact ad lib and food restricted dams, and OVX ad lib fed and food restricted dams given P and cholesterol respectively in Experiment 5.

Descriptive statistics

Treatment	Group	N	Mean	Std. Error
Oil	Virgin OVX	6	2.72	1.26
	Ad lib intact	7	11.08	3.66
	Food Restricted Intact	5	13.07	5.22
	Ad lib OVX + P	7	20.99	6.81
	Food Restricted OVX + Chol	5	14.58	4.28
E2	Virgin OVX	5	87.2	32.76
	Ad lib Intact	6	51.53	17.98
	Food Restricted Intact	6	28.89	13.25
	Ad Lib OVX + P	5	34.6	13.47
	Food Restricted OVX + Chol	6	70.72	16.29

Table 18 (cont)

ANOVA table

Source	df	SS	MS	F	p
Group	4	4498. 79	1124.7	1.05	.392
Treatment	1	25248.6	25248.6	23.57	.001
Group X Treatment	4	9691.82	2422.96	2.26	.077
Error	48	51490.94	1072.73		

Table 19

Source table for the AVOVA conducted on the total number of GnRH cells in ad lib fed and food restricted dams on days 15, 20 and 25 pp in Experiment 4.

Source	SS	df	MS	F	p
Diet	536.69	1	536.69	.80	.378
Day	535.11	2	535.11	.8	.459
Diet X Day	744.78	2	744.78	1.11	.342
Error	668.95	30	668.95		

Table 20

Source table for t-tests conducted on Dam weight changes data from ad lib fed and food restricted dams in Experiment 4.

Day 15 pp

Group	N	Mean	Std. Error	df	t	p
Ad lib	18	48.6	4.85	34	17.75	.001
Food Restricted	18	-62.35	3.94			

Day 20 pp

Group	N	Mean	Std. Error	df	t	p
Ad lib	12	51.86	3.83	22	7.06	.001
Food Restricted	12	17.96	2.9			

Day 25

Group	N	Mean	Std. Error	df	t	p
Ad lib	6	33.22	3.77	10	2.32	.043
Food Restricted	6	18.9	4.87			

Table 21

Source table for t-tests conducted on daily litter weight gain in animals nursed by either ad lib fed or food restricted dams in Experiment 4.

Day 15 pp

Group	N	Mean	Std. Error	df	t	p
Ad lib	18	14.89	.56	34	9.21	.001
Food Restricted	18	9.35	.22			

Day 20 pp

Group	N	Mean	Std. Error	df	t	p
Ad lib	12	17.02	.97	22	.95	.353
Food Restricted	12	15.95	.58			

Day 25

Group	N	Mean	Std. Error	df	t	p
Ad lib	6	40.73	2.65	10	3.1	.011
Food Restricted	6	31.46	1.4			

Table 22

Source table for t-tests conducted on overall food intake data from ad lib fed and food restricted OVX dams in Experiment 4.

Group	N	Mean	Std. Error	df	t	p
Ad lib	17	47.01	1.37	33	17.71	.001
Food Restricted	18	23.39				

Table 23

Source table for one way ANOVA conducted on the mean dam weight of intact ad lib and food restricted dams, and OVX ad lib fed and food restricted dams given P and cholesterol respectively in Experiments 5 and 6.

Descriptive statistics

Day pp	Group	N	Mean	Std. Error
15	Ad lib intact	12	47.12	4.89
	Food Restricted Intact	13	-55.44	7.19
	Ad lib OVX + P	11	32.22	3.73
	Food Restricted OVX + Chol	11	-64.25	4.98
20	Ad lib Intact	12	43.66	5.98
	Food Restricted Intact	13	17.47	4.47
	Ad Lib OVX + P	11	35.18	3.47
	Food Restricted OVX + Chol	11	22.02	4.6

Table 23 (cont)

ANOVA table

Source	df	SS	MS	F	p
Group	3	84637.78	28212.52	60.45	.001
Treatment	1	75.96	75.96	.16	.689
Group X Treatment	3	2327.51	775.84	1.66	.191
Error	39	18202.58	466.73		
Day of Lactation	1	36830.77	36830.77	275.94	.001
Day of Lactation X Group	3	37882.22	12627.40	94.6	.001
Day of Lactation X Treatment	1	850.95	850.95	6.37	.016
Day of Lactation X Group X Treatment	3	287.58	95.86	.72	.547
Error	39	5205.554	133.48		

Table 24

Source table for one way ANOVA conducted on the mean litter weight gain of pups nursed by intact ad lib and food restricted dams, and OVX ad lib fed and food restricted dams given P and cholesterol respectively in Experiments 5 and 6.

Descriptive statistics

Day pp	Group	N	Mean	Std. Error
15	Ad lib intact	12	15.99	.57
	Food Restricted Intact	13	9.14	.30
	Ad lib OVX + P	11	13.17	.66
	Food Restricted OVX + Chol	11	10.48	.3
20	Ad lib Intact	12	18.38	.81
	Food Restricted Intact	13	16.8	.59
	Ad Lib OVX + P	11	15.51	.75
	Food Restricted OVX + Chol	11	18.48	.48

Table 24 (cont)

ANOVA table

Source	df	SS	MS	F	p
Group	3	235.45	78.48	15.25	.001
Treatment	1	25.44	25.44	4.94	.032
Group X Treatment	3	19.39	6.46	1.27	.303
Error	39	200.77	5.15		
Day of Lactation	1	597.98	597.98	246.2	.001
Day of Lactation X Group	3	172.84	57.61	23.72	.001
Day of Lactation X Treatment	1	.9	.9	.37	.547
Day of Lactation X Group X Treatment	3	2.85	.95	.39	.76
Error	39	94.73	2.43		