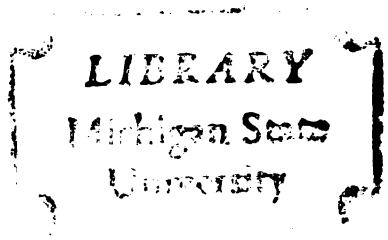

This is to certify that the

thesis entitled

STRESS EFFECTS ON PREGNANCY,
FETAL NORMALCY AND PROLACTIN RELEASE
IN THE LABORATORY RAT

presented by

Donald Wallace McKay

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Major professor

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STRESS EFFECTS ON PREGNANCY, FETAL NORMALCY AND PROLACTIN RELEASE IN THE LABORATORY RAT

By

Donald Wallace McKay

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ABSTRACT

STRESS EFFECTS ON PREGNANCY,
FETAL NORMALCY AND PROLACTIN RELEASE
IN THE LABORATORY RAT

by

Donald Wallace McKay

The effect of restraint stress on serum prolactin and reproduction was studied in pregnant Long-Evans rats. The female progeny of stressed and non-stressed dams were evaluated for developmental normalcy of growth, ovarian cyclicity, fertility and fecundity. In addition, temporal changes in serum prolactin concentrations and dopaminergic neuronal activity in selected brain regions were determined over a 24 hr period in pregnant and pseudopregnant rats.

In the first study, restraint stress treatments were administered to pregnant females at intervals during the 22 day gestation period. Pregnant rats were restrained from 0100-0400 hrs on days 4 through 7 of gestation. In contrast to non-stressed controls, no nocturnal surge of prolactin was detected in blood samples drawn at 0400 hrs on days 4 or 7 of pregnancy. The stress treatment although severe enough to interfere with the prolactin surge had no effect on gestation length, abortion rates, or live litter size. Offspring from these pregnancies experienced no deficiencies in weight or survivability when measured at 0, 14 and 28 days of age. In the next trial, restraint was administered from 1300 through 1600 hrs on days 4-7, 8-11

or 12-15 of pregnancy. Serum prolactin concentrations measured in samples collected at 0300 hrs following the first and fourth days of the respective treatment interval were not affected by restraint stress. No effect of stress was detected on the length of gestation, incidence of abortion or live litter size in any of the groups tested. Prenatal stress did not influence the age of pubertal vaginal opening in randomly selected offspring from the treatment groups. The fertility of prenatally stressed females was not different than prenatally non-stressed controls. In the third stress trial, restraint was applied twice daily from 0900-1100 hrs and 1300-1500 hrs on days 10-13, 14-17 or 18-21 of pregnancy. The treatment administered on days 10-13 of pregnancy resulted in a significant incidence of abortion, while the length of gestation was significantly increased in females restrained on days 14-17 of pregnancy. The pregnancies of animals treated on days 18-21 were unaffected by restraint. Upon maturity, offspring from these pregnancies were all capable of producing normal litters. These results demonstrate that although certain stress regimes can terminate pregnancy, the stress induced alteration of the nocturnal PRL surge is not sufficient to cause abortion. Furthermore, prenatally stressed female offspring are as developmentally normal as non-prenatally stressed controls in terms of growth, fertility and fecundity.

In the second study, temporal changes in prolactin and LH concentration and dopamine neuronal activity in selected brain regions were determined over a 24 hour period in pregnant, pseudopregnant and diestrous rats. Two daily surges of prolactin were observed on day 6 of pregnancy and pseudopregnancy, but not in diestrous rats.

Similarly, biphasic changes in the rate of DOPA accumulation were observed in the median eminence, but not in other brain regions, of pregnant and pseudopregnant rats, while there were no changes in diestrous rats. These results suggested a specific activation of tuberoinfundibular dopamine (TIDA) neurons during pregnancy and pseudopregnancy. Manipulation of the surge release of prolactin by restraint stress or ovariectomy following cervical stimulation resulted in changes in the rates of DOPA accumulation specific to the median eminence. Restraint stress during the expected nocturnal prolactin surge of pregnancy from 2300 hrs day 5 until 0300 hrs day 6 delayed the appearance of the surge between 3 and 6 hours. The peak rate of DOPA accumulation in the median eminence was likewise delayed by restraint stress. Ovariectomy following cervical stimulation eliminated both the diurnal prolactin surge and the subsequent increase in median eminence DOPA accumulation. These results suggest that specific alterations of TIDA neuronal activity during pregnancy and pseudopregnancy may be related to the surge release of prolactin from the anterior pituitary.

DEDICATION

I dedicate this thesis to my wife, Kathleen.

ACKNOWLEDGEMENTS

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INTRODUCTION

Stress is an oft used, but poorly defined concept. While commonly stress is associated with anxiety or trauma, it may be more accurately described as an organism's attempt to maintain homeostasis when confronted by novel or noxious stimuli. Since it is recognized that both psychological and physical stressors can affect many body functions, many management decisions in the livestock industry are made in an attempt to reduce stress. Yet without an understanding of the mechanisms by which stress produces alterations in the body's processes, any management decisions will be lacking a sound basis.

The stress response is mediated by a number of neuroendocrine reactions. The most thoroughly documented endocrine response to a stressor is the sustained release of adrenal corticoids. While the increased release of adrenal corticoids is a reliable index of stress, it is now apparent that stress can influence the endocrine control of most body functions.

A number of studies has confirmed that stress can interfere with reproduction, however, the mechanisms through which stress acts to inhibit reproductive processes are unknown. The identification of stress induced alterations in the hormonal regulation of reproduction will provide knowledge of the physiology of reproduction, and give as a basis from which to make decisions regarding the management of stress.

To study the effects of stress in the pregnant female, one must not only consider the deleterious consequences of stress on the health of the dam or the success of the pregnancy, but it is important to assess the normalcy of the offspring that were stressed in utero. Previous reports have indicated that such offspring suffer from various "emotionality" disturbances and altered sexual behavior, but studies on functional reproductive capabilities of these animals are lacking.

Throughout this thesis, major emphasis will be given to the effects of restraint or stress on reproduction in the rat. The rat offers several distinct advantages over other species in the study of reproduction: 1) Fertility - the laboratory rat under standard conditions is capable of producing litters of consistent size in a short time; 2) management - because of their size and short estrous cycle length, the routine husbandry and breeding of laboratory rats is simplified; and 3) background knowledge - the major reason for the use of the laboratory rat in the study of reproduction is the abundance of information available on the hormonal support of pregnancy in this species.

The present investigation had the following specific aims:

1) To examine the effects of stress on the maintenance of pregnancy, with regard to the hormonal environment sustaining the pregnancy.
2) To determine the normalcy of the development and function of offspring that were stressed prenatally.

3) To more thoroughly characterize stress effects on prolactin release during pregnancy, and to associate those changes with specific alterations in neuronal activity within the hypothalamus.

Chapter 1 will be an overview of reproduction in the rat with particular emphasis on the hormonal control of pregnancy and pseudopregnancy. Included in this chapter is a brief review of physiology to acquaint the reader with terminology and current thought.

Stress effects on pregnancy and normalcy of offspring will be the subject of Chapter 2. In addition to a review of the literature involving stress effects on hormone release, pregnancy, and offspring development, this section will include original work that addresses the first two objectives of the thesis.

The next chapter focuses on the regulation and release of prolactin during pregnancy and pseudopregnancy. The literature surveyed in Chapter 3 selectively deals with a possible catecholaminergic control of prolactin release, and the hypothesis that dopamine is a major inhibitor of pituitary prolactin secretion. The results of this section provide additional insight and explanation for the findings in the previous chapter.

Finally, Chapter 4 is a brief summary and discussion of the aforementioned studies.

CHAPTER 1

Reproduction in the Laboratory Rat

The reproductive "normalcy" of a laboratory rat is often established by observing the pattern of estrous cycles. In the laboratory, this is accomplished by the use of a vaginal smear or wet prep followed by microscopic examination of the obtained cells. Changes in vaginal histology very accurately reflect alterations in ovarian hormone secretion such that diestrus is characterized by the presence of leucocytes in the smear, while proestrus and estrus preps typically show an abundance of nucleated and cornified epithelial cells, respectively (Nalbandov, 1964). Lack of a functional corpus luteum (C.L.) accounts for the short cycle length of 4-5 days in the laboratory rat (Bone, 1979) compared to the 21 day estrous cycle of the pig or cow which form functional corpora lutea in each ovarian cycle. Stimulation of the uterine cervix during proestrus, estrus, or the first day of diestrus triggers neuroendocrine mechanisms which may result in the development of functional corpora lutea (Long and Evans, 1921). If stimulation of the cervix occurs during copulation with a fertile male, then corpora lutea of pregnancy are formed and maintained for approximately 22 days, the normal gestation period in the rat.

If cervical stimulation is via artifical means (e.g., glass rod, or electrical stimulation) or by contact with an infertile male, then

corpora lutea of pseudopregnancy are formed which usually regress 12 to 14 days later. Thus, CL regression or luteolysis can be temporarily delayed for 2 to 3 weeks by artifical cervical stimulation or natural mating, respectively.

Hormones of Pregnancy

The most pronounced alteration in hormone secretion occurring during pregnancy and pseudopregnancy is increased progesterone release from the corpus luteum. Several years ago, it was reported that the function of the corpus luteum was dependent on pituitary luteotropic stimulation (Astwood, 1941). In recent years it has been shown that the primary pituitary luteotroph is prolactin and the luteotrophic function of prolactin is dependent on the stimulatory function of two daily surges of hormone secretion (Butcher et al., 1972; Freeman et al., 1974; Smith et al., 1975). The surges of prolactin occur twice a day in pregnant and pseudopregnant rats such that one surge (nocturnal) appears in the early morning prior to the illumination of the vivarium, and a smaller peak (diurnal) is detected just prior to lights off (Butcher et al., 1972; Freeman et al., 1974).

Early studies of this endocrine control system demonstrated that increased pituitary prolactin release on the second day of diestrus could maintain functional corpora lutea (Nikitovitch-Winer and Everett, 1958) and that prolactin (PRL) supplementation could maintain pregnancy in hypophysectomized rats (Astwood, 1941). Pharmacological blockade of the prolactin surges on day 2 of pseudopregnancy resulted in the precipitous decline in serum progesterone concentrations and the regression of the corpora lutea (Smith et al., 1976). These results suggest that the surge release of prolactin on day 2 of

pregnancy or pseudopregnancy is responsible for the "rescue" of the C.L. from regression or luteolysis (Smith et al., 1975).

Maintenance of the Corpus Luteum

In addition to prolactin, other hormones are involved in the C.L. maintenance of pregnant and pseudopregnant rats. A number of studies have demonstrated a role for LH in sustaining the corpora lutea (Rothchild et al., 1974; Morishige and Rothchild, 1974a; Lam and Rothchild, 1977). Current evidence suggests that the function of LH is to stimulate the luteal production of androgen, which is subsequently aromatized to estrogen within the C.L. (Gibori et al., 1978). The resultant estrogen synergizes with prolactin to sustain luteal progesterone (Gibori et al., 1977; Gibori and Keyes, 1978; Gibori and Richards, 1978; Gibori et al., 1979).

Hormone Regulation of Prolactin Surges

The importance of prolactin for the initiation and maintenance of luteal function necessary to sustain pregnancy in the rat cannot be overstated. The interrelationships between ovarian steroids and prolactin release were recognized early. The induction of pseudopregnancy is most readily accomplished during stages of the estrous cycle when estrogen and progesterone are elevated (Greep and Hisaw, 1938). Additionally, pseudopregnancy could be achieved by single injections of estrogen or progesterone, or multiple prolactin injections in the absence of cervical stimulation (Alloiteau, 1957; Alloiteau and Vignal, 1958a,b).

Maintenance of the surge release of prolactin is affected by ovarian steroid secretion. Ovariectomy (OVX) following cervical stimulation causes the abolition of the diurnal prolactin surge and an attenuation of the nocturnal surge and its premature disappearance after day 7 (Freeman et al., 1974). Replacement therapy of progesterone restores the nocturnal surge release of prolactin in the OVX rat, but the diurnal surge remains absent (Freeman and Sterman, 1978). Estradiol implants in the OVX cervically stimulated female reinstate the diurnal surge but lessen the magnitude of the nocturnal surge. These findings demonstrate the importance of the ovary in the normal secretion of prolactin of pseudopregnancy. Steroid hormones, however, are not required for the initiation of prolactin surges induced by cervical stimulation, as surge concentrations of serum prolactin have been achieved in ovariectomized-adrenalectomized rats (Smith and Neill, 1976a).

Uterine and Placental Influences on Pregnancy and Pseudopregnancy

Although the successful maintenance of pregnancy depends upon the secretion of progesterone from the corpus luteum (Csapo and Wiest, 1969), pituitary release of prolactin is only necessary to maintain pregnancy the first eleven days of gestation (Pencharz and Long, 1931).

Rat placental luteotrophin (rPL) a prolactin-like compound from the placenta is first measurable on day 8 of pregnancy. During the latter half of the 22 day pregnancy in the rat, rPL is the predominant luteotrophin (Gibori and Richards, 1978). The diurnal and nocturnal pituitary surges disappear coincident with the development of placental

luteotrophin secretion. It is speculated that rPL may feedback to the hypothalamus to cause the early suppression of prolactin surges during pregnancy (Yogev and Terkel, 1978). During pregnancy, the nocturnal surge is apparent for 10 days, while the diurnal surge endures for only the first 8 days (Smith and Neill, 1976b). However, in pseudo-pregnant rats, the nocturnal surge persists through day 11 while the diurnal surge disappears a day earlier.

Earlier findings demonstrated that hysterectomy (HYST) prevented the regression of the C.L. (Hashimoto et al., 1968; Hausler and Malven, 1971), which suggested that uterine or placental factors may regulate the disappearance of the prolactin surges. Removal of the uterus but not the ovaries of cervically stimulated rats results in the maintenance beyond 16 days of both nocturnal and diurnal surges (Freeman, 1979). Although the uterus produces a substantial amount of prostaglandin $F_{2\alpha}$, a potent luteolytic agent that has its action directly on the C.L., there appears to be a uterine factor that acts within the CNS to suppress the surge release of prolactin. OVX-HYST rats supplanted with progesterone experienced both surges at day 16, while the diurnal surge was absent in progesterone treated OVX rats with intact uteri. In pregnant rats, progesterone is capable of reinitiating the nocturnal PRL surge up to 4 days after its normal cessation provided the uterine-placental unit is removed (Voogt, 1980). Thus, it appears that the uterus produces a substance that works independently of any luteolytic action on the C.L., and that this factor acts to terminate the nocturnal release of PRL via an undetermined neuronal mechanism.

CHAPTER 2

Stress Effects on Reproduction and Developmental Normalcy

Endocrine Response to Stress

Numerous observations have shown that the scope of the endocrine response to stress is not limited to the hypothalamo-hypophysial-adrenal axis (Ajika et al., 1972; Brown-Grant, 1954; Krulich et al., 1974). It was suggested that the adrenals may act to modify the release of the pituitary hormones (e.g., LH, PRL) as pharmacological doses of glucocorticoids (50 µg dexamethasone/100 g body wt) have been shown to lessen the stress induced release of some pituitary hormones (Euker et al., 1975; Harms et al., 1975). However, the adrenal glands are not necessary for the manifestation of many other stress responses such as decreased TSH secretion or inhibition of ovulation, and they may have little physiological function in the mediation of acute stress effects on pituitary hormones other than ACTH (Brown-Grant, 1954; McKay et al., 1975).

A number of studies have demonstrated that the magnitude and direction of the hormonal changes due to stress are dependent upon such factors as age, reproductive status, circadian cyclicity, and the intensity and duration of the stressor (Dunn et al., 1972; Morishige and Rothchild, 1974b; Riegle and Meites, 1976a,b; Tache et al., 1976).

Stress Effects on Prolactin and LH Release

Since prolactin release was demonstrated to be "stress susceptible" (Grosvenor et al., 1965), a number of studies have been conducted to

determine the effects of acute stress on serum prolactin concentrations. Such routine laboratory procedures as blood sampling were shown to result in significant elevations of prolactin concentrations in female rats (Neill, 1970). Acute ether exposure and blood sampling evoked dramatic increases in serum PRL and LH in intact male rats, while the same stress in gonadectomized males resulted in a decrease in LH levels and a concomitant elevation of serum PRL concentrations (Euker et al., 1975).

The reported increases of adenohypophysial hormones other than ACTH, which followed "stress" were in apparent contradiction to Selye's "hypophyseal shift theory" (Selye, 1946). Based on morphological and functional criteria, it was predicted that the "general adaptation syndrome" or stress response would result in depressed release of all pituitary hormones other than ACTH, as such functions as growth, sexual development, and thyroid activity were shown to be decreased when animals were subjected to a chronic stress treatment.

Indeed, it was subsequently demonstrated, that repeated exposures to the stressor, over a period of days resulted in decreased release of LH, PRL, growth hormone (GH), and follicle stimulating hormone (FSH), while corticosterone concentrations were elevated (Tache et al., 1976; Riegle and Meites, 1976b). These results demonstrate profound differences in the pituitary response to short or acute stressors versus a repeatedly or chronically stressful environment.

Stress Effects on Reproduction

A number of reproductive processes in the female are believed to be affected by stress. Stress has been shown to decrease or block

ovulation (Terman, 1973; Hagino et al., 1969; McKay et al., 1975), while fertilizability of oocytes appears to be relatively stress resistant (Ulberg and Sheean, 1973; Sod-Moriah, 1971). Studies considering its effects of stress on egg and embryo development after fertilization indicate variable alterations in reproductive function. Euker and Riegle (1973) studied the effects of restraint stress administered over a number of days on pregnancy. Reproduction was affected by stress in an "all or none" fashion; the females aborted, or they gave birth to a litter of normal size.

It was demonstrated that increased adrenal activation was detrimental to pregnancy, as increased body temperature caused embryonic degeneration in intact females, but not adrenalectomized rats (Fernando-Cano, 1958) or ewes (Tilton et al., 1972). Decreased numbers of implantation sites were evident when ACTH or corticoids were administered prior to nidation in rats (Yang et al., 1969). The maintenance of ACTH at high levels regardless of blood glucocorticoid concentrations resulted in decreased pup and litter size in mice (Kittinger et al., 1980). This suggests that ACTH may affect reproductive function independent of its effect on the adrenals. In contrast to the study of Kittenger et al. (1980), we found that adrenalectomized pregnant rats maintained a normal pregnancy (McKay and Riegle, 1978). These results indicate that increased ACTH, due to adrenalectomy, was not sufficient to disrupt pregnancy.

Restraint stress administered to adrenalectomized rats on days 1-4 of pregnancy resulted in a total loss of pregnancy associated with a precipitous decline in serum progesterone concentrations (McKay and

Riegle, 1978). Similar stress treatment of intact pregnant females had no effect on pregnancy, and resulted in increased serum progesterone levels. These findings suggest that adrenal progesterone may have an important function in the maintenance of pregnancy.

The aforementioned findings demonstrated drastic alterations of pituitary and adrenal hormones due to stress. Whether or not these hormonal alterations act centrally to affect pregnancy or have a peripheral mode of action has not been established. Experimental alteration of the uterine vasculature has resulted in embryonic malformations (Brent and Franklin, 1960), and increased embryonic mortality (George et al., 1967; Senger et al., 1967). However, it appears that the uterus is relatively resistant to sudden changes in circulation during pregnancy (Bruce, 1972). The resistance may be explained in part by the development of collateral circulation (Bruce, 1967), or by circulatory changes within the fetus to aid its continued survival (Assali and Brinkman, 1973).

Prenatal Stress and Developmental Normalcy

In addition to stress induced embryonic mortality that may arise during pregnancy, it is important to consider prenatal induced stress anomalies manifest in fetal or postnatal development (Euker and Riegle, 1973). Since Thompson (1957) first demonstrated the effects of prenatal psychological stress on offspring emotionality, many investigators have attempted to study the phenomenon. Archer and Blackmun (1971) reviewed the literature on this subject, and summarized that due to differences in: 1) sex, strain and species; 2) the prenatal manipulation; and 3) the completeness of the description of

behavioral changes, very few conclusions could be drawn except that "some change in activity-reactivity may be induced".

Behavioral abnormalities coincident to prenatal immobilization stress have been reported. Adult prenatally stressed males exhibit less mounting behavior (i.e., demasculinized) (Ward, 1972; Herrenkohl and Whitney, 1976), and display a higher incidence of lordosis in response to other males (i.e., femininized) (Ward, 1972; Dahlof et al., 1977). Some of the observed changes of offspring development, however, may be due to stress effects on the mothers' nursing ability (Herrenkohl and Whitney, 1976).

Prenatal physical stress or glucocorticoid treatment have been implicated in such physical changes as delayed postnatal growth (Barlow et al., 1978; Reinisch et al., 1978), cleft palate (Barlow et al., 1975) and decreased ano-genital distances of male rat pups (Dahlof et al., 1978a,b). Neuroendocrine reflexes have been reported altered by prenatal stress, as ether stress administered to adult prenatally stressed males resulted in only a small increase in prolactin secretion in contrast to control animals (Politch et al., 1978), some investigators believe that developmental behavioral changes such as feminization and demasculinization may be due to an altered androgenization of the fetal rat brain (Ward and Weisz, 1980), as maternal restraint stress during late gestation alters the occurrence of peak levels of testosterone within the male fetus (Ward and Weisz, 1980; Weisz and Ward, 1980).

Chapman and Stern (1978, 1979) issued a caveat to the interpretation of maternal stress studies: Failure to control for the litter

variable, e.g., litter size, male/female pup ratio, genetic lineage, may account for previously reported effects of prenatal stress. In studies that followed similar stress regimen as that of Ward (1972) and Herrenkohl and Whitney (1976), it was found that litter variance in the analysis accounted for most of the observed changes. Under conditions of controlled litter size, balanced for sex, the removal of litter variance eliminated any statistical significance that otherwise would have been attributed to the stress treatment. These investigators further cautioned against the use of low animal numbers and suggested that the use of littermates in the same treatment group be avoided.

While less data exist for prenatal stress effects on the subsequent development of females, one laboratory has demonstrated decreased fertility and fecundity in such animals (Herrenkohl, 1979; Herrenkohl and Gala, 1979). Adult prenatally stressed females displayed a significant inability to nurse litters past day 10 of lactation, even though the litter size in these animals was smaller than in non-stressed control females. Behaviorally, prenatally stressed female mice appeared to be less aggressive toward intruder males, and it was speculated that this is to compensate for a decreased attractivity to males (Politch and Herrenkohl, 1979).

Taken together, the findings presented in this literature review do not provide a clear understanding of stress effects on hormone release or reproductive control mechanisms. These discrepancies that seem to characterize the entire study of stress relate to major problems: 1) the use of a poorly defined stressor and lack of a complete description of stress effects and 2) the individual and

strain differences in the responsiveness to stressors. To overcome these problems, it is necessary that investigators utilize more carefully controlled experiments in which a measurable and repeatable stressor is administered to the animal. Objective measures of the stress response must be used, such that individual variation may be more easily partitioned.

The purpose of this series of studies is to 1) examine the effects of a chronic restraint stress on maternal prolactin concentrations, and on the ability of pregnant rats to sustain a normal pregnancy, and 2) assess the development and/or reproductive competence of prenatally stressed female offspring.

Materials and Methods

Animals, Housing and Routine Handling

Long-Evans rats were housed in a temperature (23±2°C) and light controlled vivarium (lights on from 0600 to 1800 hrs) for at least two weeks prior to experimentation. Male and female rats were housed 3 and 4 rats per box, respectively, and were allowed free access to food (Wayne Lablox) and water except during restraint treatments. Females for any given experiment were of similar age and background. Daily vaginal lavage and cytological evaluation were used to determine each rat's reproductive status with regard to pregnancy, breeding, or ovarian cyclicity. All female rats were handled daily for this purpose a minimum of two estrous cycles prior to experimentation, and continued until day 16 of pregnancy.

Breeding

On the afternoon of proestrous as determined by the vaginal wet prep, one adult female was placed in a box of males housed 3 per cage. The next morning upon return to her home cage, a vaginal wet prep was obtained and observed microscopically for the presence of sperm. The detection of sperm established Day 1 of pregnancy.

Blood Collection

Blood samples were collected via orbital sinus puncture under light ether anesthesia at various times as specified in Results. Control samples were collected within 90 seconds of cage disturbance to minimize the effects of bleeding on serum hormone concentrations.

The resultant blood samples were allowed to clot and were refrigerated at 4°C overnight. The clotted samples were centrifuged at 4°C for ten minutes to separate serum from cellular elements. Individual samples were stored in disposable glass culture tubes at -20°C for several days until radiommunoassays were performed for the measurements of prolactin.

Restraint Treatment

Restraint stress was achieved by securing ether anesthetized rats with tape to a stainless steel counter top. The rats were thus maintained in a supine position for intervals as specified in Results. During the stress interval, the animals were physically disturbed every 10-20 minutes to prevent acclimation to the restraint. Non-stressed controls remained undisturbed in their cages during the same time interval. Any restraint or blood sampling conducted between

1800-0600 hrs was facilitated by the illumination of two red 25 watt incandescent bulbs.

Radioimmunoassay

Double antibody RIA determinations of PRL and LH serum concentrations were made with the aid of kits supplied by Dr. A.I. Parlow of the NIAMDD, and a second antibody, ovine anti-rabbit gamma globulin, developed at the Endocrine Research Unit. Hormone values measured in sera, are expressed in terms of NIAMDD rat prolactin RP-1 and NIAMDD rat LH RP-1. All blood samples were run in duplicates of two dilutions and samples from any particular experiment were processed in the same assay to limit variability.

Statistical Analyses

Frequency of abortion was determined by Chi-square adjusted for small n (Sokal and Rohlf, 1969; Rohlf and Sokal, 1969). Differences in the length of gestation, litter size comparisons, number of days until vaginal opening and comparisons of serum prolactin concentrations were evaluated by use of Student's *t*-test (Sokal and Rohlf, 1969; Rohlf and Sokal, 1969). Weight of litters was evaluated by a two-way analysis of variance by use of the Program Balanova supplied by the MSU computer laboratory.

Results

The Effect of Restraint Stress on the Nocturnal Surge of Prolactin and Reproduction in Long-Evans Rats

In the first study, the restraint was administered between 0100 and 0400 hrs on days 4-7 of pregnancy. (Preliminary data in our

laboratory established the existence of an early morning (nocturnal) surge of prolactin at this time.) Serum prolactin concentrations were decreased ($p<.001$) from control when measured at the end of the restraint period on days 4 and 7 of pregnancy (Figure 1). The four day stress treatment did not block the occurrence nor the magnitude of the diurnal prolactin surge as measured at 0100 hrs on day 7 of pregnancy. Although the acute effect of stress on serum prolactin was pronounced, this particular stress regimen had no effect on abortion, litter size or gestation length (Table 1). Within 16 hours of parturition, the litters were weighed and sexed. The litters were reduced to 3 males and 3 females. No significant differences of weight were detected between control and prenatally stressed litters when measured at birth 14 or 28 days of age (Figure 2).

The Effect of a 3 hr Restraint Stress Administered on Days 4-7, 8-11, or 12-15 of Pregnancy

It is established that restraint stress administered in the afternoon during early pregnancy or pseudopregnancy is capable of decreasing the magnitude of the diurnal prolactin surge (Riegle and Meites, 1976b; Freeman et al., 1974). Although previous work suggested that the nocturnal surge of prolactin was not affected by stress (Freeman et al., 1974), our work demonstrated that restraint stress administered during the time of the expected surge could interfere with its occurrence. To further explore the effects of restraint stress on reproduction and the nocturnal surge of prolactin, the following experiment was conducted.

Pregnant female rats were subjected to restraint stress from 1300-1600 hrs on days 4-7, 8-11, or 12-15 of pregnancy. A blood

Figure 1. **The effect of 4 days of 3 hr restraint stress treatments** initiated on day 4 of pregnancy on serum prolactin concentrations.

Serum prolactin concentrations were determined in blood samples collected by orbital sinus puncture before and after stress on the first and fourth days of treatment. The open bars represent mean prolactin concentrations of non-stress controls (n=14), and the shaded bars depict the mean prolactin levels of restraint stressed animals (n=13). The vertical line atop each bar represents 1 SE.

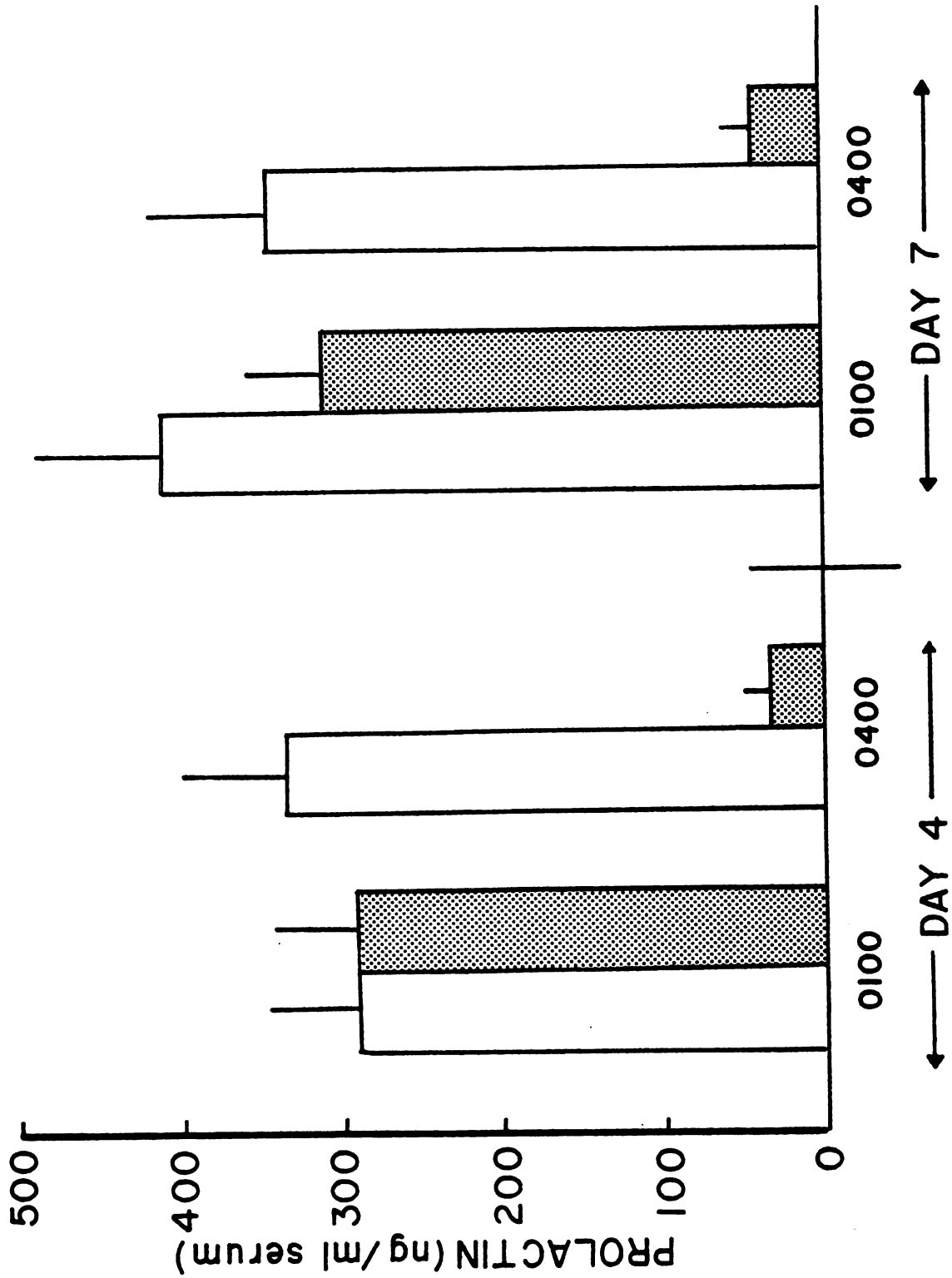

PROLACTIN (ng/ml serum)
500
400
300
200
100
0
0100
0400
DAY 4
0100
0400
DAY 7

TABLE 1

Effect of Restraint Stress Administered from 0100-0400 hrs on Days 4-7 of Pregnancy

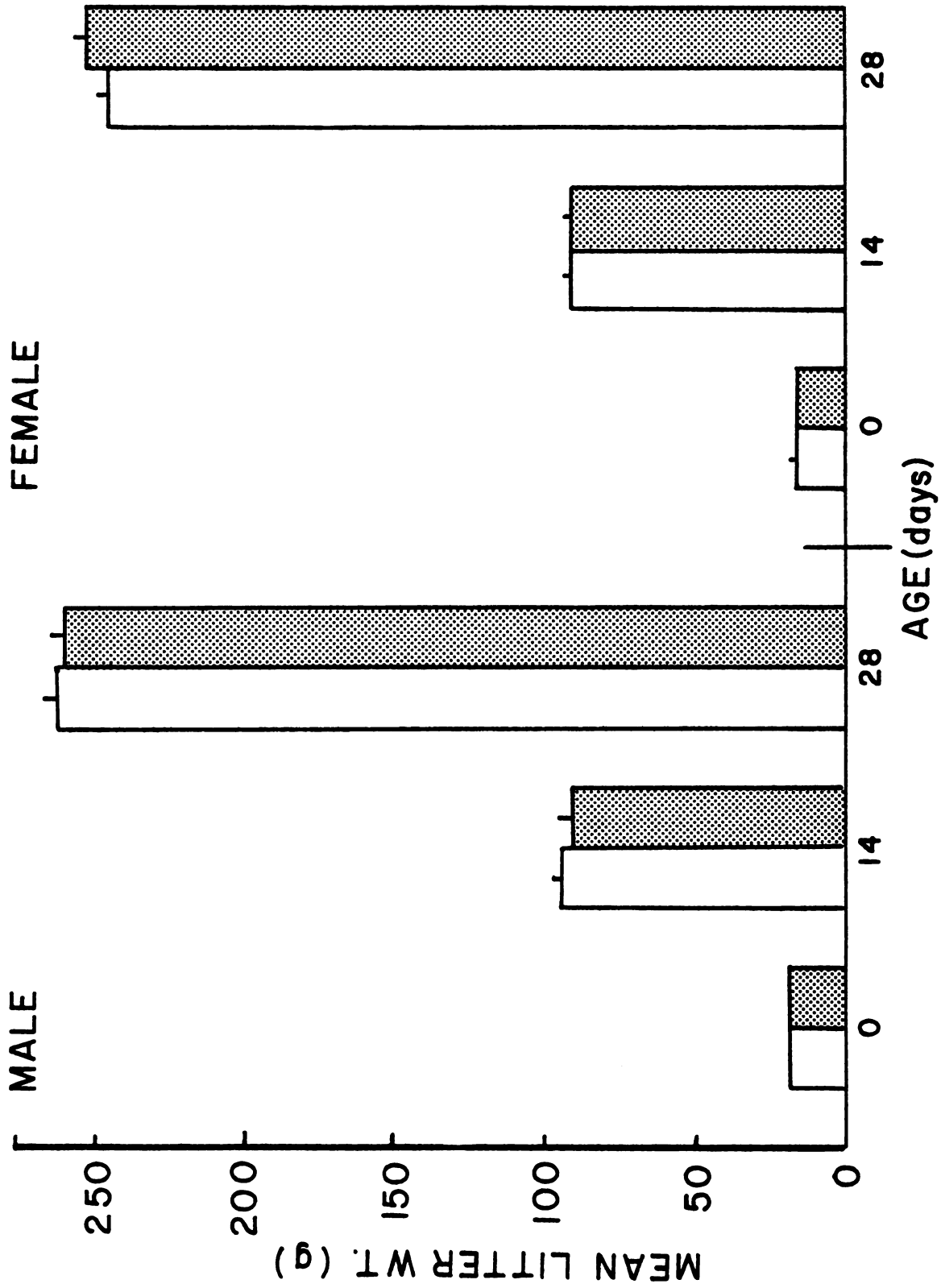
	n	n abort	Gestation length (days)[b]	Litter Size
Control	14	1	22.50±.04[a]	11.69±.73
Restrained	13	1	22.14±.08	10.29±.98

[a]Mean ± S.E.

[b]Gestation length of those pregnancies terminating with live birth.

Figure 2. The effect of prenatal stress on the growth of offspring.

On the day of birth, litters from non-stressed control dams or pregnant females stressed from 0100-0400 hrs on days 4-7 of pregnancy were sexed and paired to 3 males and 3 females. Weights of the litters were then obtained and again when the pups were 14 and 28 days of age. Open bars represent mean weights of control litters (n=8) and shaded bars represent mean weights of prenatally stressed litters (n=7). The vertical lines atop the bars represent 1 SE. Where no lines are shown, the SE was too small to be graphically portrayed.


MEAN LITTER WT. (g)
0
50
100
150
200
250
MALE
FEMALE
0
14
28
AGE (days)
0
14
28

sample was collected by orbital sinus puncture at 0300 hrs following the first and last day of treatment, and was subsequently assayed for prolactin. Results presented in Figure 3 show that restraint stress had no measurable effect on serum prolactin concentrations when measured at 0300 hrs following 1 or 4 consecutive days of stress treatment. Restraint treatments administered on days 4-7, 8-11, or 12-15 did not cause any significant changes in frequency of abortion, gestation length, or live litter size (Table 2).

Randomly selected females were weaned from each litter at 21 days of age. No more than two females were saved from any particular litter. Each female was checked daily for vaginal opening and the results of that study are presented in Table 3. When compared with respective control females, prenatal stress did not result in any significant change in the age at which vaginal opening was detected. Subsequent vaginal lavage revealed no noticeable differences in ovarian cyclic characteristics between prenatally stressed females and controls.

Table 4 illustrates the fertility of the same female rats. When mated to control males, female rats prenatally stressed from days 4-7, 8-11, or 12-15 of gestation showed no significant inability to bear normal litters of healthy offspring. In fact, the only animal that did not successfully complete pregnancy was a 12-15 control female.

The Effect of Intermittent Restraint Stress on Reproduction During Late Pregnancy in the Rat

In a final study, the restraint interval was separated into two 2 hr periods, administered four days consecutively. Rats were

Figure 3. The effect of 4 days of 3 hr of restraint stress treatments initiated on days 4, 8 or 12 of pregnancy on serum prolactin concentrations.

Serum concentrations of prolactin were determined in blood samples collected via orbital sinus puncture at 0300 hr following the first and fourth days of treatment. Each bar represents the average of 5-8 determinations. Open bars represent non-stressed controls (C), and shaded bars depict restraint stressed (RS) females. The vertical line atop each bar represents 1 SE.

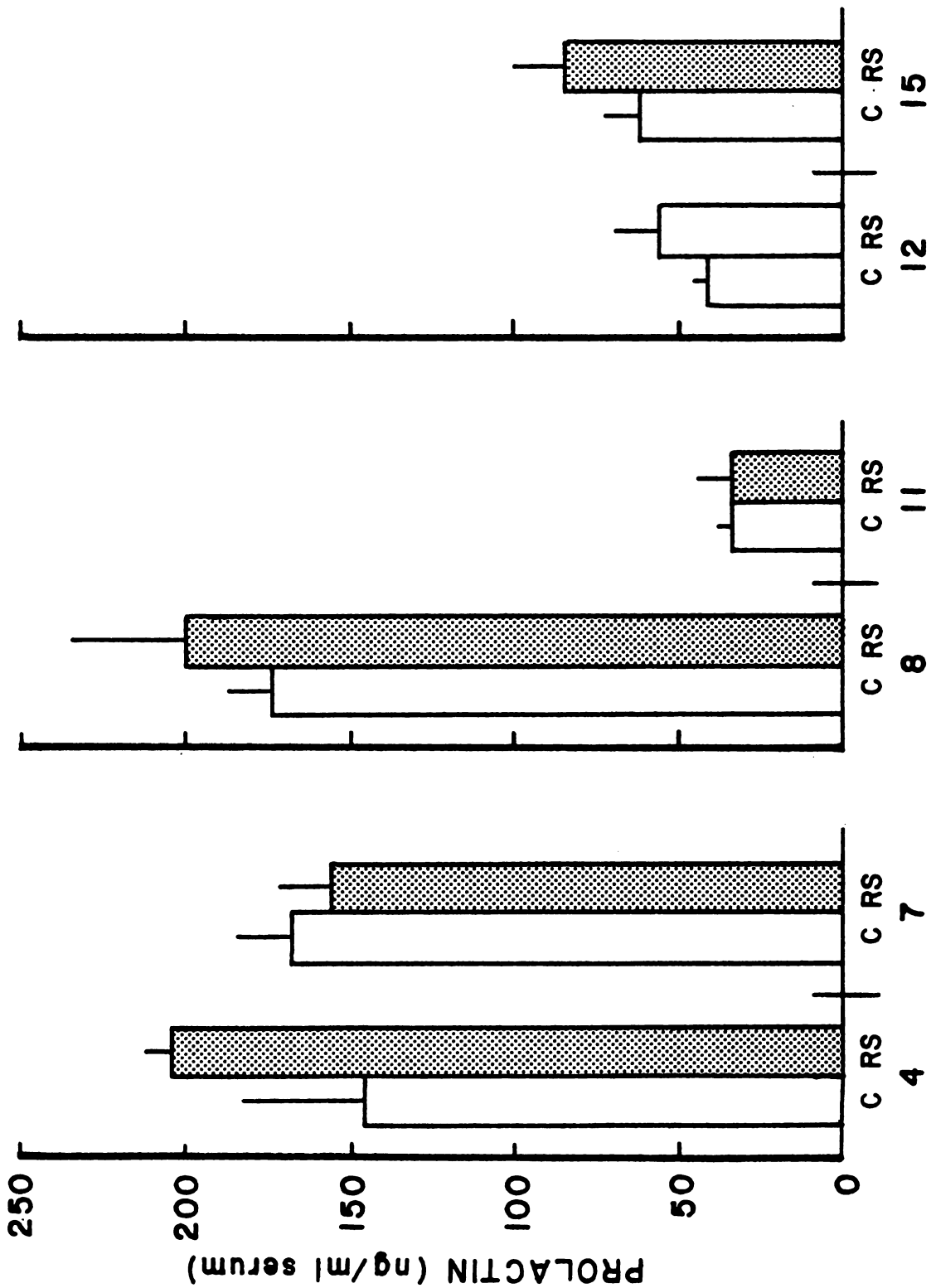

PROLACTIN (ng/ml serum)
250
200
150
100
50
0
C RS
4
C RS
7
C RS
8
C RS
11
C RS
12
C RS
15

TABLE 2

Effect of Restraint Stress Administered from 1300-1600 hrs on Days 4-7, 8-11, or 12-15 of Pregnancy[c]

Restraint Interval (days)	n		# Abort		Gestation Length[b]		Live Littersize	
	C	RS	C	RS	C	RS	C	RS
4-7	8	8	0	2	22.13±.12[a]	22.5 ±.22	10.63±1.27[a]	11.33±1.18
8-11	8	9	1	3	22.36±.15	22.57±.17	10.14±1.05	11.83± .60
12-15	8	8	0	2	22.5 ±.16	22.67±.13	11.13± .61	9.83± .98

[a]Mean ± S.E.

[b]Gestation length of those pregnancies terminating with live birth.

[c]Abbreviations: C = Control; RS = Restraint Stressed.

TABLE 3

Days to Vaginal Opening of Prenatally Stressed Female Rats

Restraint Interval	n		Age at Vaginal Opening	
	Control	Stressed	Control	Stressed
4-7	9	10	39.89± .81[a]	40.30±1.39
8-11	10	10	40.90±1.66	40.30±1.18
12-15	10	10	38.90±1.14	37.40±1.29

[a]Mean ± S.E.

[b]Pregnant rats were restraint stressed between 1300-1600 hrs on days 4-7, 8-11 or 12-15 of pregnancy

TABLE 4

Fertility of Prenatally Stressed Female Rats[b]

	Litter Size	
	Control	Stressed
4-7	12.30±.60[a]	12.1±.43
8-11	13.2 ±.79	11.5±.62
12-15	12.33±.53	13.4±.58

[a]Mean ± S.E.

[b]Pregnant rats were restraint stressed between 1300-1600 hrs on days 4-7, 8-11 or 12-15 of pregnancy.

stressed from 0900 through 1100 hrs and 1300 through 1500 hrs on either days 10-13, 14-17, or 18-21 of pregnancy. Table 5 shows the effect of restraint stress on abortion, gestation length and litter size. The frequency of abortion was significantly ($p<.05$) increased in rats subjected to restraint on days 10-13 of pregnancy. Gestation length and litter size were not affected in those females that delivered live young.

Rats subjected to restraint on days 14-17 of pregnancy had a significantly greater ($p<.01$) length of gestation (23.3±.28 vs. 22.19±.13 days), but live litter size remained the same.

No differences were detected in length of gestation, litter size, or frequency of abortion between control and restraint stressed rats handled on days 18-21 of pregnancy.

Randomly selected progeny from each treatment group were bred as adults, and allowed to litter. Results of this breeding are presented in Table 6. No differences in mean gestation length, nor litter size were detected except in the group prenatally stressed from days 18-21. While gestation length is significantly less ($p<.001$) for treated versus control females, the difference is magnified by the precise littering of the prenatally stressed group. Certainly, prenatal stress did not act to lengthen gestation in any circumstance.

These females were permitted to suckle their offspring for 14 days to determine lactational sufficiency. While no attempt was made to balance the litter size, only one control litter and one treated litter were not maintained. Pups were at comparable weight when measured at birth, 7, and 14 days of age (Table 6).

TABLE 5

Effect of Restraint Stress Administered Twice Daily from 0900-1100 hrs and 1300-1500 hrs on Days 10-13, 14-17, or 18-21 of Pregnancy

Restraint Interval (days)	n		# Abort		Gestation Length[b]		Live Littersize	
	C	RS	C	RS	C	RS	C	RS
10-13	7	9	0	6*	22.07±.08[a]	22.67±.34	10.86± .69	11.0 ±1.73
14-17	8	10	0	2	22.19±.13	23.30±.28**	10.38±1.15	9.0 ±1.08
18-21	7	7	0	0	22.43±.13	22.57±.28	9.71±1.54	9.57±1.29

[a]Mean ± S.E.

[b]Gestation length of those pregnancies that terminated with live young.

*$p<.05$

**$p<.001$

TABLE 6

Fertility and Fecundity of Prenatally Stressed Female Rats

Prenatal Treatment Interval		n	Gestation Length[b]	Live Littersize	Litter Wt (gm)		
					Birth	7 Days	14 Days
10-13	C[d]	10	22.11±.07[a]	11.22± .76[a]	5.94[c]	12.43	21.68
	S	7	22.0 ±.00	10.13± .72	6.27	12.58	21.69
14-17	C	10	22.25±.08	10.7 ± .60	5.95	13.50	22.87
	S	8	22.29±.15	10.29±1.63	6.01	12.63	21.40
18-21	C	9	22.5 ±.08	9.89± .68	5.82	13.85	23.36
	S	8	22.0 ±.00*	10.5 ± .28	5.9	13.05	22.48

[a]Mean ± S.E.

[b]Gestation length of those pregnancies which terminated with live young.

[c]Average weight (gm) per pup.

[d]Abbreviations: C = control; S = prenatally stressed.

[e]Pregnant rats were restraint stressed twice daily from 0900-1100 hrs and 1300-1500 hrs on days 10-13, 14-17 or 18-21 of pregnancy.

*$p<.01$

Discussion

The nocturnal surge of prolactin during early pregnancy is thought to be necessary for the maintenance of the corpus luteum of pregnancy (Smith et al., 1975). The results of the first experiment demonstrated that stress was able to interfere with the nocturnal surge of prolactin, however, no changes were detected in the frequency of abortion, gestation length or size of litters in female rats subjected to 4 days of stress treatment during early pregnancy. These results suggest that either the large nocturnal surge of prolactin is not necessary to maintain a successful pregnancy, or that the surge, although interrupted, may have been delayed in appearance for the duration of the stress. Additionally, the stress did not delay nor decrease implantation, as the length of gestation was not influenced by the 4 day stress regime, nor was the litter size affected.

These results are in agreement with those of Castro-Vazquez et al. (1975), in that immobilization did not affect the number of embryos that successfully implanted. In that study, however, no attempt was made to assess the normalcy of the offspring, as the mothers were sacrificed on day 6 of pregnancy. In the present study, when litters were pared to 3 males and 3 females each, no difference in litter weight was detected throughout the 28 day postnatal measurement period. These data indicate that although the mother was subjected to a stresser of sufficient intensity to alter pituitary hormonal support of pregnancy during the stress treatment, it was not sufficient to cause adverse effect, on the pre- or postnatal growth of her offspring.

Earlier studies have demonstrated increased abortion and fetal malformation when restraint stress was administered for several days (Euker and Riegle, 1973).

In the second study of this experimental sequence, restraint stress treatment in the afternoon did not appear to alter the occurrence or magnitude of the nocturnal prolactin surge following one or four days of treatment. Thus, it would appear that the nocturnal surge is relatively insensitive to stress administered earlier in the day. This phenomenon may explain why Freeman et al. (1974) suggested that the nocturnal surge was insensitive to stress as surgical and handling procedures in their study were performed during the daylight hours.

Although restraint stress treatment administered in the afternoon on four consecutive days during midpregnancy tended to increase the number of litters that aborted, the change was not significant. In agreement with Euker and Riegle (1973), the effect of stress on litter size was all or none, as no difference was measured in litter size among those females that successfully delivered live young. Gestation length was not altered by restraint stress administered on days 4-7, 8-11, or 12-15 of pregnancy. These data are also consistent with those of Barlow et al. (1978) in that restraint stress during midpregnancy did not affect gestation length.

In this experiment, litters were not culled to balance litter effects, however, at day 21 following parturition, the pups were weaned, and only one or two pups per litter were saved for developmental studies. The date of vaginal opening, which signified the

first estrus, was not altered by prenatal stress treatment. When these same prenatally stressed females were bred to control males, no effect of maternal stress was detected on ovarian cycles or fertility as each female produced a healthy litter of pups.

In the third experiment, the restraint interval was altered such that pregnant females were subjected to two hours of restraint twice daily. The four day treatment periods were chosen to reflect such times during which specific developmental processes were occurring. Differentiation of the gonads begins at mid-pregnancy in the rat, and testosterone is first measurable in fetal males around day 14 of pregnancy (Gladue, 1979). Peak testosterone concentrations in the fetal and maternal circulation are found around day 18-19 of pregnancy (Weisz and Ward, 1980).

An increased incidence of abortions in rats stressed from days 10-13 of pregnancy, again affected litter size in an "all or none" fashion. Non-aborted pregnancies exhibited normal gestation length and litter size. During the latter half of pregnancy, i.e., days 11-22, the corpora lutea are no longer dependent upon the pituitary for support (Pencharz and Long, 1942). Instead, C.L. function is maintained by rat placental luteotrophin (rPL). At present, the effects of stress on placental luteotrophin are unknown. Thus, the increased abortion shown with this treatment may be due to altered concentrations of rPL or perhaps due to some non-endocrinological phenomenon such as decreased uterine blood flow.

While the incidence of abortion was not influenced by restraint stress administered on days 14-17 of pregnancy, the gestation length

was significantly increased. The increased gestation length experienced by the restraint stressed females may have contributed to the higher number of litters that included 1 or more stillborn pups.

Restraint stress administered on days 18-21 of pregnancy had no effect or incidence of abortion, length of gestation or live litter size. These data suggest that stages of pregnancy are differentially sensitive to stress, and that a stressor of sufficient intensity administered in the latter half of pregnancy can interrupt or delay the reproductive process. In that regard, our results are in agreement with those of Barlow et al. (1978). In contrast to the study of Barlow et al. (1978), we did not find that restraint stress administered on days 18-21 resulted in a shortened gestation period or increased perinatal death.

In the present study, there were no significant differences in the reproductive performance between prenatally stressed and control females except that when mated, the 18-21 prenatally stressed group delivered their litters significantly sooner than controls. In all cases, prenatally stressed females were able to successfully nurse pups when measured at 7 and 14 days following parturition. This finding is in direct conflict with the work of Herrenkohl (1979) and Herrenkohl and Gala (1979), who claim that prenatally stressed female rats are less fertile and unable to sustain lactation past 10 days following parturition.

This discrepancy may be due in part to the different nature of the stress employed, or possibly due to differences in the strain of rats. However, there is another possible explanation of the

conflicting results. Herrenkohl does not mention the incidence of abortion which followed the restraint stress, nor does she indicate the source of prenatally stressed female offspring. Chapman and Stern (1978) have conducted behavioral studies using an identical stressor to that employed by Herrenkohl, and have found that all treatment effects are eliminated if variation due to litter effects are removed. Therefore, if an investigator was not careful to either balance litters throughout treatments or control for such effects by random selection as was done in the present study, the influence of an individual litter may overwhelm the results of a particular stress treatment. In the behavioral studies cited, one might expect to find a difference in masculinity or femininity if offspring were from litters dominated by one sex or the other (Clemens et al., 1976). This is a problem inherent in the use of polytocous animals for such studies.

In the present investigation, attempts were made to minimize litter effects by utilizing not more than one or two female litter mates whenever possible, in subsequent experimentation. In all cases, adult female rats that were stressed prenatally demonstrated complete reproductive competence when compared with non-stressed control animals. While laboratory conditions and strain differences may explain significant findings in other laboratories, these results suggest that investigators must guard against litter effects contributing a major source of variation to the results.

CHAPTER 3

Prolactin Release and Tuberoinfundibular Dopaminergic Nerve Activity During Pregnancy and Pseudopregnancy

During pseudopregnancy and early pregnancy in the rat, the pattern of prolactin release is characterized by two daily surges, one diurnal and the other nocturnal (Butcher et al., 1972; Freeman et al., 1974). Both surges are thought to be required for the initiation and maintenance of luteal function in the intact rat (Smith et al., 1975). In Chapter 2, we reported that stress was able to interfere with either surge, yet in those same studies we were not able to demonstrate an increased incidence of abortion during early pregnancy when the prolactin surges are manifest. Due to this somewhat unexpected finding, we set out to more fully characterize the release of prolactin during pregnancy and pseudopregnancy and to explore in more detail, the effects of restraint stress on the surge release of prolactin.

Very little is known about the neural mechanisms that regulate the surges or the effects of stress on prolactin secretion during pregnancy or pseudopregnancy. A variety of noxious stimuli such as aortic cannulation, sham surgery, and serial blood sampling have been shown to temporarily suppress the diurnal, but not the nocturnal prolactin surge of pseudopregnant rats (Freeman et al., 1974). A similar effect on the diurnal surge of pregnancy has also been reported (Riegle and Meites, 1976b). Whether these changes are mediated

by decreased release of a prolactin releasing factor (PRF) or the increased action of an inhibitory factor (PIF) is unknown. As an additional aspect of the present study, we studied the activity of the tuberoinfundibular (TI) dopaminergic neurons that are thought by many to be involved in the regulation of pituitary prolactin and LH release.

Prolactin, Dopamine and Tuberoinfundibular Dopaminergic Neurons

Studies that have demonstrated a tonically negative influence of the hypothalamus over prolactin release date back to 1954 (Everett, 1954). Since that time, the quest for hypothalamic factors that either inhibit or promote the release of prolactin from the anterior pituitary, has generated many studies, and their results have been covered by several thorough reviews (Meites et al., 1972; MacLeod, 1976; Fernstrom and Wurtman, 1977). Dopamine (DA), and pharmacological agonists of DA were demonstrated to have potent inhibitory effects on PRL secretion, in vitro and in vivo. Dopamine is thought to act directly on the adenohypophysis, as physiological concentrations of DA suppressed the release of prolactin from cultured pituitaries (Shaar and Clemens, 1974). Hypothalamic extracts stripped of catecholamine content by alumina absorption lost their ability to inhibit PRL release from identically cultured pituitaries (Shaar and Clemens, 1974). The absence of DA receptors in the medial basal hypothalamus and the presence of such receptors in the pituitary further suggested that hypothalamic DA must be released and transported to exert its effects directly on the pituitary (Brown et al., 1976).

The source of the pituitary prolactin inhibiting dopamine is thought to be the tuberoinfundibular dopaminergic (TIDA) neurons, whose cell bodies lie in the periventricular-arcuate nuclei of the hypothalamus. In the median eminence the TIDA terminals juxtapose the median eminence capillary plexus of the hypothalamo-hypophyseal portal vessels that course down the pituitary stalk, and are the major vascular supply for the pituitary (Figure 4) (Renaud et al., 1978; Fuxe et al., 1976). Thus, the TIDA neurons may exert major effects on the pituitary by the release of dopamine directly into the hypophyseal portal vessels.

Dopamine in the Portal Vasculature

The actual measurement of dopamine in hypophyseal portal plasma of the rat at concentrations higher than peripheral plasma (Ben-Jonathan et al., 1977) and the demonstration that DA, at portal blood concentrations, was able to suppress prolactin release in vivo also suggest that dopamine is an endogenous PIF (Gibbs and Neill, 1978).

Compatible studies have shown that pharmacological manipulation of dopaminergic neurotransmission resulted in an inverse relationship between serum prolactin and hypophyseal portal plasma concentrations of DA (Horowski and Graf, 1976; Gudelsky and Porter, 1979). However, attempts to simultaneously measure physiological changes of peripheral PRL and portal DA in the same animal have failed to demonstrate such a "mirror image" relationship (DeGreef and Neill, 1979).

Despite the failure to prove the reciprocal model of the DA-PRL release, recent work has further demonstrated association between the

two substances within the pituitary. Approximately 75% of all pituitary DA is internalized specifically within prolactin granules of lactotrophs, while no such connection appears to exist for the control of LH, growth hormone, follicle stimulating hormone, adrenocorticotrophic hormone or thyroid stimulating hormone (Nansel et al, 1979). During the estrous cycle, pituitary DA content was recently found to vary inversely with peripheral prolactin concentrations (Chiocchio et al., 1980). These results suggest a functional relationship between anterior pituitary dopamine content and pituitary prolactin release.

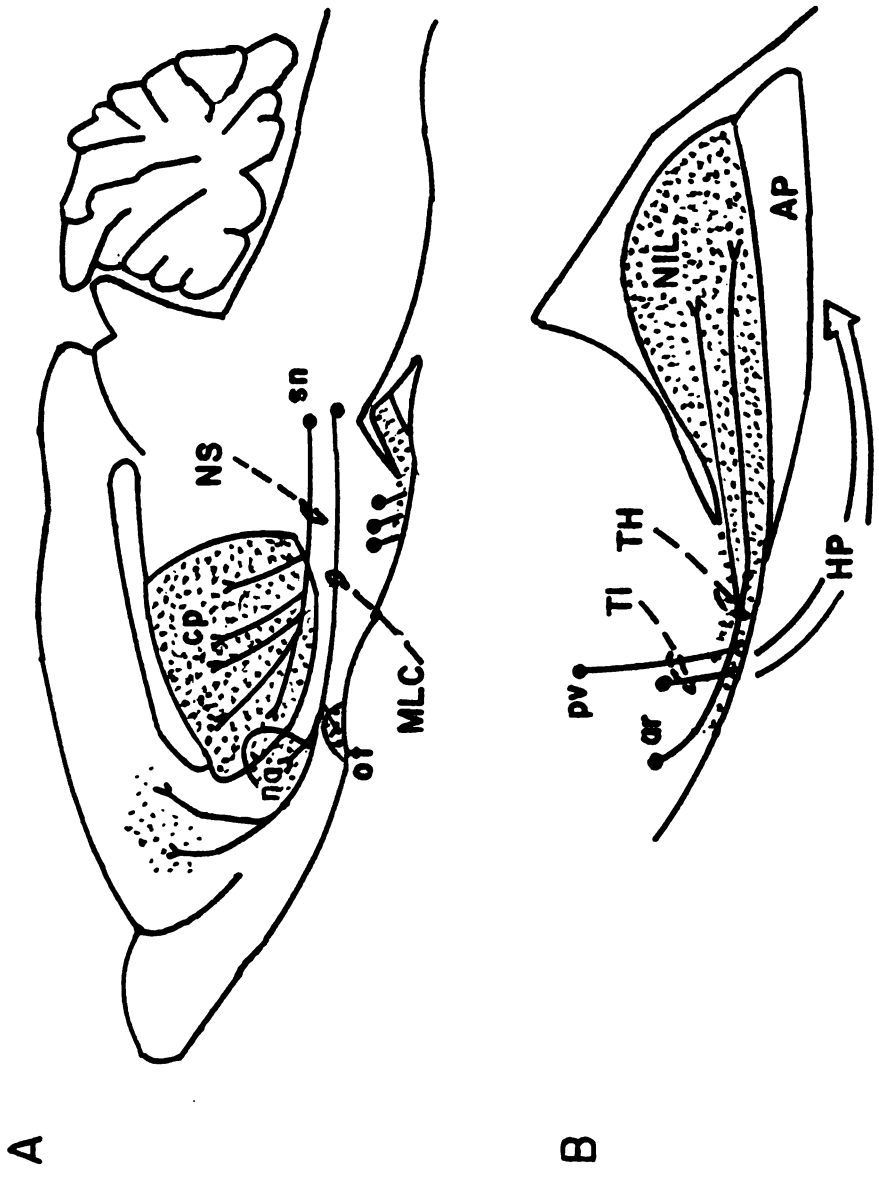
Hormonal Effects on TIDA Neuronal Activity

In the attempt to understand the neuronal-hormonal control of prolactin release, it is necessary to characterize changes in neuronal activity with alterations of physiological states. Histofluorescence techniques demonstrate that castration as well as ovarian steroid supplementation can alter the activity of TIDA neurons (Fuxe et al., 1969). When specific anterior pituitary hormones were injected into rats, it was found that PRL, but not LH, FSH or ACTH caused an increase in TIDA neuronal activity. These results suggested that PRL may act, in part, as its own regulator via a feedback system from the pituitary to the TIDA neurons. These findings were substantiated when it was shown by a sensitive radioenzymatic assay that PRL induced a specific activation of TIDA neurons 10-26 hours following treatment in OVX rats (Gudelsky et al., 1976), and induced an increase of DA into hypophyseal portal blood in male rats (Gudelsky and Porter, 1980).

When estimates of TIDA neuronal activity were obtained by the measurement of the accumulation of DOPA after the administration of a

Figure 4. Distribution of major DA neuronal systems in the rat brain.

Shaded areas depict regions of DA nerve terminals. A. Sagittal section of rat brain. NS, nigrostriatal neurons; MLC, mesolimbic neurons; sn, substantia nigra; cp, corpus striatum; na, nucleus accumbens; ot, olfactory tubercle. B. Sagittal section and expanded view of the basal hypothalamus and pituitary. TI, tuberoinfundibular neurons; TH, tuberohypophyseal neurons; NIL, neurointermediate lobe of the pituitary; AP, anterior pituitary; HP, representation of hypophyseal portal system; pv, periventricular nucleus; ar, arcuate nucleus.


A
NS
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decarboxylase inhibitor (Demarest and Moore, 1980) the results following pharmacological and endocrinological manipulations were similar to those in the aforementioned studies in that: 1) the administration of haloperidol, a dopaminergic antagonist known to increase serum PRL concentrations, resulted in an increase of TIDA neuronal activity 16 hours following treatment, and 2) the estrogen-induced increase in neuronal activity within the median eminence was abolished in the hypophysectomized rat, which suggested that the effect of the steroid was due to its facilitory action on pituitary prolactin release.

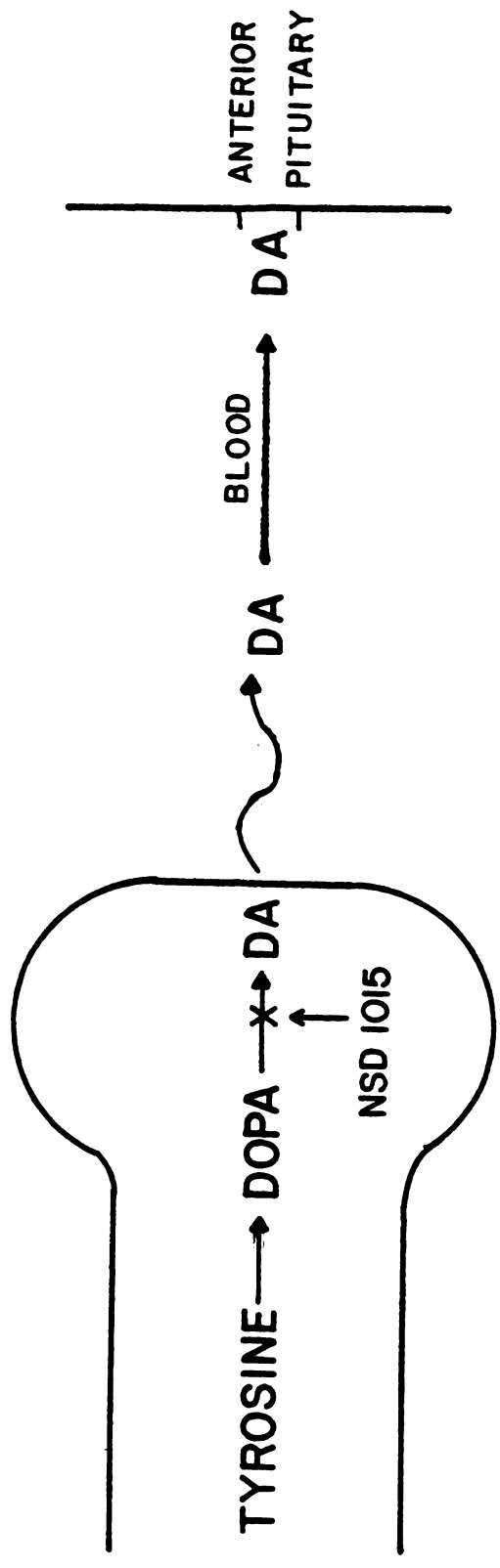
The exact mechanism by which prolactin causes the latent activation of DA synthesis within the terminals of tuberoinfundibular neurons is not known. Recent studies have shown that prolactin induced a delayed increase in the activity of tyrosine hydroxylase selectively within the median eminence (Nicholson et al., 1980). Since tyrosine hydroxylase is the rate limiting enzyme in the synthesis of DA (see Figure 5), it is possible that the latent period may reflect the time required to synthesize and transport new enzyme to the TIDA nerve terminals within the median eminence. Consistent with this hypothesis is the finding that cycloheximide, a protein synthesis inhibitor, administered up to 4 hours following icv PRL treatment blocked the expected increase of TIDA neuronal activity (Johnston et al., 1980).

Stress and Biogenic Amines

The effect of stress on pituitary hormone release could also involve alterations in hypothalamic hypophysiotropic factors. Stress-induced changes of catecholamine content have been shown within the

Figure 5. Schematic diagram of a tuberoinfundibular dopaminergic neuron.

DA is synthesized for release into the hypophyseal portal system and transport to the pituitary. The synthesis of DA can be blocked by the administration of a decarboxylase inhibitor (NSD 1015) which will allow the accumulation of DOPA to measurable quantities.


ANTERIOR PITUITARY
DA
BLOOD
DA
DA
DOPA
TYROSINE
NSD 1015

whole brain (Moore and Lariviere, 1964; Corrodi et al., 1968) and the hypothalamus (Palkovits et al., 1975; Keim and Sigg, 1976). The intensity, duration, and type of stressor affect the magnitude of the catecholaminergic response (Keim and Sigg, 1976) as does the reproductive status (e.g., pregnant) of the animal (Moyer et al., 1977).

There is general agreement that hypothalamic monoamine neurons exert an influence on the release of gonadotropins (Kordon and Glowinski, 1972; Fuxe et al., 1976). However, results of early attempts to correlate stress-induced changes in pituitary and peripheral hormone release with whole brain concentrations of dopamine were not consistent (Carr et al., 1968; Corrodi et al., 1971). The development of sensitive assay techniques permitted the measurement of catecholamines in discrete hypothalamic nuclei (Kventnansky et al., 1977) that may be associated in the stress-induced changes of hormone release.

The study of acute stress revealed that at least two neuronal systems were operational in the control of prolactin release. The initial rise in serum prolactin concentrations was not due to the inhibition of dopamine, but to enhanced activity of such substances as norepinephrine, serotonin, or TRH (Blake, 1974; Marchlewska-Koj and Kralich, 1975; Collu et al., 1979; Mueller et al., 1976). The administration of DA agonists blocked the stress-induced increase or prolactin, but treatment with DA antagonists was permissive to increased prolactin secretion (Meltzer et al., 1976).

Immobilization stress produced a biphasic effect on serum prolactin levels (Riegle and Meites, 1976b; Tache et al., 1976). At the onset of stress, prolactin release is stimulated. If the stress is continued beyond one hour, prolactin release declines to very low

levels. Concentrations of another hormone commonly associated with stress, corticosterone, were increased throughout the stress interval, and remained elevated when the stress was administered over a period of days (Riegle, 1973; Tache et al., 1979; Kawakami et al., 1979), suggesting that PRL and ACTH secretion are controlled by different neuroendocrine mechanisms.

The administration of TRH or noxious stimuli following six hours of restraint in male rats had no effect on stress depressed serum PRL concentrations (Kawakami et al., 1979). The injection of a dopamine receptor blocker resulted in the elevation of prolactin levels to that of controls. These results indicated that a dopaminergic mechanism was involved in the depressed serum concentrations of prolactin which followed a lengthy 6 hour stress.

Unlike the chronically stressed rat, the lactating rat is characterized by sustained high levels of prolactin and low concentrations of corticosterone (Stern and Voogt, 1973/74). Serum prolactin concentrations are decreased when the lactating female is pup-deprived. A variety of stressors can induce a modest increase in serum prolactin levels following pup removal. These stress-induced increases of prolactin last for less than 60 minutes in the pup-deprived rat. The return of the pups and normal suckling results in greatly elevated and sustained prolactin values. Grosvenor has hypothesized a multi-phase process in which prolactin is released from the pituitary following various neurogenic stimuli (e.g., suckling) that transform the storage pools of PRL into a releasable form (Grosvenor et al., 1979). In the normal animal, the releasable PRL pool is small, and TRH and/or ether

stress cause only a brief (<60 min) increase in serum PRL concentrations. Once the pituitary prolactin content is transformed by the suckling stimulus, TRH or ether stress can sustain elevated PRL concentrations for extended periods of time (Grosvenor et al., 1979; Grosvenor et al., 1980). The "depletion-transformation" process appears to be regulated by DA, as 5-bromo-α-ergocryptine (a dopamine agonist) administered before but not after suckling, prevented depletion of PRL (Grosvenor et al., 1980). These results suggest that pituitary DA is a major inhibitor of sustained elevations in PRL release, and are consistent with the depressed TIDA activity and decreased anterior pituitary DA content seen during suckling and lactation (McKay et al., 1980). While it is generally agreed that DA released from TIDA neurons tonically inhibits the release of prolactin, very little is known about the activity of these neurons during pregnancy. The demonstration of elevated concentrations of DA in the hypophyseal portal blood during pregnancy and parts of the estrous cycle was necessary to establish tuberoinfundibular DA as a major PIF (Ben-Jonathan et al., 1977), but procedural problems such as the depressive effect of anesthesia on PRL release and the time and stress involved in obtaining a blood sample preclude the usefulness of this technique to establish the exact relationship between TIDA neuronal activity and pituitary PRL release during pregnancy and pseudopregnancy (Ben-Jonathan et al., 1977; DeGreef and Neill, 1979). The objectives of the present study include characterization of TIDA neuronal activity during normal pregnancy and pseudopregnancy in the rat by determining within the median eminence the rate of DOPA accumulation following the inhibition of DOPA decarboxylase. This

technique has been shown to be a reliable technique to estimate TIDA neuronal activity (Demarest and Moore, 1980). If tuberoinfundibular DA is a hypophysiotropic hormone with PIF capabilities, it is reasonable to suspect that the surge release of pituitary prolactin may be accompanied by alterations in TIDA neuronal activity.

In the previous chapter, it was shown that restraint stress interfered with the diurnal and nocturnal surge release of PRL during early pregnancy. A second objective of this thesis will be to examine more fully the effects of a 4 hr restraint stress on the release of prolactin, and to measure related changes in DOPA accumulation. In the previously described studies, it could not be determined whether the surge release of PRL was blocked or delayed by restraint stress. In this study, the release of PRL was characterized for over 24 hours after the initiation of restraint.

If a functional feedback loop is operational between the surge release of prolactin during pregnancy and pseudopregnancy and TIDA neuronal activity, then the stress-induced alteration of PRL should be reflected in changes in the accumulation of DOPA within the median eminence.

A final study will examine the feedback hypothesis in pseudopregnant animals that have been ovariectomized. The diurnal surge of PRL is absent in the OVX cervically stimulated rat (Freeman et al., 1974); thus, any PRL feedback effects on TIDA neurons would be attributable to the slightly attenuated nocturnal surge.

Materials and Methods

Animals - Housing and Handling

The animals were caged and maintained under the same conditions as those described in Chapter 2. In studies that required pregnant rats, the breeding was conducted as previously described. Pregnancy was confirmed during necropsy by the presence of implantation sites. Pseudopregnancy was induced by electrical stimulation of the cervix with a probe (10 sec with 240 pulses/sec and a pulse duration of 1 msec at 25 volts) at 0930 hr and 1630 hr estrus and at 0930 diestrus. The first day of cervical stimulation was considered day 1 of pseudopregnancy. Pseudopregnancy was confirmed by the observation of leucocytic vaginal cytology for six days. In those cervically stimulated animals subjected to ovariectomy, vaginal cytology was no longer a useful indicator of the successful induction or maintenance of pseudopregnancy. Instead the presence of the 0300 hr prolactin surge ($Y \geq \overline{X}$ - 1 S.D.) on Day 5 served as an index of successful cervical stimulation.

Ovariectomy was performed under ether anesthesia via a mid ventral incision. Both uterine horns were exteriorized through a 1 cm incision and the ovaries were teased away from the uterus and connective tissue. After the uterus was repositioned, the peritoneum was restored with gut suture, and the exterior was closed by stainless steel clips. Sham ovariectomies for the control group followed the same procedure except that ovaries remained intact.

Restraint stress as described in Chapter 2 was administered to groups at intervals outlined in Results.

Tissue Handling and Assay - DOPA Accumulation

The in vivo rate of DA synthesis was estimated by measuring the rate of DOPA accumulation following the administration of a decarboxylase blocker (Figure 5). Each rat was injected with 3-hydroxybenzylhydrazine (NSD 1015, 100 mg/kg i.p.; Sigma Chemical Co., St. Louis, MO) 30 minutes prior to decapitation. The brains were quickly removed from the skull and placed on a cold plate. The median eminence was removed with the aid of a dissecting microscope. The striatum, olfactory tubercle, nucleus accumbens and posterior pituitary were dissected by published experimental techniques (Demarest et al., 1980; Glowinski and Iverson, 1966; Horn et al., 1974). The median eminence and posterior pituitary were homogenized in 20 µl, and the striatum, olfactory tubercles and nucleus accumbens were homogenized in approximately 10 volumes of cold 0.2 N perchloric acid containing EGTA (10 mg/100 ml). Following centrifugation 10 ml of the supernatant from individual tissue samples were assayed for DOPA assays by radioenzymatic procedure (Demarest and Moore, 1980) and the tissue pellets were analyzed for protein by the method of Lowry (Lowry et al., 1951). DOPA concentrations were expressed as ng DOPA/mg protein. The preceding technique is performed routinely in the laboratory of Dr. K.E. Moore, Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI. All assays reported in this report were performed in that laboratory under the direct supervision of Dr. K.T. Demarest, Departments of Pharmacology and Toxicology, and Physiology, Michigan State University.

DA Steady-State Concentration

DA content of the anterior pituitary was measured in rats by a radioenzymatic technique as described (Umezu and Moore, 1979). Immediately following sacrifice by decapitation, the brains were removed to expose the pituitary. The bi-lobed anterior pituitary was separated from the neuro-intermediate lobe and immediately homogenized in 30 µl of 0.2 N perchloric acid containing EGTA (10 mg/100 ml). Protein was measured as previously stated and the data was expressed in ng DA/mg protein.

Blood Collection

Blood samples were collected following decapitation or by orbital sinus puncture as specified in Results. Radioimmunoassay for PRL and LH was conducted as described in Chapter 2.

Statistical Analysis

Initial studies designed to characterize PRL, LH, and DOPA accumulation during pregnancy, pseudopregnancy, and diestrus were analyzed by computer using a one-way analysis of variance. Between group comparisons were made by the Student-Newman-Keuls' test (Nie et al., 1975). Only differences with a probability of error less than 5% were considered significant. Prolactin data were transformed to natural log values to provide homogeneity of variances.

Further studies involving restraint stress, bleeding stress and ovariectomy were analyzed by computer using the two-way analysis of variance program Balanova (Coyle and Frankmann, 1980). The prolactin data were subjected to a natural log transformation to achieve

homogeneity of variance. Selected a priori comparisons were made using the Student's t-test based upon the mean square error (within) from the two-way analysis of variance (Sokal and Rohlf, 1969; Rohlf and Sokal, 1969).

Results

Characterization of Tuberoinfundibular Dopaminergic Neuronal Activity During the Daily Surges of Prolactin Secretion in Pregnant and Pseudopregnant Rats

Serum prolactin and LH concentrations were analyzed at specified times on day 6 of pregnancy and pseudopregnancy and on the second day of diestrus. To avoid a possible increase in prolactin secretion related to stress associated with the handling and injecting of the animals, blood samples were collected from pregnant and pseudopregnant animals 1 day prior to the determination of DOPA accumulation and from diestrous animals on the corresponding day of the cycle preceding the experiment. Thus, on day 6 of pregnancy or pseudopregnancy the rats were randomly subdivided into 8 groups of 8 rats each. A single blood sample (approximately 1 ml) was collected from each rat by orbital sinus puncture under light ether anesthesia at one of the following times: 0300, 0600, 0900, 1200, 1500, 1800, 2100, and 2400 hr. Rats included in the diestrous study were similarly divided into 8 groups of 8 animals each and subjected to a blood collection at one of the same time points. During the dark period all manipulations were carried out under a red light.

During day 6 of pregnancy, two peaks of prolactin were detected; a nocturnal surge which peaked at 0300 hr and a diurnal surge which

peaked at 1800 hr, just prior to the dark period (Figure 6). On day 6 of pseudopregnancy, the pattern of prolactin secretion was similar to that observed during pregnancy; a nocturnal peak was detected at 0300-0600 hr and a diurnal peak at 1800 hr (Figure 7).

There was also a cyclic variation in the rate of DOPA accumulation in the median eminence of the pregnant and pseudopregnant rats. In the pregnant rats there were two daily low points in the rate of DOPA accumulation with the nadirs at 0600 and 2100 hr (Figure 9). In the pseudopregnant rats the nadirs occurred at 0300 and 1800 hr (Figure 10). On the second day of diestrus the serum concentrations of prolactin and the rate of accumulation of DOPA in the median eminence did not change throughout the 24 hr period (Figure 8 and 11). While there were no significant changes in the serum concentrations of LH over the 24 hr period in pregnant, or diestrous animals (Figures 6 and 8, the pseudopregnant group exhibited a slight but significant change with peak values at 1200 and 1500 hr and a nadir at 0600 hr.

Changes in DOPA accumulation throughout the 24 hr period of pregnant and pseudopregnant rats were restricted to the median eminence; there were no consistent changes in DOPA accumulation in any other brain regions (Tables 7 and 8).

A comparison of the rates of DOPA accumulation in the median eminence observed in pregnant, pseudopregnant, and diestrous rats (Figures 9, 10 and 11) might suggest differences in DA synthesis rates during these different reproductive states. Because of a number of variables (e.g., between radioenzymatic assays, time of year, etc.) it is not possible to make direct comparisons between the results obtained in these different studies. Accordingly, an additional study

Figure 6. The pattern of prolactin and LH in the pregnant rat.

Serum concentrations of prolactin ( ) and LH ( ) were determined in blood samples collected by orbital sinus puncture at 3 hour intervals on day 6 of pregnancy. Each point on the graph represents the mean of eight determinations and the vertical line through each point represents 1 SE. Where no lines are shown, the SE is less than the radius of the symbol.

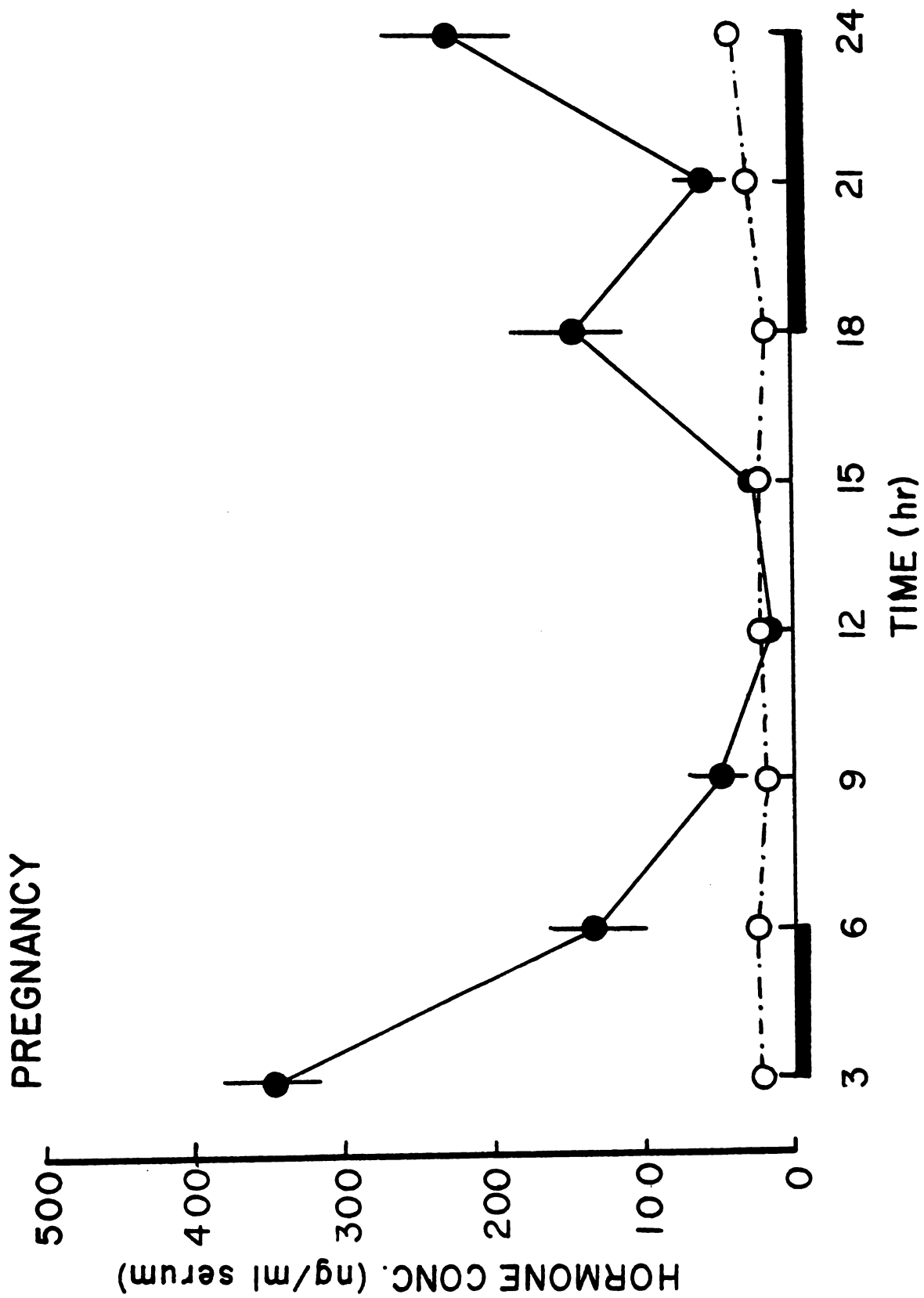

PREGNANCY
HORMONE CONC. (ng/ml serum)
500
400
300
200
100
0
3
6
9
12
15
18
21
24
TIME (hr)

Figure 7. The pattern of prolactin and LH in the pseudopregnant rat. See legend to Figure 6 for additional details.

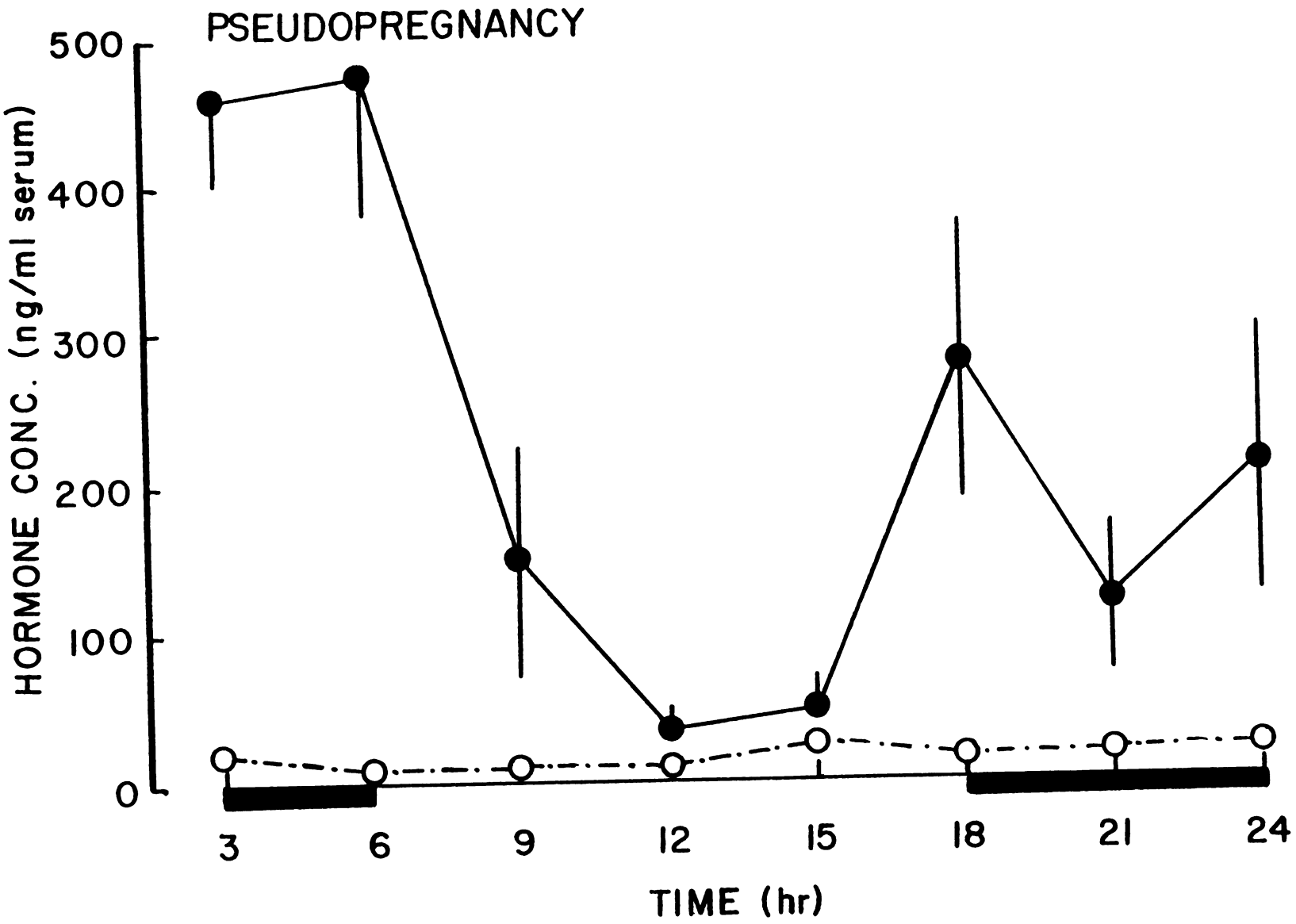

PSEUDOPREGNANCY
HORMONE CONC. (ng/ml serum)
500
400
300
200
100
0
3
6
9
12
15
18
21
24
TIME (hr)

Figure 8. The pattern of prolactin and LH on the second day of diestrous. See legend to figure 6 for details.

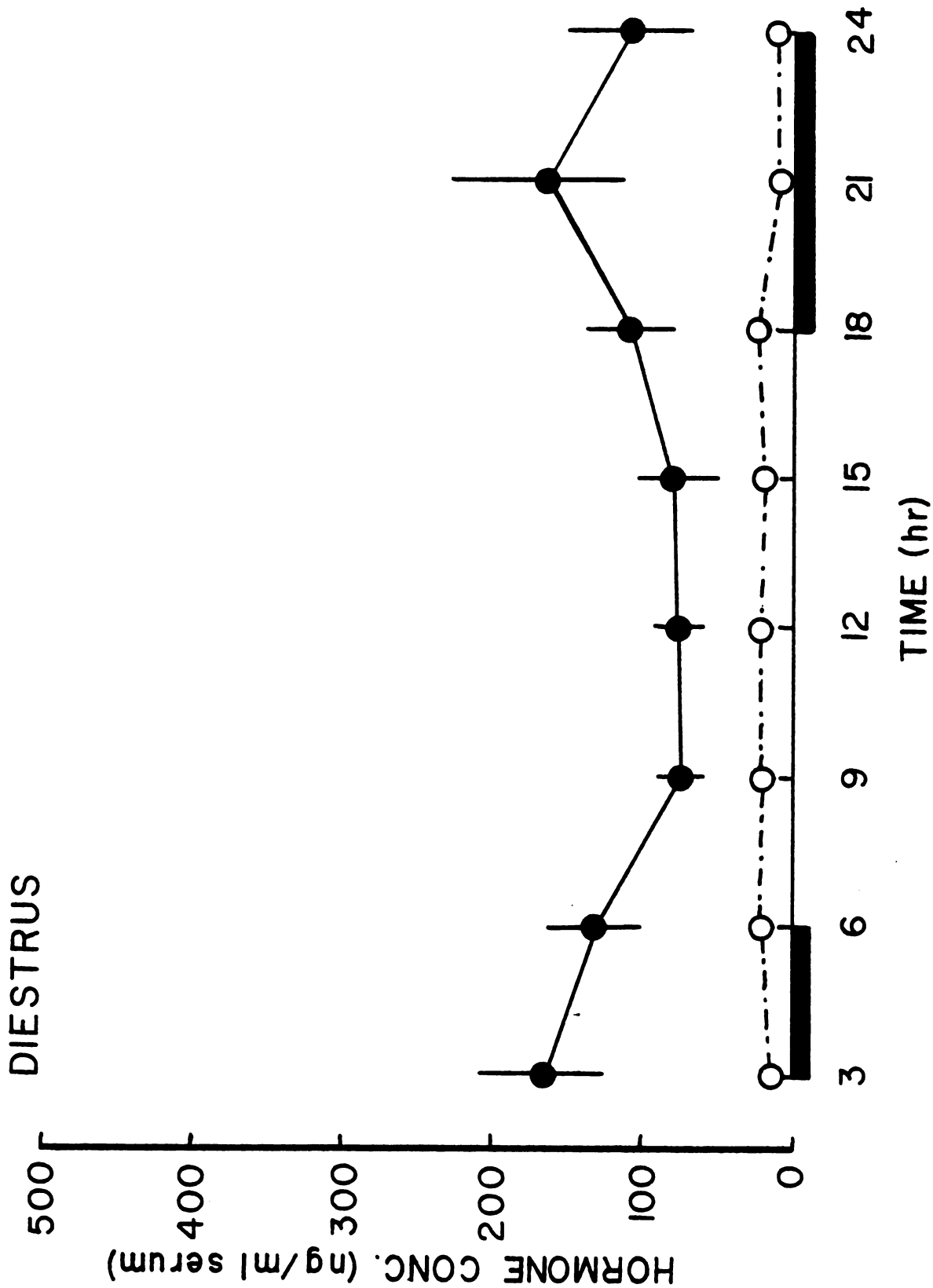

DIESTRUS
HORMONE CONC. (ng/ml serum)
500
400
300
200
100
0
3
6
9
12
15
18
21
24
TIME (hr)

Figure 9. The pattern of DOPA accumulation in the median eminence in the pregnant rat.

The accumulation of DOPA in the median eminence was determined 30 minutes after the administration of NSD 1915 (100 mg/kg, i.p.) at 3 hour intervals on day 7 of pregnancy. Each point represents the mean of 8 determinations. Vertical lines represent 1 SE.

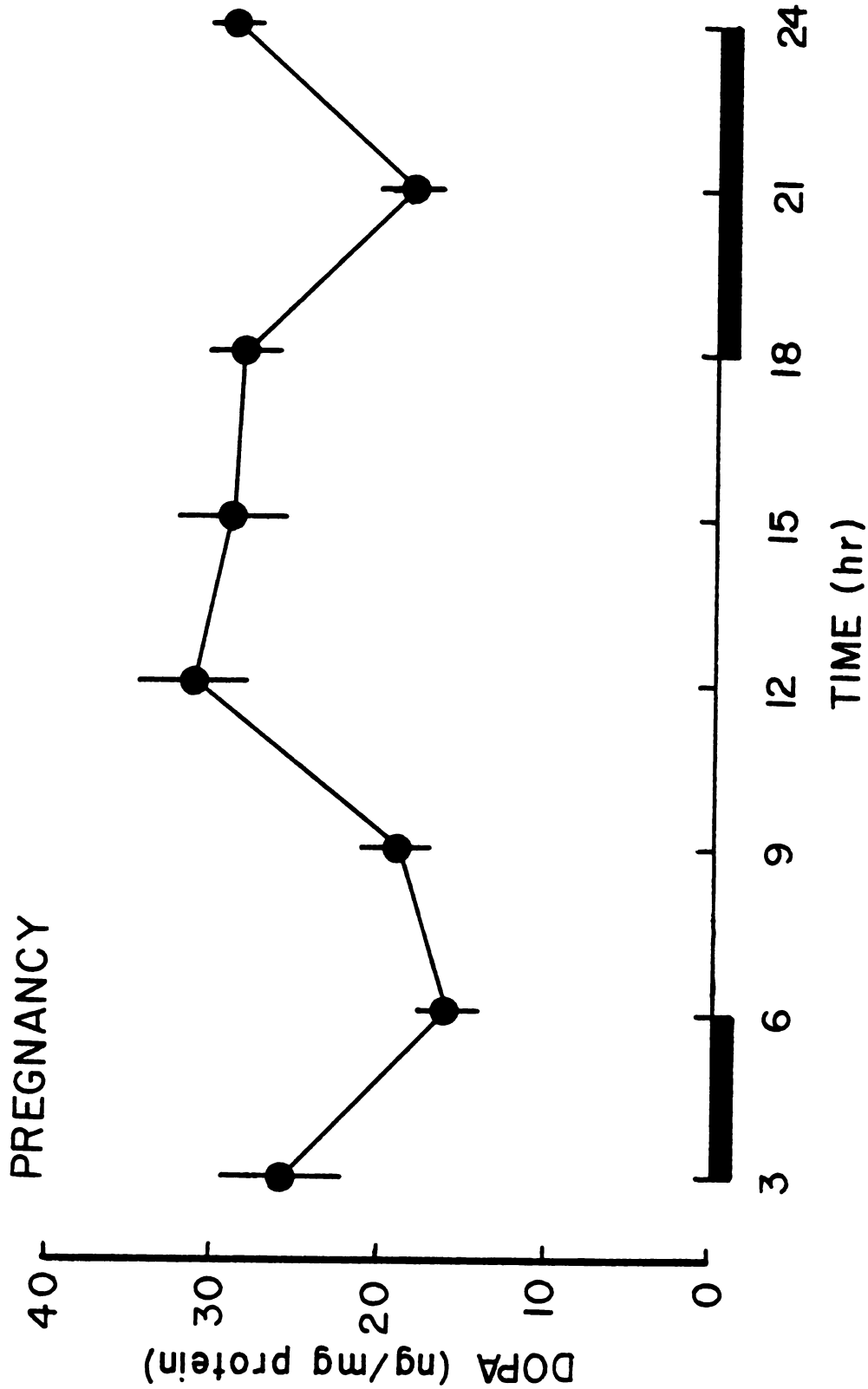

PREGNANCY
DOPA (ng/mg protein)
40
30
20
10
0
3
6
9
12
15
18
21
24
TIME (hr)

Figure 10. The pattern of DOPA accumulation in the median eminence in the pseudopregnant rat.

See legend to Figure 9 for additional details.

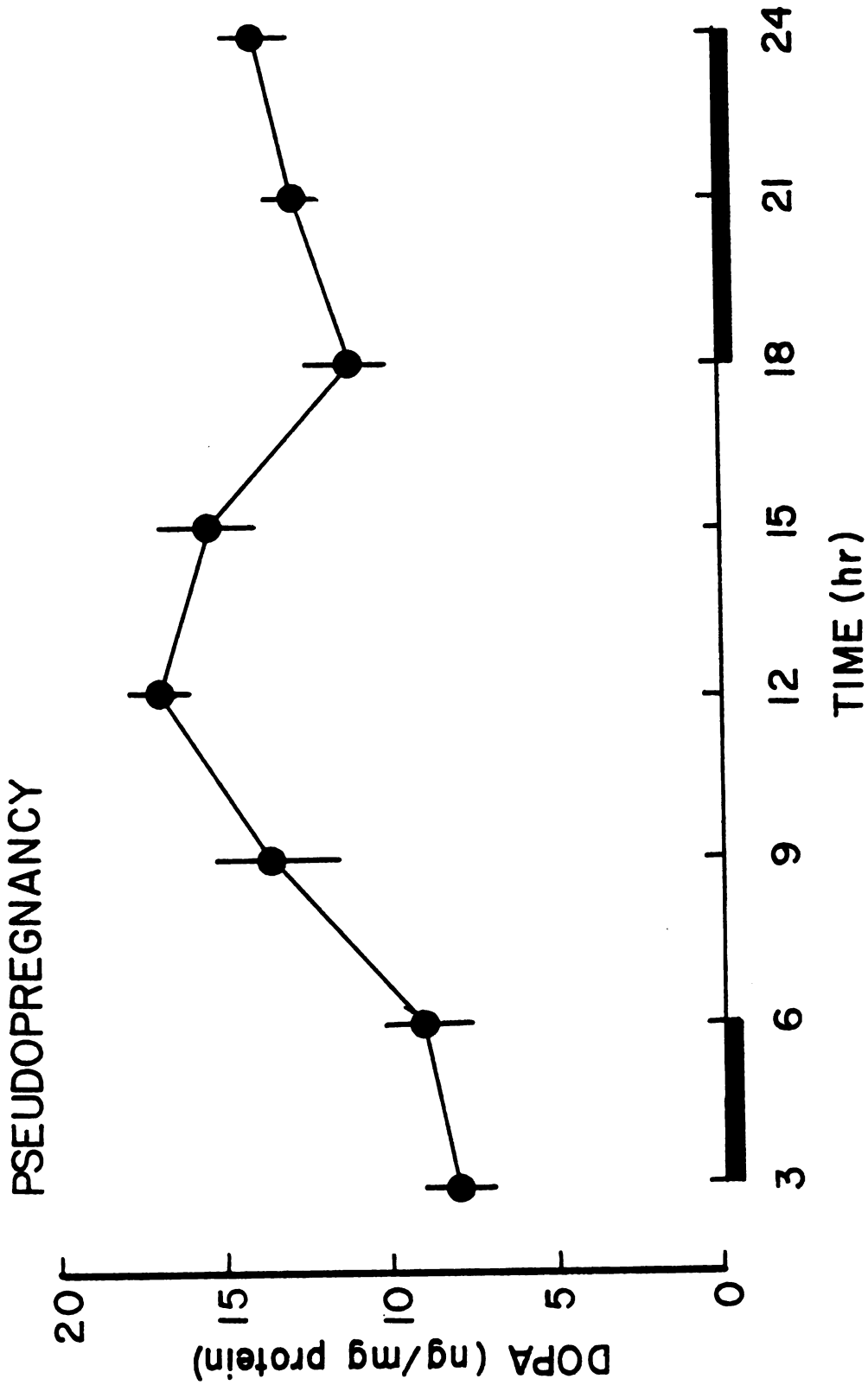

PSEUDOPREGNANCY
DOPA (ng/mg protein)
20
15
10
5
0
3
6
9
12
15
18
21
24
TIME (hr)

Figure 11. The pattern of DOPA accumulation in the median eminence on the second day of diestrous.

See legend to Figure 9 for additional details.

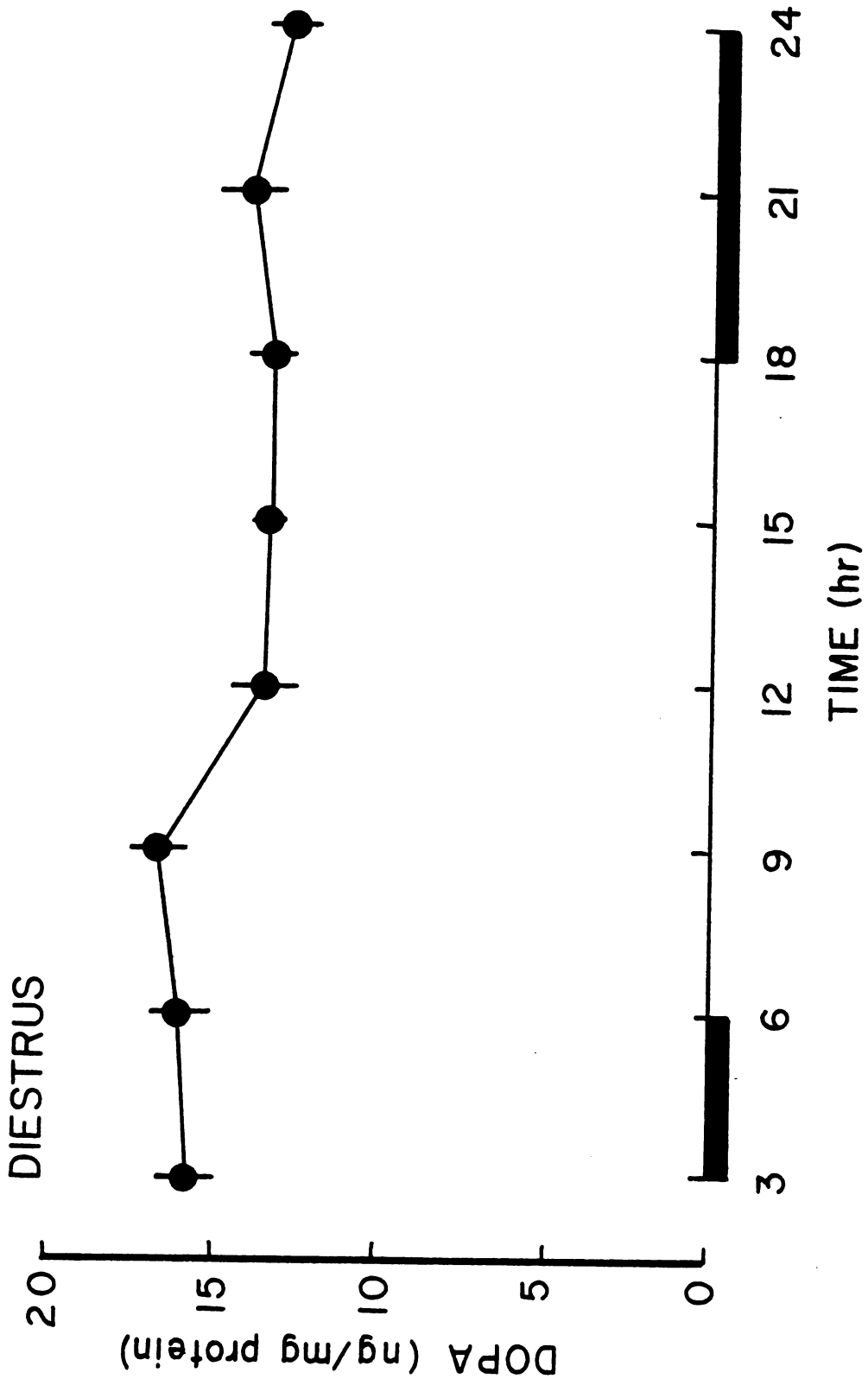

DIESTRUS
DOPA (ng/mg protein)
20
15
10
5
0
3
6
9
12
15
18
21
24
TIME (hr)

TABLE 7

DOPA Accumulation in Selected Brain Regions on Day 7 of Pregnancy

TIME	CORPUS STRIATUM	NUCLEUS ACCUMBENS	OLFACTORY TUBERCLE	POSTERIOR PITUITARY
0300	10.8±0.9	8.0±1.1	10.4±0.9	1.10±0.08
0600	9.1±0.8	7.3±0.7	10.9±0.7	0.75±0.05
0900	9.0±0.5	8.8±1.1	11.3±1.0	0.86±0.11
1200	10.7±0.5	8.1±0.5	11.5±1.1	0.80±0.07
1500	9.6±0.6	7.3±0.8	11.1±0.4	0.86±0.06
1800	11.4±0.8	11.5±1.2	10.9±1.1	0.70±0.04
2100	10.6±0.3	9.6±1.3	12.1±0.8	0.74±0.09
2400	12.1±1.3	9.8±1.5	12.1±0.7	0.59±0.20

Pregnant rats (Day 7) were administered NSD 1015 (100 m/gkg, i.p.) 30 min prior to sacrifice. Tissue concentrations of DOPA were analyzed by radioenzymatic assay and expressed as ng/mg protein. Each value represents the mean ± 1 SE of data obtained from eight animals.

TABLE 8

DOPA Accumulation in Selected Brain Regions on Day 7 of Pseudopregnancy

TIME	CORPUS STRIATUM	NUCLEUS ACCUMBENS	OLFACTORY TUBERCLE	POSTERIOR PITUITARY
0300	7.2±0.7	7.0±0.7	6.4±0.4	0.62±0.07
0600	9.3±0.5	9.1±0.6	6.0±0.5	0.75±0.13
0900	8.6±0.5	8.4±0.7	5.8±0.4	0.53±0.04
1200	8.3±0.4	9.0±0.6	6.6±0.6	0.66±0.06
1500	8.2±0.6	8.9±0.7	6.8±0.3	0.70±0.08
1800	7.7±0.5	7.3±0.8	6.5±0.4	0.63±0.04
2100	8.0±0.6	7.8±0.6	6.7±0.5	0.63±0.09
2400	9.6±0.5	7.9±0.6	7.1±0.3	0.60±0.06

Pseudopregnant rats (Day 7) were administered NSD 1015 (100 m/gkg, i.p.) 30 min prior to sacrifice. Tissue concentrations of DOPA were analyzed by radioenzymatic assay and expressed as ng/mg protein. Each value represents the mean ± 1 SE of data obtained from eight animals.

was undertaken to compare the rate of DOPA accumulation in selected brain regions on day 7 of pregnancy and pseudopregnancy and on the second day of diestrus. All animals were sacrificed between 1140 and 1220 hr, a time of maximal rates of DOPA accumulation in the pregnant and pseudopregnant rats. Samples of each brain region were analyzed in a single assay so that a direct comparison could be made between the 3 groups of animals. Rates of DOPA accumulation in the median eminence of pregnant and pseudopregnant rats were similar and both were significantly greater than that in diestrous rats (Table 9). There were no differences in the rates of accumulation of DOPA in any other brain regions.

Effects of Blood Sampling on Prolactin During Pregnancy

Serum prolactin concentrations were measured at specified times on day 7 of pregnancy. In the previous experiment, animals were subjected to blood sampling on day 6 of pregnancy and DOPA accumulation determination in the same rat 24 hours later. To assess if blood sampling and handling on day 6 of pregnancy affected serum PRL concentrations 24 hours later, blood samples were collected from animals on day 6 of pregnancy and again 24 hours later and in control rats subjected to blood sampling on day 7 only. Thus, on day 6 of pregnancy, half of the rats were randomly subdivided into 8 groups of 6 rats each. A single blood sample (approximately 1.0 ml) was collected from each rat via orbital sinus puncture under light ether anesthesia at one of the following times: 0300, 0600, 0900, 1200, 1500, 1800, 2100 and 2400 hr. Twenty-four hours later the same rat was again subjected to orbital sinus puncture. The remaining rats were similarly divided

TABLE 9

DOPA Accumulation at 1200 hr in Pregnant, Pseudopregnant and Diestrous Female Rats

	Pregnant	Pseudopregnant	Diestrous
Median Eminence	17.6±1.3[a]	18.1±1.14[a]	11.5±0.8
Corpus Striatum	9.0±0.5	8.4±0.4	8.2±1.2
Nucleus Accumbens	10.1±0.9	9.9±0.3	9.4±0.7
Olfactory Tubercle	7.4±0.4	7.8±0.5	7.6±0.4
Posterior Pituitary	0.59±0.04	0.49±0.03	0.60±0.06

Pregnant (Day 7), pseudopregnant (Day 7) and diestrous rats received NSD 1015 (100 mg/kg, i.p.) 30 min prior to sacrifice at 1200 hr ± 20 min. DOPA concentrations are expressed as ng/mg protein, n = 8-11.

[a]Values that are significantly different from values in corresponding brain regions of diestrous rats ($p<0.05$).

into 8 groups of 6 animals, but were subjected to orbital sinus puncture at one at the same time points on day 7 only. As in the previous experiment, all manipulations of the rats during the interval when the colony lights were out were carried out under a red incandescant light.

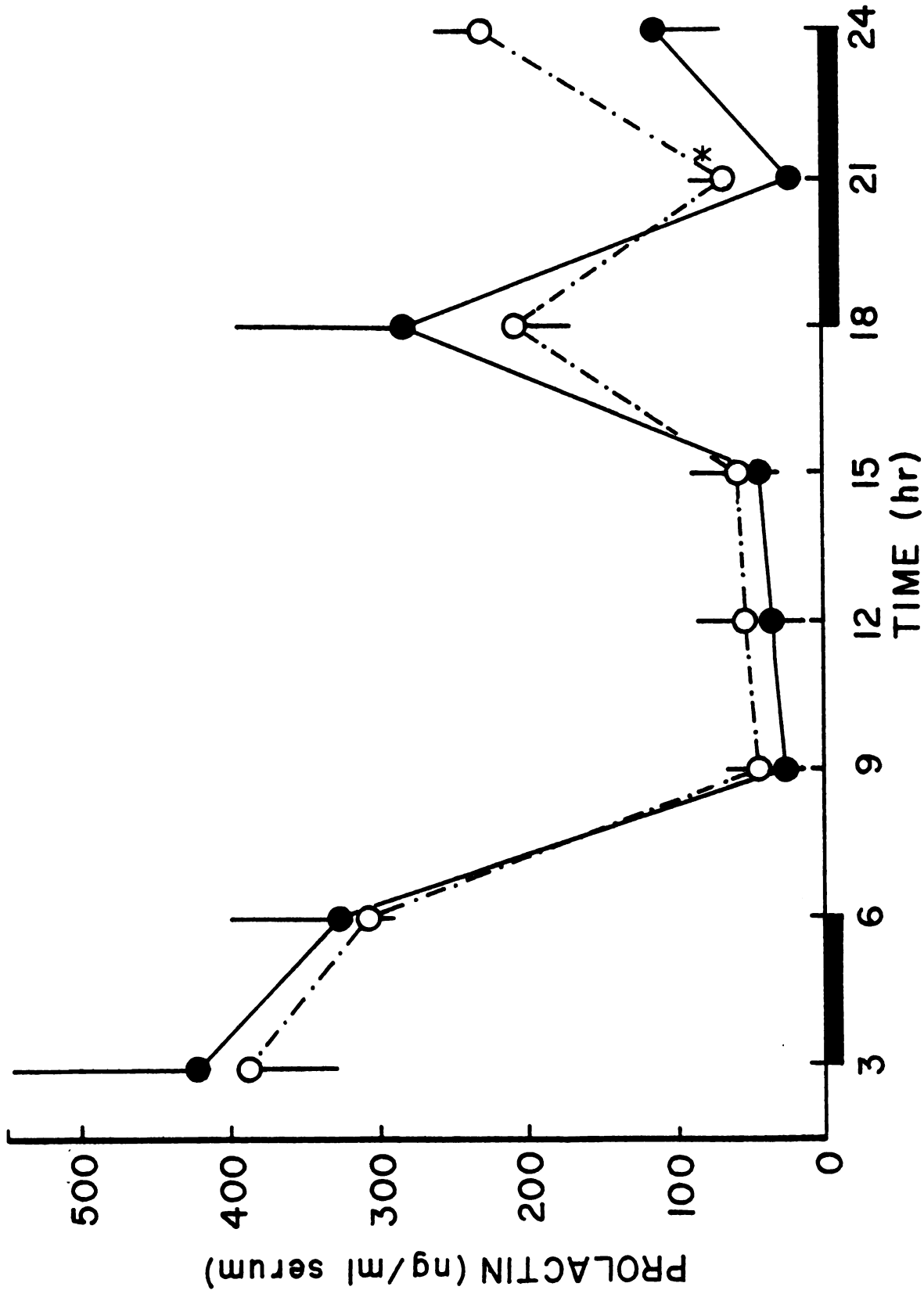
Two peaks of prolactin were measured in both treatment groups with a nocturnal surge that peaked at 0300 hrs and a diurnal surge that peaked at 1800 hrs (Figure 12). The concentrations of serum prolactin were very similar throughout the twenty-four hour period, however, a statistically significant difference ($p<.05$) occurred at 2100 hours.

The Effect of an Acute 4 hr Restraint Stress Administered During the Nocturnal or Diurnal Surge of Prolactin and DOPA Accumulation in Pregnant and Pseudopregnant Rats

Pregnant and pseudopregnant rats were subjected to a 4 hr restraint stress administered during the expected nocturnal or diurnal surge of prolactin. In this preliminary experiment, serum prolactin and LH concentrations were analyzed in samples collected immediately preceding the restraint period and at the end of the 4 hr treatment. Thus, on day 5 of pregnancy or pseudopregnancy, rats were randomly subdivided into 4 groups of 8 rats each. At 2300 hrs on day 5, 2 groups of rats were subjected to orbital sinus puncture under light ether anesthesia. One group was then restrained until 0300 hrs day 6, while animals in the second group were returned to their home cages and remained undisturbed during the four hour interval. Another blood sample was collected from the same animals at 0300 hr on day 6. Both groups were then returned to their respective cages until 1200 hrs day 6 when they

Figure 12. Effect of orbital sinus puncture on serum prolactin concentrations during early pregnancy.

Serum concentrations of prolactin were determined in blood samples collected via orbital sinus puncture at 3 hour intervals on day 7 of pregnancy. The open circles represent those animals that had been subjected to orbital sinus puncture 24 hrs earlier, and the closed circles represent pregnant rats that were bled on day 7 only. Each point on the graph represents the mean of 6 determinations and the vertical line through each point represents 1 SE. Where no lines is shown the SE is less than the radius of the symbol.


PROLACTIN (ng/ml serum)
500
400
300
200
100
0
3
6
9
12
15
18
21
24
TIME (hr)
*

were sacrificed for DOPA accumulation determination. The remaining two groups were undisturbed until 1430 hrs day 6 when they were likewise subjected to orbital sinus puncture. From 1430-1830 hrs one group was restraint stressed, while the other animals rested in their home cages. At 1830 hrs, animals of both remaining groups were again handled for blood collection, and were returned to their cages until determination of DOPA accumulation at 2400 hrs.

Restraint stress from 2300 hrs of day 5 of pregnancy until 0300 hrs on day 6 interfered with the appearance of the nocturnal surge of PRL ($p<.001$) (Figure 13). A similar result was observed in pseudopregnant rats (Figure 14). There were no significant differences due to restraint stress in the rate of DOPA accumulation within the median eminence when measured at either 1200 hrs day 6 of pregnancy or pseudopregnancy (Figures 13 and 14).

The diurnal surge of PRL was absent at 1830 hr in pregnant and pseudopregnant rats restraint stressed from 1430-1830 hr on day 6 (Figures 15 and 16). No significant changes in the rate of DOPA accumulation within the median eminence were measured at 2400 hrs in either pregnant or pseudopregnant rats (Figures 15 and 16). Serum LH concentrations were not altered by restraint stress in either pregnant or pseudopregnant rats.

The Effect of Restraint Stress During the Nocturnal Prolactin Surge of Pregnancy on the Pattern of Prolactin Release

Serum prolactin concentrations were analyzed at specified times on day 6 of pregnancy following a 4 hr restraint stress administered during the expected nocturnal PRL surge. Although results of the preliminary experiment demonstrated that restraint stress could

Figure 13. The effect of restraint stress on the nocturnal surge of prolactin and DOPA accumulation in the pregnant rat.

Serum concentrations of prolactin were measured in blood samples collected by orbital sinus puncture at 2300 hr on day 5 of pregnancy and immediately following the 4 hr treatment interval at 0300 hr on day 6. Control (C) and restraint stressed (RS) rats were sacrificed at 1200 hr for the measurement of DOPA accumulation. Each rat received NSD 1015 (±00 mg/kg, i.p.) 30 min prior to sacrifce. Each bar represents the mean value of 5-8 determinations. Vertical lines atop each bar represent 1 SE. * = $p<.01$.

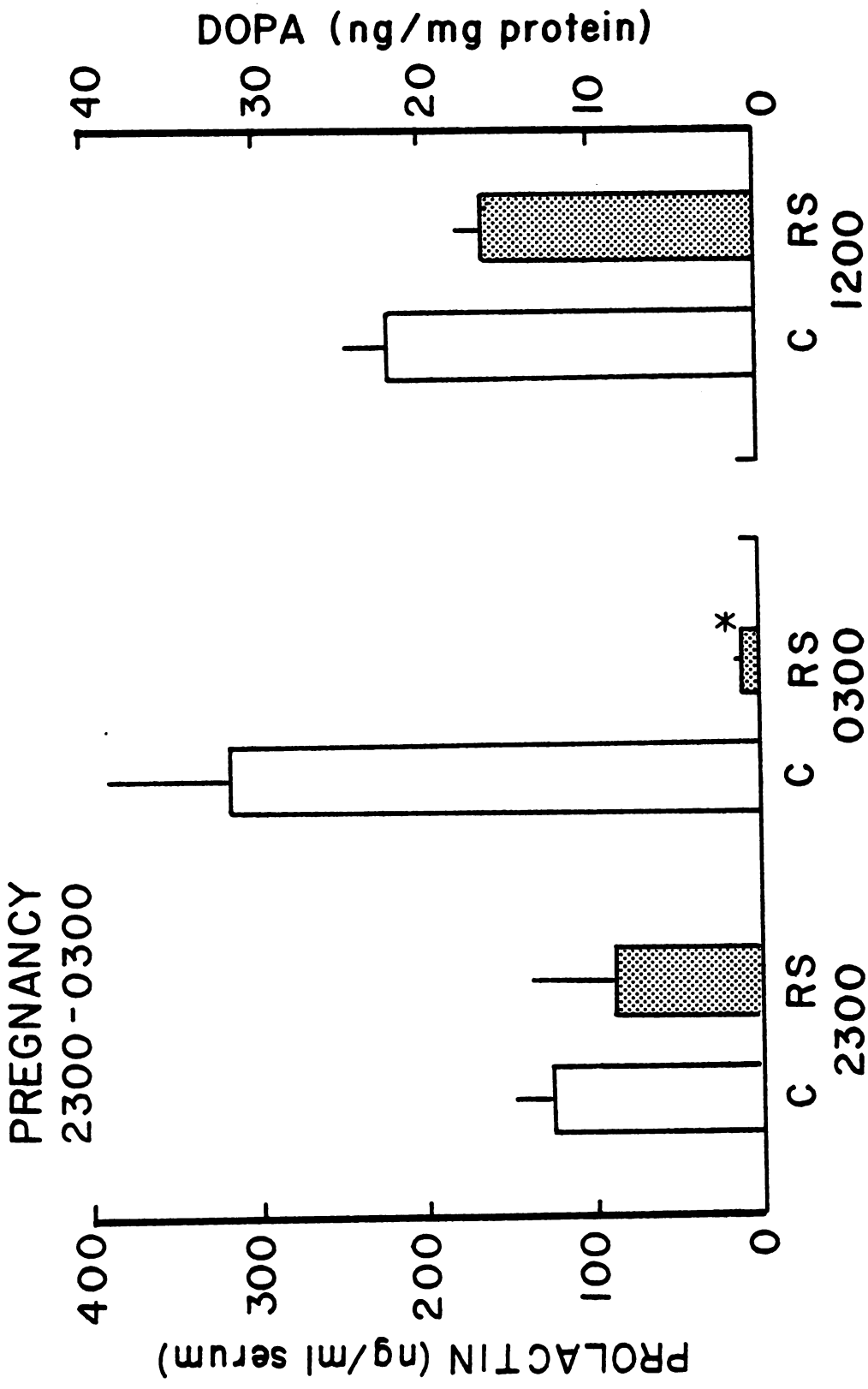

DOPA (ng/mg protein)
140
30
20
10
0
C RS
1200
PREGNANCY
2300-0300
400
300
200
100
0
PROLACTIN (ng/ml serum)
*
C RS
0300
C RS
2300

Figure 14. The effect of restraint stress on the nocturnal surge of prolactin and DOPA accumulation in the pseudopregnant rat.

See legend to Figure 13 for additional details.

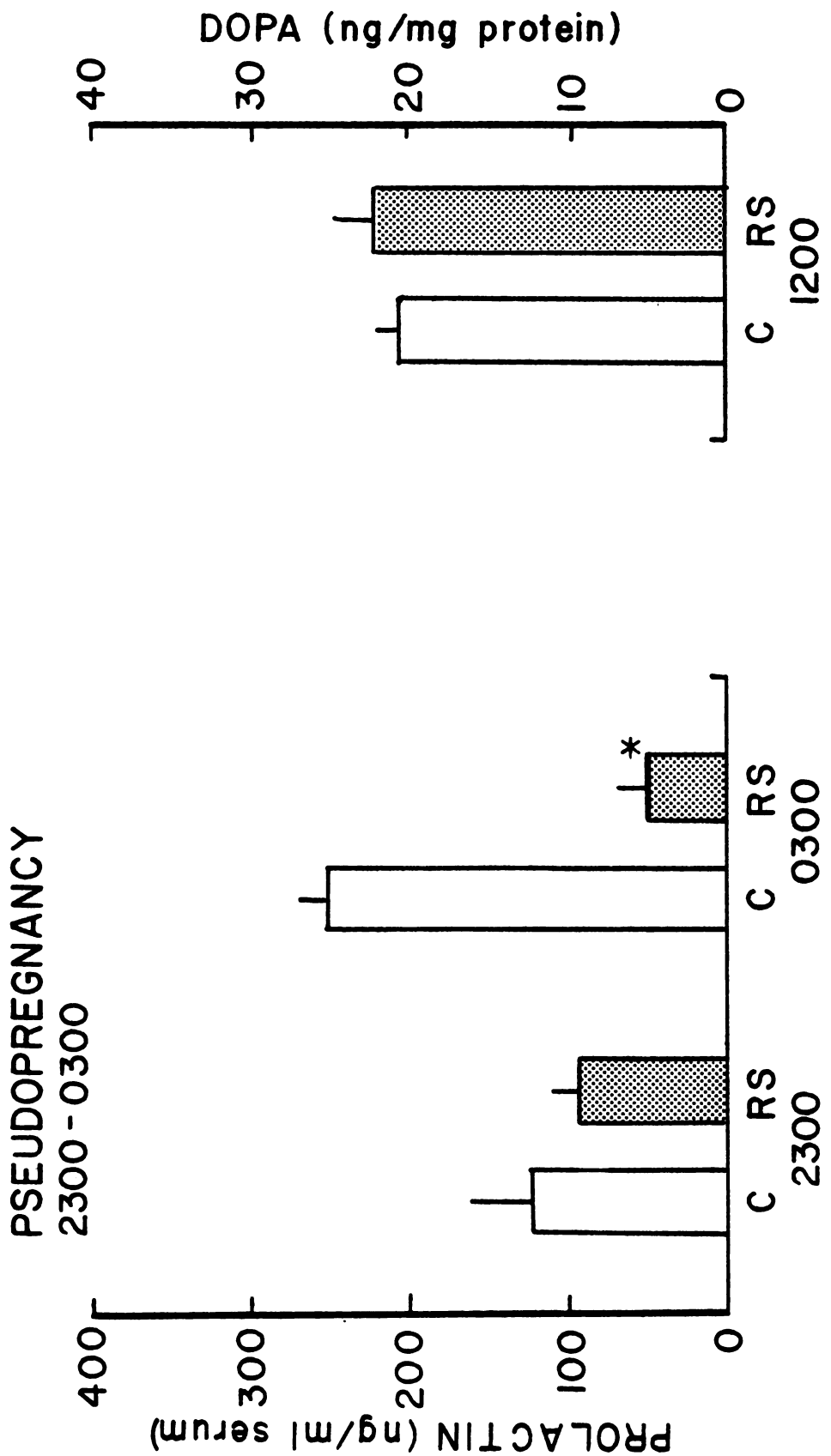

PSEUDOPREGNANCY
2300-0300
PROLACTIN (ng/ml serum)
400
300
200
100
0
C RS
2300
C RS
0300
*
DOPA (ng/mg protein)
40
30
20
10
0
C RS
1200

Figure 15. The effect of restraint stress on the diurnal surge of prolactin and DOPA accumulation in the pregnant rat.

Serum concentrations of prolactin were measured in blood samples collected by orbital sinus puncture at 1430 hr and immediately after the 4 hr stress interval at 1830 hr on day 6 of pregnancy. Control (C) and restraint stressed (RS) rats were sacrificed for the measurement of DOPA accumulation at 2400 hr. See legend to Figure 13 for additional details.

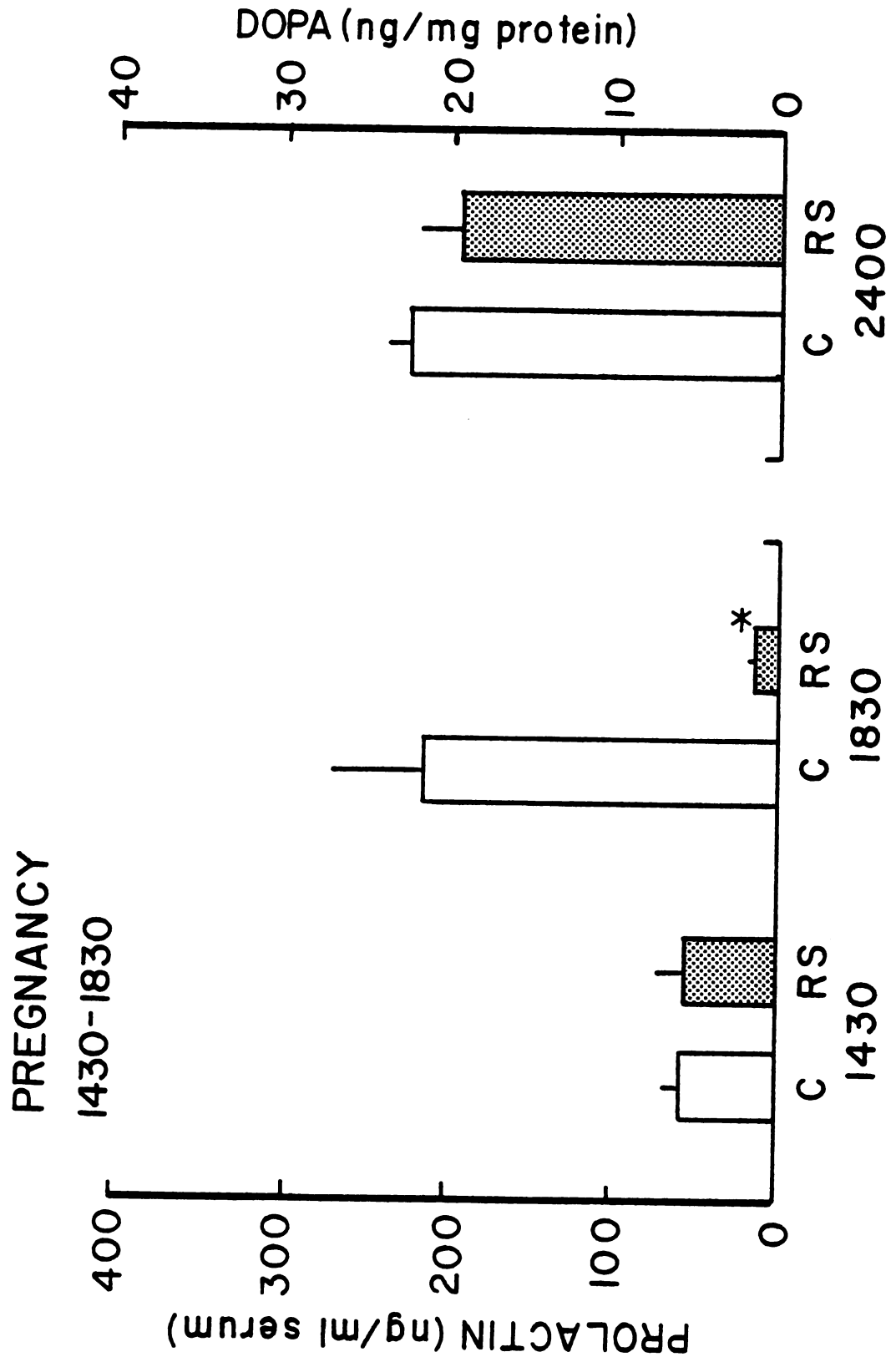
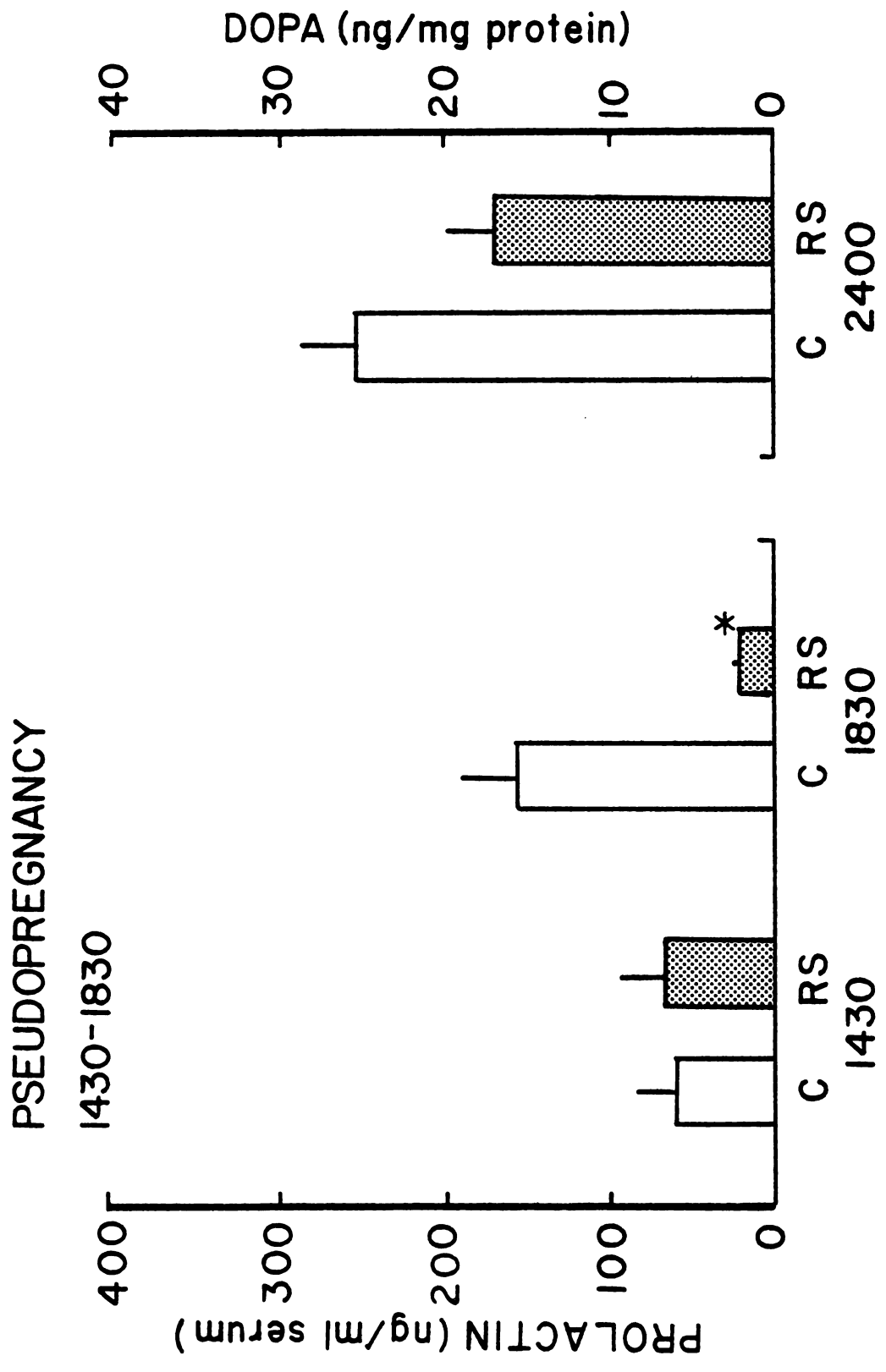

DOPA (ng/mg protein)
40
30
20
10
0
C RS
2400
PREGNANCY
1430-1830
400
300
200
100
0
PROLACTIN (ng/ml serum)
*
C RS
1830
C RS
1430

Figure 16. The effect of restraint stress on the diurnal surge of prolactin and DOPA accumulation in the pseudopregnant rat.

See legend to Figure 15 for additional details.


DOPA (ng/mg protein)
40
30
20
10
0
C
RS
2400
PSEUDOPREGNANCY
1430-1830
PROLACTIN (ng/ml serum)
400
300
200
100
0
*
C
RS
1830
C
RS
1430

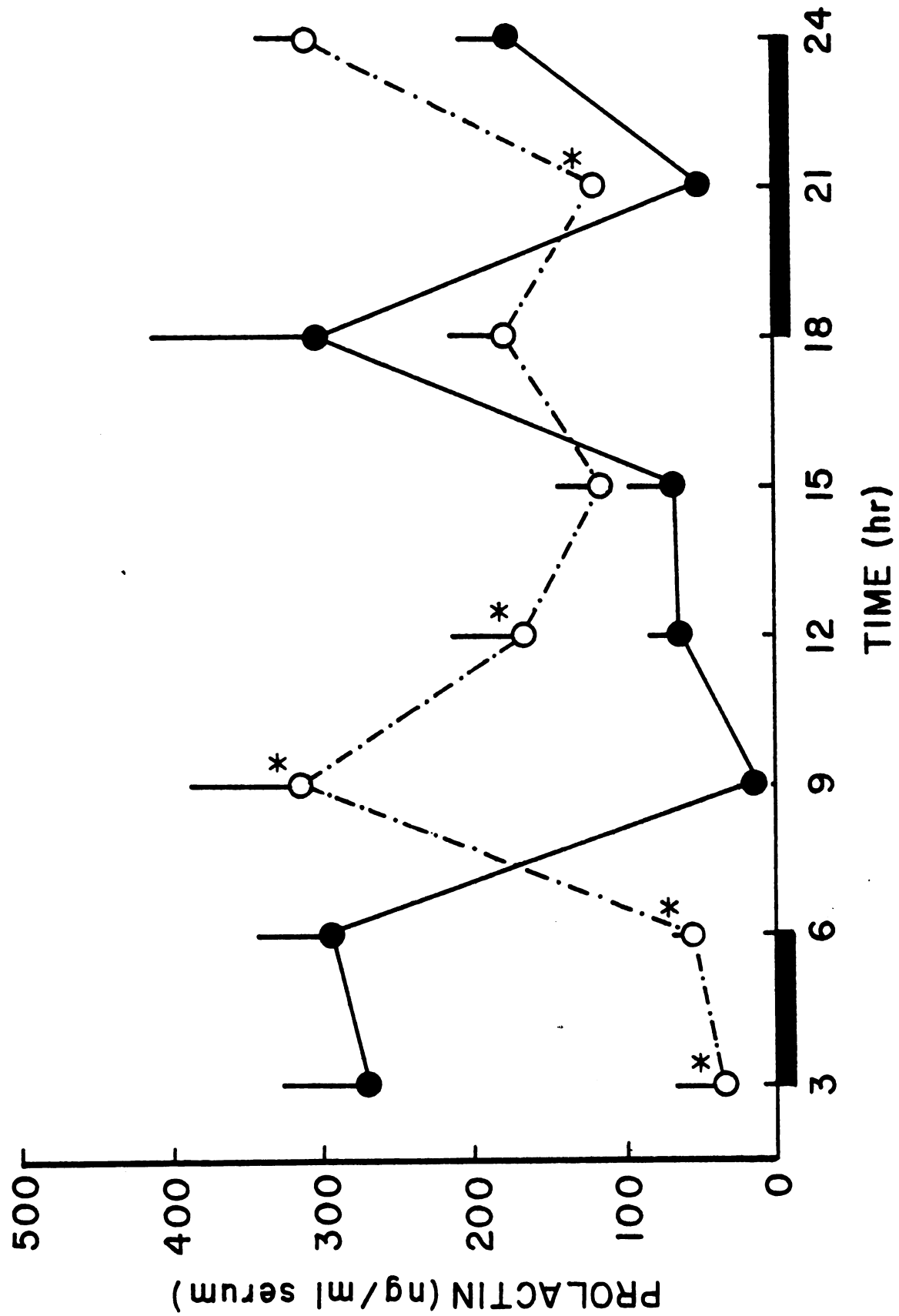
interfere with the occurrence of the nocturnal surge of prolactin, it was not known if the surge was blocked or merely delayed. Therefore, in the first experiment to be reported here, day 5 pregnant rats were randomly subdivided into 16 groups of 6 rats each. A 4 hr restraint stress was administered to 8 groups of rats from 2300 hrs day 5 until 0300 hrs day 6 of pregnancy. The other 8 groups remained in their respective cages undisturbed during this time interval. A single blood sample (approximately 1 ml) was collected from each treated and control rat by orbital sinus puncture under light ether anesthesia at one of the following times on day 6 of pregnancy: 0300, 0600, 0900, 1200, 1500, 1800, 2100 or 2400.

Restraint stress significantly lowered serum prolactin levels when measured at 0300 and 0600 hrs (Figure 17). However, the nocturnal surge was delayed rather than blocked by restraint stress, as serum PRL concentrations were significantly elevated over control levels at 0900 and 1200 hrs. Although the prolactin concentrations of restraint stressed rats at 1800 hrs (diurnal surge) tended to be lower than 1800 hr values in non-stressed females, the difference was not significant.

PRL concentrations at 2100 hrs of restraint stressed rats were significantly elevated over non-stressed controls, which might suggest that the 3 hr sampling interval was not adequate to detect a slight delay in the diurnal PRL peak.

Figure 17. The effect of a 4 hr restraint stress on the pattern of prolactin release in the pregnant rat.

Day 5 pregnant rats were subjected to a 4 hr restraint stress initiated at 2300 hr. Serum concentrations of prolactin were determined in blood samples collected by orbital sinus puncture at 3 hr intervals on day 6 of pregnancy. Each point on the graph represents the mean of 6 determinations: = contro; = restrained. The vertical line through each point represents 1 SE, and where no lines are shown, the SE is less than the radius of the symbol. * = $p<.05$.


PROLACTIN (ng/ml serum)
500
400
300
200
100
0
3
6
9
12
15
18
21
24
TIME (hr)
*
*
*
*
*

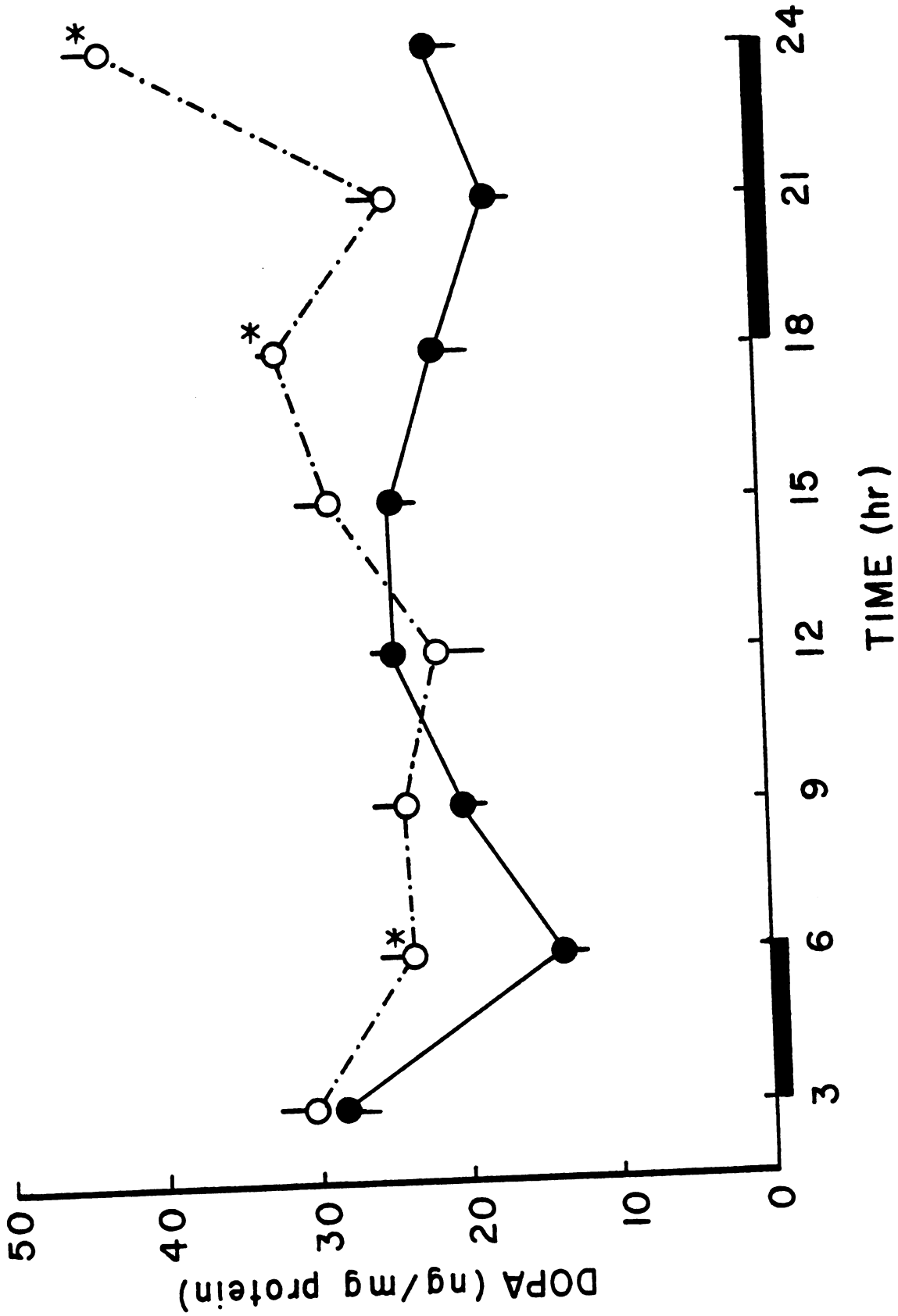
The Effects of Restraint Stress During the Nocturnal Prolactin Surge of Pregnancy on the Rate of DOPA Accumulation

To further investigate the effects of restraint stress on PRL release and TIDA neuronal activity during pregnancy, a second experiment was conducted. As in the previous study, 16 groups of 6 pregnant rats each were evenly divided between restraint stress and control treatments. In this experiment 2 blood samples were collected from each rat. The first at 2300 hrs of day 5 and the second at 0300 hr on day 6 of pregnancy. The rats designated as the treatment group were subjected to restraint stress between those blood collection times, while the remaining control rats were returned to their home cages. The rate of DOPA accumulation within the median eminence was measured in each rat at one of the specified times on Day 6 of pregnancy as listed in the previous study. Only those restraint stressed rats in which the 0300 hr PRL surge was interfered with were included in the results.

The rate of DOPA accumulation within the median eminence was significantly elevated from non-restrained controls at 0600, 1800 and 2400 hrs (Figure 18). In accordance with the preliminary study, no significance between group difference in the rate of DOPA accumulation in the median eminence was observed at 1200 hrs. The significantly elevated rates of DOPA accumulation measured within the median eminence of restraint stressed rats at 1800 hrs are consistent with the restraint stress induced delay of prolactin release.

Figure 18. The effect of a 4 hr restraint stress on the pattern of DOPA accumulation in the median eminence of the pregnant rat.

Day 5 pregnant rats were subjected to a 4 hr restraint stress initiated at 2300 hr. DOPA accumulation was determined in rats sacrificed at 3 hr intervals on day 6 of pregnancy. Prior to sacrifice, each animal received an injection of NSD 1015 (100 mg/kg, i.p.). See legend to Figure 17 for additional details.

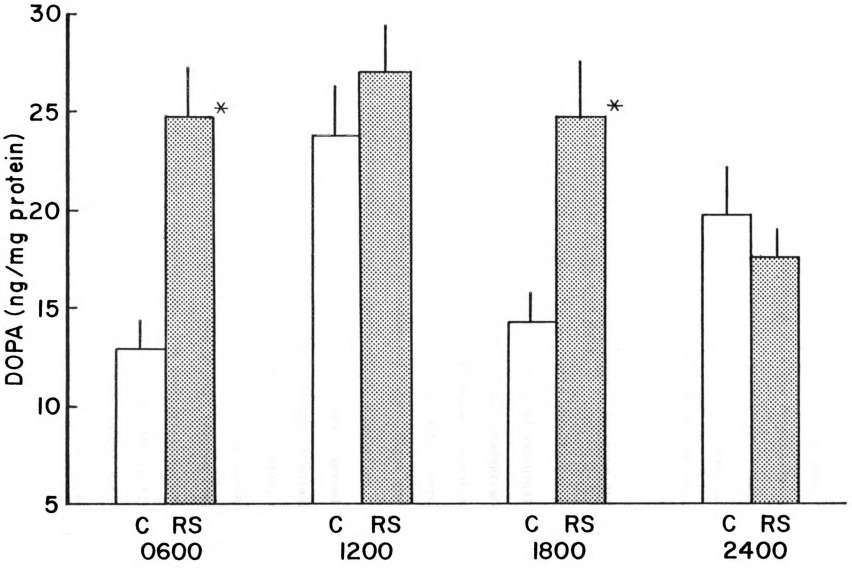

DOPA (ng/mg protein)
50
40
30
20
10
0
3
6
9
12
15
18
21
24
TIME (hr)
*
*
*

The Effect of Restraint Stress During the Nocturnal Prolactin Surge of Pregnancy on the Rate of DOPA Accumulation at Selected Times After Stress

An additional study was undertaken to examine the rates of DOPA accumulation within the median eminence of stressed and non-stressed rats on day 6 of pregnancy. In the previous experiment, the rate of DOPA accumulation within the median eminence was altered from control values during the 24 hr period following restraint stress (see Figure 18). Since this was the first study to suggest that restraint stress alters the activity of TIDA neurons, it was necessary to confirm that the observed differences were due to stress treatment rather than the normal variability of TIDA neuronal activity that is detected throughout the day in pregnant rats. Accordingly, this study was conducted to compare the rates of DOPA accumulation within the median eminence of selected times on day 6 of pregnancy following restraint stress administered from 2300 hrs on day 5 of pregnancy until 0300 hrs on day 6. Restraint stressed females and non-stressed controls were sacrificed for determination of DOPA accumulation at one of the following times: 0600, 1200, 1800 or 2400. Rates of DOPA accumulation in the median eminence of rats killed at 0600, 1200, and 1800 hours were similar to those of the previous study (Figures 18 and 19). However, when measured at 2400 hrs, DOPA accumulation values between stressed and non-stressed rats were not significantly different as previously measured (Figures 18 and 19). These results might suggest that the 2400 hr sampling point falls at a time of transition of PRL release and TIDA neuronal activity. The large standard error bars depicted in Figure 17 indicated a large variation in the release of PRL at 2400 hrs. That data indicated that some rats had initiated their nocturnal

Figure 19. Effect of a 4 hr restraint stress on DOPA accumulation at selected times.

Day 5 pregnant rats were subjected to a 4 hr restraint stress initiated at 2300 hr. Following the administration of NSD 1015 (100 mg/kg, i.p.) DOPA accumulation was determined in the median eminence of day 6 pregnant rats at 0600, 1200, 1800 or 2400 hr. Each bar represents the mean of eight control (C) or eight restraint stressed (RS) determinations. Vertical lines atop each bar represent 1 SE. * = $p<.05$.


30
25
20
15
10
5
DOPA (ng/mg protein)
C RS
0600
C RS
1200
C RS
1800
C RS
2400
*
*

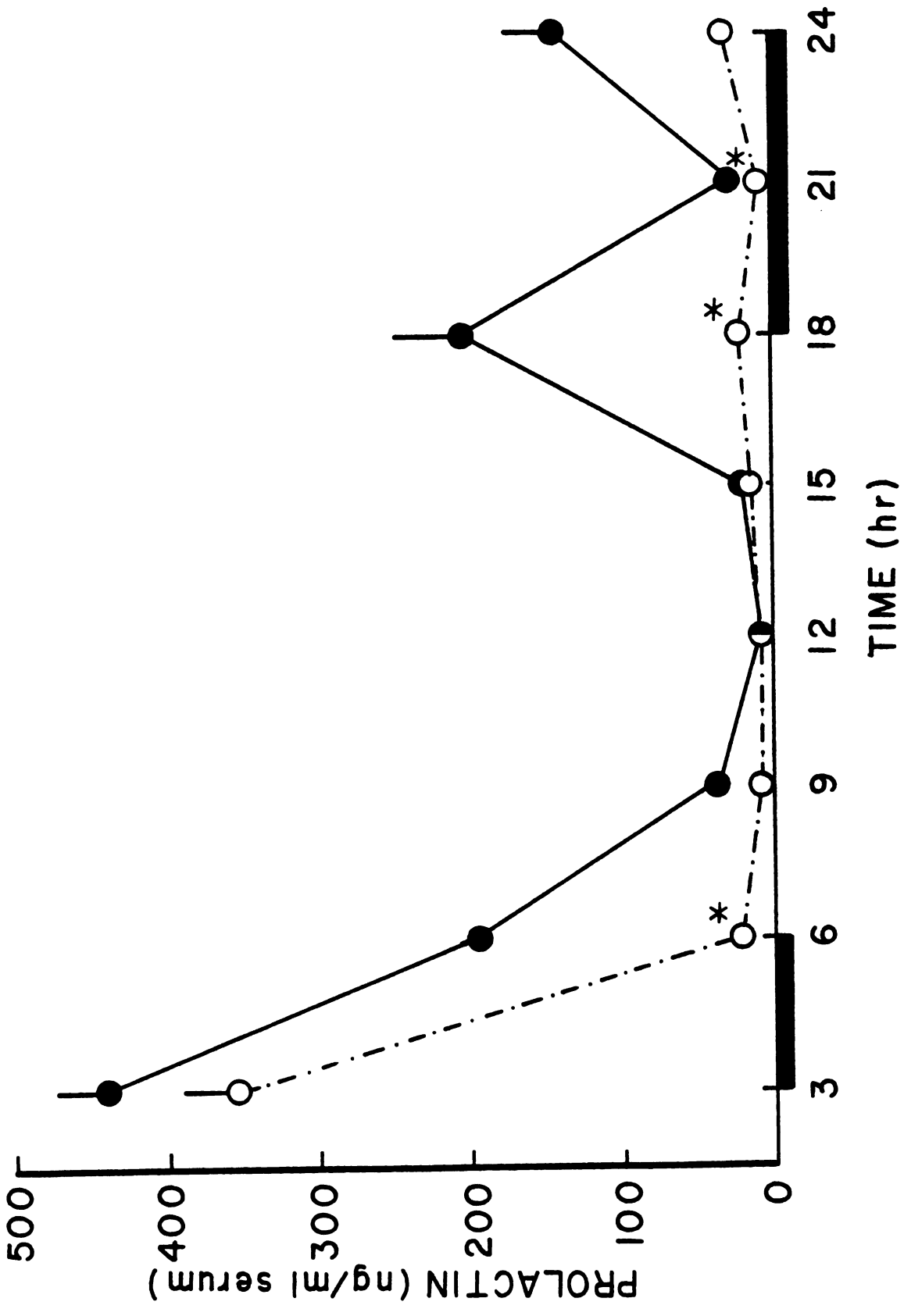
surge of prolactin while others had not. Thus, the discrepancy between the rates of DOPA accumulation values at 2400 hrs between this study and the one previous, are consistent with the hypothesis of a functional relationship between TIDA neuronal activity and pituitary prolactin release, and may be explained by the number of rats that had initiated the nocturnal surge of PRL by the 2400 hr sampling point.

The Effect of Ovariectomy on Prolactin Secretion and Anterior Pituitary Dopamine Content in Cervically Stimulated Rats

The pattern of prolactin release of cervically stimulated rats following ovariectomy is characterized by an attenuated nocturnal surge and the absence of the diurnal surge typical of pseudopregnancy (Freeman et al., 1974). In this study, serum prolactin concentrations and steady-state DA concentrations in the anterior pituitary were measured in cervically stimulated-ovariectomized rats. Eight groups of 6 rats were ovariectomized on day 2 postcervical stimulation. Sham surgery was performed on a similar number of animals which served as the control group. On day 6 postcervical stimulation, each animal was sacrificed by decapitation at one of the following times: 0300, 0600, 0900, 1200, 1500, 1800, 2100, or 2400 hrs. Trunk blood was collected from each rat, and the anterior pituitary was quickly removed from the brain for measurement of DA concentration. The concentrations of serum PRL analyzed at specific times on day 6 are presented in Figure 20. In the cervically stimulated (CS) sham operated control rats there were two peaks of prolactin release, one nocturnal (measured at 0300 hrs) and the other diurnal (measured at 1800 hrs). The nocturnal

Figure 20. The effect of ovariectomy following cervical stimulation on the pattern of prolactin release.

Ovariectomy ( ) or sham surgery ( ) was performed on day 2 following cervical stimulation. Serum concentrations of prolactin were measured in trunk blood samples collected on day 5. Each point on the graph represents the mean of 5-6 samples, and the vertical line through each point represents 1 SE. Where no lines are shown, the SE is less than the radius of the symbol. * = $p<.05$.


PROLACTIN (ng/ml serum)
500
400
300
200
100
0
3
6
9
12
15
18
21
24
TIME (hr)
*
*
*

surge of CS ovariectomized rats was attenuated in length, but not magnitude, and the diurnal surge was totally absent.

Adenohypophysial DA content of the same rats is presented in Figure 21. Cervically stimulated sham operated controls showed peak concentrations at midday (0900-1500 hrs) and again at 2400 hrs. A significant nadir was measured at 0300 hrs and concentrations tended to be decreased in the evening hours (1800-2100 hours).

There was also a cyclic variation in the adenohypophysial DA content of CS-OVX rats. DA concentrations in the pituitary were low in the early morning (0300-0600 hrs) and were elevated throughout the remainder of the day in ovariectomized rats.

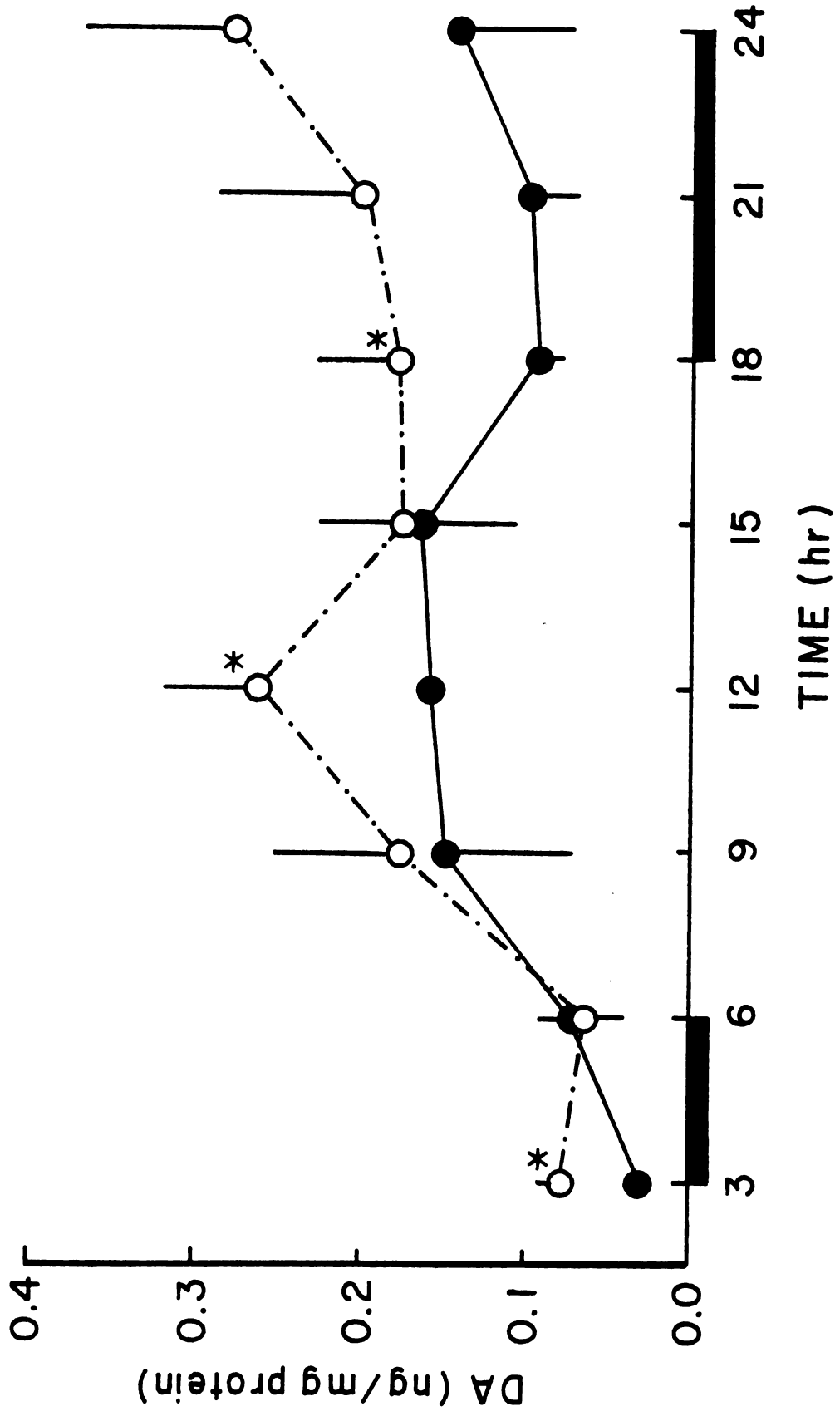
Significant differences in anterior pituitary DA concentration between sham and OVX rats were detected at 0300, 1200 and 1800 hrs. Throughout the evening hours (i.e., 1800-2400 hrs) OVX anterior pituitary dopamine concentrations tended to be higher than sham concentrations although only the 1800 hr difference was significant ($p<.05$). These results suggest that pituitary DA content was decreased following the surge release of prolactin.

The Effect of Ovariectomy Following Cervical Stimulation on DOPA Accumulation in the Median Eminence

It has been suggested that PRL may cause a selective feedback activation of TIDA neurons (Gudelsky et al., 1976; Hökfelt and Fuxe, 1972). Thus, the elimination of a surge of prolactin may be reflected in decreased TIDA neuronal activity. To test this possibility, cervically stimulated rats were ovariectomized to eliminate the diurnal

Figure 21. Anterior pituitary dopamine concentrations in ovariectomized-cervically stimulated rats.

Dopamine steady-state concentrations were measured in the anterior pituitary of ovariectomized cervically stimulated rats. See legend to Figure 20 for details.


DA (ng/mg protein)
0.0
0.1
0.2
0.3
0.4
3
6
9
12
15
18
21
24
TIME (hr)
*
*
*

surge of prolactin (Freeman et al., 1974), and DOPA accumulation in the median eminence was measured at selected times. On day 2 following cervical stimulation, rats were randomly subdivided into 16 groups of 8 rats each. Half of the groups were ovariectomized while the remaining 8 groups received sham ovariectomy. A single blood sample (approximately 1 ml) was collected from each rat by orbital sinus puncture under light ether anesthesia at 0300 hrs day 5 post CS. The following day, rats were sacrificed for determination of DOPA accumulation at one of the following times: 0300, 0600, 0900, 1200, 1800, 2100, or 2400 hrs. Rats in which the 0300 hr day 5 PRL concentration was not greater than or equal to the mean minus 1 standard deviation ($\bar{X}$ - 1 S.D.) were excluded from the study. Table 10 lists the respective numbers of rats per treatment included in the analysis. Results of the rate of DOPA accumulation in the median eminence are presented in Figure 22.

The pattern of DOPA accumulation in the median eminence is similar for both sham and OVX rats except at 2100 hrs, when OVX values were significantly decreased from control.

These results suggest that the absence of the diurnal surge of prolactin at 1800 hr may result in decreased TIDA neuronal activity due to a lack of prolactin feedback.

Discussion

An overwhelming amount of experimental evidence supports the concept that the hypothalamus exerts a tonically negative control over prolactin in release from the anterior pituitary. A major hypothalamic inhibitory substance is DA, which is released from

TABLE 10

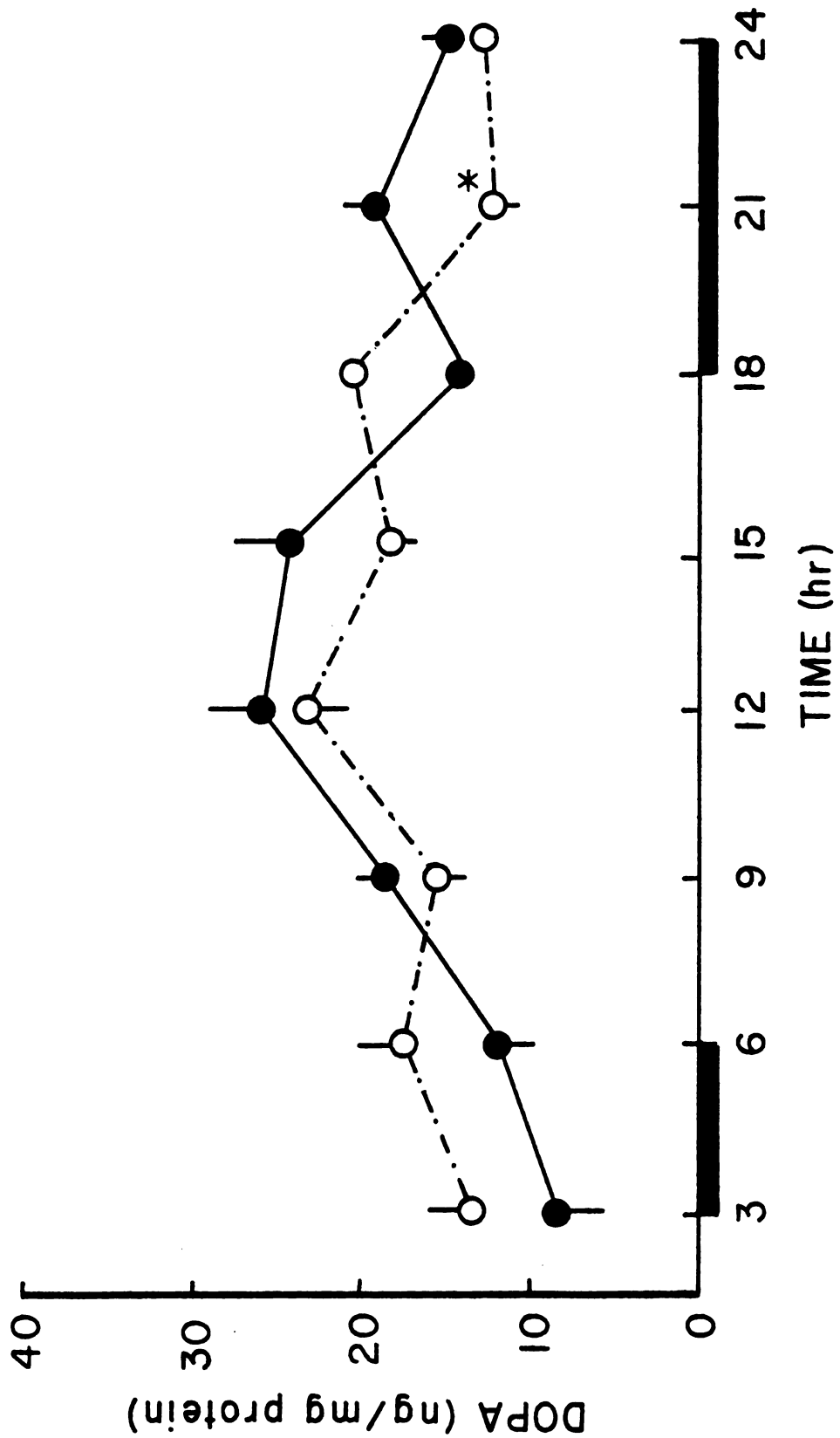
Number of Rats Per Treatment Cell for Determination of DOPA Accumulation Following Cervical Stimulation-Ovariectomy[a]

TIME	Treatment	
	SHAM	OVX
0300	3	8
0600	8	7
0900	7	7
1200	7	9
1500	7	5
1800	8	7
2100	7	7
2400	6	6

[a]Rats were cervically stimulated on the day of estrus, and were ovariectomized during the afternoon of the following day. A blood sample was collected by orbital sinus puncture from each rat at 0300 hrs day 5 following cervical stimulation and rats were subjected to DOPA accumulation determination at the listed times on Day 6.

Figure 22. The effect of ovariectomy following cervical stimulation on the pattern of DOPA accumulation in the median eminence.

DOPA accumulation was determined in the median eminence of sham-operated ( ) and ovariectomized ( ) rats on day 5 following cervical stimulation. Rats were sacrificed at 3 hr intervals following the administration of NSD 1015 (100 mg/kg, i.p.). Each point on the graph represents the mean of 3-9 determinations (see Table 10). The vertical line through each point represents 1 SE. Where no lines are shown, the SE is less than the radius of the symbol. * = $p<.05$.


DOPA (ng/mg protein)
40
30
20
10
0
3
6
9
12
15
18
21
24
TIME (hr)
*

tuberoinfundibular nerve terminals in the median eminence, directly into the hypophyseal portal vessels for transport to the pituitary (Ben-Jonathan, 1977). Consequently, alterations in tuberoinfundibular dopaminergic neuronal activity may regulate the release of pituitary prolactin. It has also been suggested that prolactin may in part regulate its own release by activating the tuberoinfundibular nerves through a sluggish feedback mechanism (Gudelsky et al., 1976). The present experiment describes pituitary prolactin release during pregnancy and pseudopregnancy, and characterizes alterations in TIDA neuronal activity when prolactin release is manipulated by restraint stress or ovariectomy.

The results of the present study confirmed the biphasic release of prolactin previously noted in early pregnancy (Butcher et al., 1972) and pseudopregnancy (Freeman et al., 1974). Freeman et al. (1974) also reported that the stress of surgery, blood collection, or anesthesia resulted in the complete disappearance of the diurnal prolactin surge of pseudopregnancy, although the nocturnal surge was insensitive to such stressors. In contrast to those findings, results of this study showed that both the nocturnal and diurnal prolactin surges of pregnancy and pseudopregnancy are sensitive to stress. Under our conditions, a 4 hr restraint stress imposed during the time of the expected nocturnal surge of prolactin delayed the appearance of the surge until later in the morning, when a surge of comparable length and magnitude was detected. Furthermore, blood collection via orbital sinus puncture did not diminish the appearance of the diurnal surge of prolactin. The differences between the findings of the

present study and those of Freeman et al. (1974) may be due to the more chronic nature of the stressors employed in the latter study.

A biphasic pattern of tuberoinfundibular dopaminergic neuronal activity was also observed during pregnancy and pseudopregnancy. Increased periods of neuronal activity occurred at times between the nocturnal and diurnal surges of prolactin. The observed changes in neuronal activity were specific for the tuberoinfundibular system since no consistent changes were observed in other brain regions containing dopaminergic neurons examined. During a period when there was no surge of prolactin (i.e., on the second day of diestrus), the accumulation of DOPA in the median eminence did not change. This finding suggests that there may be some relationship between the surges of prolactin and changes in the TIDA neuronal activity which occur in pregnant and pseudopregnant rats.

Alterations in the surge release of prolactin are reflected in changes of TIDA neuronal activity. When the administration of restraint stress delayed the nocturnal surge of prolactin in pregnant rats, a shift in the peak activity of tuberoinfundibular dopaminergic nerves was also observed. Furthermore, only one peak of TIDA neuronal activity was detected in ovariectomized cervically stimulated rats in which the diurnal prolactin surge was absent. These findings suggest a causal role of the surge release of prolactin during pregnancy and pseudopregnancy in the activation of TIDA neurons.

Attempts by other investigators to demonstrate a reciprocal relationship between hypophyseal portal blood DA concentrations and peripheral prolactin levels have repeatedly failed (DeGreef and Neill,

1979; Ben-Jonathan et al., 1980). However, definite differences in hypophyseal portal DA concentrations have been measured throughout the day in cervically stimulated rats (DeGreef and Neill, 1979) and between stages of pregnancy (Ben-Jonathan, 1980). The limited number of sample points, the time requirement to obtain a sample, and non-specific anesthesia effects have all been suggested as possible reasons for the failure to show reciprocity between portal DA and peripheral prolactin concentrations. However, decreased portal DA concentrations were measured during lactation when serum PRL concentrations are elevated and the reverse was measured during late pregnancy (Ben-Jonathan, 1980). Although these findings suggest that an inverse relationship may exist between TIDA neuronal activity and prolactin release, the feedback of placental luteotrophin on tuberoinfundibular DA neurons is unknown. Recently, it has been suggested that rPL may act at the hypothalamus to inhibit the release of prolactin (Yogev and Terkel, 1978). Although the neuronal mechanism of this proposed feedback is unknown, it may involve the activation of TIDA neurons. Limitations in our experimental design do not allow for the correlation of DOPA accumulation values to serum prolactin concentrations, however, our results suggest that a "mirror image" relationship indeed may not exist. It is possible that the TIDA nerve activity may follow the surge release of PRL by a certain lag time dependent on other factors.

Chiocchio et al. (1980) found that adenohypophyseal dopamine content was inversely related to PRL release. In the present study, steady-state concentrations of DA in the anterior pituitary of

cervically-stimulated rats also reflected prolactin release. In sham operated controls, decreased concentrations of DA were measured at times commensurate with the surge release of prolactin. Ovariectomized animals, in which the diurnal surge was absent, showed only one such nadir at 0300-0600, a time when the nocturnal surge was manifest.

The results of the present study support the hypothesis of an operational feedback loop between the surge release of prolactin which follows cervical stimulation and TIDA neuronal activity. Although the exact function of this relationship is not clear it is possible that the prolactin induced neuronal activity may act to inhibit the release of other hypophysiotropic hormones as well as supply the necessary DA to inhibit the release of prolaction from the pituitary. Further studies are required to determine if TIDA neurons have a causal role in the initiation and maintenance of the daily prolactin surges induced by cervical stimulation.

CHAPTER 4

Summary

Stress Effects on Pregnancy and Offspring Development

A primary hypothesis tested in these experiments was that maternal stress would produce alterations in endocrine regulation of reproduction leading to fetal wastage and possible alterations in reproductive function of young rats exposed to maternal stress treatment. The effect of maternal stress on fetal survival in this experiment was much less than that of preliminary studies. In only one treatment group was restraint stress sufficient to induce significant incidence of abortion. In that situation, the females that did not abort produced litters comparable in size to control females. Consistent with this finding, Euker and Riegle (1973) reported that restraint stress affected reproduction in an "all or none" fashion such that females either aborted the pregnancy, or produced a litter of normal size.

Results of several studies suggest that significant behavioral disturbances develop in offspring of laboratory rodents which were subjected to "psychological stress", e.g., conditioned response, while pregnant (see review by Archer and Blackmun, 1971). Physical stress e.g. immobilization or heat, of the pregnant rat has been associated with a variety of measurable behavioral, anatomical and endocrinological changes in male offspring (Ward, 1972; Dahlof et al., 1978a;

Politch et al., 1978), however, few data are available concerning the effects of prenatal physical stress on the developmental normalcy of female rats. Results of the present study showed no abnormality in development of prenatally stressed female rats. Prenatally stressed females were found to develop normally in terms of weight, onset of puberty, ovarian cyclicity, fertility and fecundity.

In contrast to these findings, it has been reported that prenatal stress does result in decreased fertility and fecundity of female offspring (Herrenkohl, 1979; Herrenkohl and Gala, 1979). Such conflicting reports are not unusual in the study of prenatally induced stress effects. Archer and Blackmun (1971), addressed problems arising from strain differences of rodents and treatment differences concerning the type and duration of the stressor. They found that existing data were difficult to interpret and concluded that few if any statements could be made from the dozens of studies that they reviewed. Additionally, since negative results tend not to be published, the existing data are probably biased in favor of any debilitating effects of stress.

Another source of bias may be introduced into stress studies by the use of littermates within the same treatment group. When using siblings of polytocous species (e.g., rats, swine, dogs) in the same treatment group, their similar genetic and environmental backgrounds may result in a more uniform response to a treatment than if group members were selected at random. It has been suggested that failure to account for such a littermate effect could explain all of the reported behavioral deficiencies of prenatally stressed male rats

(Chapman and Stern, 1978). The studies from Herrenkohl's laboratory in which restraint stress was reported to reduce fertility and fecundity of female offspring, do not address the allocation of pups to treatment groups. It is doubtful that litter effects were considered in her studies. Additionally, Herrenkohl's reports make no mention of stress induced abortion. If the abortion rate due to stress was high, one might assume that a greater chance exists for the use of surviving littermates in subsequent studies. In the present study, the litter effect was reduced by the minimization of littermates allocated to any particular treatment group.

Prolactin Release and TIDA Neuronal Activity

The diurnal surge of prolactin during pregnancy is stress sensitive (Riegle and Meites, 1976b), however, this study provided the first demonstration that the nocturnal surge of pregnancy could be altered by stress. Restraint stress administered during or before either the nocturnal or diurnal prolactin surges of pregnancy can block either PRL surge without affecting pregnancy. Results from a time-course study following restraint stress administered from 2300 hrs day 5 of pregnancy until 0300 hrs day 6 showed that the nocturnal surge of prolactin was not blocked, but rather delayed until later in the morning.

Smith and Neill (1976a) suggested that the nocturnal prolactin surge of pseudopregnancy was programmed to occur at a "critical period", necessary to maintain the functional corpora lutea. Results of the present study suggested that restraint stress reset the timing

of the surge. The resultant "nocturnal surge" was detected at 0900 hrs and appeared to be of normal magnitude and duration.

While little is known of the neuronal mechanisms that regulate the surge release of PRL during pregnancy or pseudopregnancy, it is generally accepted that dopamine released from tuberoinfundibular neurons has a potent inhibitory effect on pituitary prolactin release (MacLeod, 1976).

DA is released from the terminals of these nerves in the median eminence where it enters the hypophyseal portal blood (Ben-Jonathan et al., 1977) and is carried to the anterior pituitary. In the pituitary, DA associates with prolactin granules (Nansel et al., 1980). It is thought that this DA then inhibits the release of prolactin from the pituitary. In the present study, DA content of the anterior pituitary reflected the surge release of prolactin in ovariectomized and sham-ovariectomized cervically stimulated rats. These results are consistent with the work of Chiocchio et al. (1980) who demonstrated an inverse relationship between serum prolactin levels and anterior pituitary DA concentrations throughout the estrous cycle in the rat.

Results of the present study also indicated that there is a relationship between the surges of prolactin and activity changes of TIDA neurons in pregnant and pseudopregnant rats. In control pregnant and pseudopregnant rats, two surges of prolactin were measured, a nocturnal surge which peaked near 0300 hrs and a smaller diurnal surge detected at 1800 hrs. TIDA nerve activity of pregnant and pseudopregnant animals showed two peaks, one near midday, the other near midnight. When restraint stress during the expected nocturnal surge of

prolactin delayed the appearance of the surge by 3-6 hours, the apogee of TIDA activity was likewise delayed. Ovariectomy of cervically stimulated rats eliminated the diurnal surge of prolactin, and also the late night peak of TIDA neuronal activity. Thus, the delay or elimination of the surge release of prolactin, resulted in the delay or absence of peak TIDA activity. These findings are consistent with the current understanding of prolactin feedback on tuberoinfundibular DA neurons (see Moore et al., 1980), and provide the basis for future studies on the neuronal control of prolactin release during pregnancy.

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