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Insulin withdrawal effects on female sexual behavior and cell nuclear estrogen receptor levels in diabetic rats.

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INSULIN WITHDRAWAL EFFECTS ON FEMALE SEXUAL BEHAVIOR AND BRAIN CELL NUCLEAR ESTROGEN RECEPTOR LEVELS IN DIABETIC RATS

A Thesis Presented
By
LINDA IRENE SIEGEL

Submitted to the Graduate School of the University of Massachusetts in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE
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Department of Psychology
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ABSTRACT

Acute withdrawal of exogenous insulin significantly impairs estradiol and progesterone-induced sexual receptivity and brain cell nuclear estrogen receptor levels in ovariectomized, streptozotocin-diabetic rats. Insulin withdrawal for the first 24 hr, the first 18 hr, or the last 18 hr of a 24-hr estradiol benzoate-conditioning period resulted in significant reductions in mean lordosis ratings. Omission of insulin for any 12-hr period during conditioning was without effect on female sexual receptivity. Twenty-four hr insulin withdrawal resulted in a significant reduction of cell nuclear estrogen receptor levels in hypothalamus-preoptic area of rats sacrificed 26 hr after estradiol benzoate administration. Withdrawal of insulin during the middle 12 hr of conditioning had no significant effect on the quantities of brain cell nuclear estrogen receptors.

These results are in agreement with the hypothesis that decreased cell nuclear binding of estradiol by neural target tissues may contribute to the reproductive dysfunctions of diabetics. Additionally, these data provide strong support for the concept that estradiol acts at the cell nucleus to facilitate lordosis.
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Reproductive deficiencies in female diabetics. Impairments of reproductive function associated with diabetes mellitus have been well documented in human beings (Gabbe, Mestman & Hibbard, 1976; Schöffling, Federlin, Ditschuneit & Pfeiffer, 1963; Williams & Porte, 1974) and laboratory animals of both sexes (Lawrence & Contopoulos, 1960; Foglia, Rosner, Peralta-Ramos & Lema, 1969). Sterility is a deficit frequently observed in diabetic women. Abortion, intrauterine death, excessive body weight and neonatal hypoglycemia are common fetal complications of pregnancies among diabetic women (Greene, 1975; Williams & Porte, 1974) who, as a group, suffer a maternal mortality risk 20 times higher than that of the nondiabetic population (Gabbe et al., 1976). Insulin treatment largely corrects these problems.

Female rats, rendered diabetic by subtotal (95%) pancreatectomy or by selective destruction of pancreatic beta-cells following alloxan or streptozotocin administration, show a variety of reproductive deficits. Sexual maturity is delayed in alloxan-diabetic prepubertal rats, as evidenced by diminished uterine and ovarian weights, closed vaginas and absence of mature follicles in the ovaries of these animals compared to food-restricted and ad libitum-fed controls (Lawrence & Contopoulos, 1960). In rats made
diabetic at maturity, the cessation or lengthening of estrous cycles with prolonged periods of diestrus is commonly seen (Davis, Fugo & Lawrence, 1947; Foglia, Chieri & Peralta-Ramos, 1970; Lawrence & Contopoulos, 1960; Levi & Weinberg, 1949). Uterine and ovarian atrophy accompany these disruptions. Lawrence and Contopoulos (1960) found decreased gonadal weights and a marked reduction in ovarian size in diabetic rats. The ovarian tissue as well as all uterine components were atrophic, but a number of well-maintained corpora lutea were observed although the rats had been in diestrus for seven to eight weeks. The administration of olive oil to diabetic rats allowed free access to laboratory chow (carbohydrate 50%, protein 25%, fat 6%) has been shown to aid in the restoration of estrous cycles, but complete return of cyclicity to that viewed prior to induction of diabetes is found only after insulin treatment (Levi & Weinberg, 1949).

When ovulation does occur, a significantly lower number of eggs are found in the fallopian tubes of pancreaticectomized rats. Chieri and associates (1969) have reported three successive periods that take place after subtotal pancreatectomy: (1) prediabetes, characterized by normal fasting blood sugar levels, normal growth and aglycosuria, which lasts two to three months; (2) incipient diabetes, during which diabetic symptoms are noted only after feeding, with a duration of approximately one month;
and (3) manifest diabetes, with all diabetic characteristics present and which lasts until death (six to seven months). In rats rendered diabetic by this procedure, diminished numbers of eggs were observed at the third day of pregnancy, even during the prediabetic stage when the rats were normoglycemic. When blood sugar levels were elevated, the decline in numbers of ova shed was apparently independent of the duration and level of hyperglycemia. Insulin therapy restored the number of eggs to control levels (Chieri, Pivetta & Foglia, 1969).

Alterations in the estrous cycles of diabetic rats consequently result in decreased fertility (Davis et al., 1947; Foglia et al., 1970; Lawrence & Contopoulos, 1960; Levi & Weinberg, 1949), and various dysfunctions are also found once pregnancy occurs. Decreased uterine and ovarian weights are commonly observed in pregnant or pseudopregnant diabetic rats (Farina, Chieri, Basabe & Foglia, 1971; Foglia et al., 1970; Lawrence & Contopoulos, 1960). Fetal death and resorption at days 10-12 of pregnancy have been reported, and insulin treatment at the beginning of pregnancy generally prevents these deficiencies (Davis et al., 1947; Foglia et al., 1970; Lawrence & Contopoulos, 1960). Levi and Weinberg (1949), however, found pregnancy to be uninfluenced by diabetes (although estrous cycles were disrupted). Body weights of pups were not reported in this study, but Lawrence and Contopoulos (1960) observed lower
body weights in viable fetuses of diabetic rats in which gestation had been maintained for 21 days, and no correlation was found between the severity of hyperglycemia in rats that maintained pregnancy and in those that did not. In the same study, Lawrence and Contopoulos investigated the lactational performances of eight diabetic rats which were maintained on insulin throughout mating and most of gestation, but had insulin gradually withdrawn from day 17 of pregnancy until day 21 (at which time insulin was totally withheld). Three of these animals delivered still-born fetuses, and the remaining rats delivered normal young (as did nondiabetic control animals). When all young were replaced by newborns from normal mothers, the diabetic animals showed typical maternal behavior although two failed to lactate (one had borne healthy young and the other, dead fetuses). The survival rate and weaning weight of the young were less than those of young nursed by nondiabetic mothers.

Much of the work seeking to explain these reproductive deficiencies has centered on investigations of altered sexual hormone levels and/or diminished sensitivity of target organs to these hormones. Decreased numbers of ova shed as well as reductions of uterine and ovarian weights (Farina et al., 1971; Howland & Zebrowski, 1974; Liu, Lin & Johnson, 1972) are consistent with these explanations, although gonadal atrophy and cessation or estrous cycles are similarly observed under conditions of malnutrition
(Howland, 1972; Leathem, 1961). Liu and associates, however, found ovarian, uterine and body weights of acutely diabetic, immature rats to be significantly reduced compared to the weights of ad libitum-fed and matched-fed animals. Insulin treatment restored weights to control levels. In addition, when follicle stimulating hormone (FSH) plus human chorionic gonadotropin (hCG) were administered, the ovarian weights of the diabetics were still significantly lower than those of food-restricted rats, although there was no difference between groups in terms of the percentage increase in ovarian weights after treatment. Thus, the decreased ovarian weights of the diabetic rats may have been due to the insulin deficiency rather than to the decreased food intake that occurred shortly after alloxan was given and that lasted throughout most of the study.

There is disagreement in the literature concerning gonadotropin secretion in diabetic rats. Some of these disparities may be a result of procedural differences such as the age of the animal, and the method of induction and duration of diabetes. Sufficient levels of circulating gonadotropins have been reported in immature (Liu et al., 1972) and mature rats (Howland & Zebrowski, 1974), lending support to the concept that ovarian responsiveness to gonadotropins is reduced in diabetic rats (Farina et al., 1971). Howland and Zebrowski (1974), however, observed alterations
in gonadotropin levels in immature rats and similar disruptions have also been shown in underfed rats (Howland, 1972). Additionally, normal follicular development and ovarian weights have been found in immature diabetic rats given pregnant mare's serum gonadotropin (PMSG), but none of these animals ovulated, whereas 96% of the nondiabetic rats ovulated in response to PMSG (Kirchick, Keyes & Frye, 1977; 1978). The anovulation observed in the diabetic rats was not attributed to a lack of ovarian responsiveness to exogenous gonadotropins or to decreased secretion of estradiol, since 90% of the animals ovulated after hCG, and serum estradiol levels did not differ between groups. Ovulation was not impaired in insulin-treated diabetic rats. Basal serum luteinizing hormone (LH) concentrations also did not differ between groups; however, since only the nondiabetic animals displayed a preovulatory LH surge on the day of proestrus, anovulation in the diabetic rats was likely due to the absence of the LH surge.

A possible explanation for the lack of the LH surge and other reproductive deficits in diabetic rats may be that of decreased binding of estradiol by brain areas mediating these processes. Denari and Rosner (1972) have reported significantly reduced in vivo uptake of (3H)estradiol in the anterior hypothalamus and pituitary of diabetic rats after acute (24-hr) insulin withdrawal. No significant differences in uptake by skeletal muscle, uterus or vagina were shown.
Gentry and associates (Gentry, Wade & Blaustein, 1977) found decreased cell nuclear uptake of \(^{(3)H}\)estradiol in the hypothalamus, preoptic area and pituitary of ovariectomized, diabetic rats after a similar insulin withdrawal period. No deficits were noted in whole homogenate uptake of \(^{(3)H}\)estradiol or in levels of cytoplasmic estrogen receptors. Additionally, when insulin was withheld from ovariectomized, diabetic rats for the first 24 hr of a 30-hr or 54-hr estradiol benzoate (EB)-conditioning period, significant decreases in lordosis ratings were observed; although insulin withdrawal for the last six hr of a 30-hr conditioning period was without apparent behavioral effect. These observations are consistent with the concept that diminished binding of estradiol may contribute to the reproductive failures of female diabetics.

**Steroid hormones: induction of female sexual behavior in rodents and neural sites of action.** Sexual receptivity in female mammals shows a strong dependence upon levels of steroid sex hormones, particularly estradiol and progesterone (Young, 1961). In the ovariectomized rat, sexual receptivity is induced by sequential injections of estradiol-17\(\beta\) (given as EB) and progesterone (Boling & Blandau, 1939; Beach, 1942), or by the systemic injection of estradiol alone (Green, Luttage & Whalen, 1970). Sequential EB-progesterone injections also induce receptivity in
intact rats (Powers, 1970). Injection of progesterone 20 to 48 hr after EB administration will facilitate receptivity in ovariectomized rats within four to six hr (Boling & Blandau, 1939). Estrous behavior is observed 18 to 20 hr after estradiol treatment alone (Green et al., 1970). Sexual receptivity is similarly induced by injections of EB and progesterone in other rodents such as guinea pigs (Joslyn, Feder & Goy, 1971), hamsters (DeBold, Martin & Whalen, 1976) and mice (Young, 1961).

Estradiol-concentrating cells are found in brain regions at which lesions, electrical stimulation or implants of estradiol have been found to affect reproductive behavior and physiology. The techniques of autoradiography and scintillation counting reveal that peak sites of estradiol uptake overlap with neural areas in which implants of estradiol facilitate female sexual behavior (see Pfaff & Keiner, 1973). Although the precise sites of action are unknown, the preoptic area (Powers & Valenstein, 1972b; Rodgers & Schwartz, 1976) and regions of the hypothalamus, particularly the ventromedial hypothalamus (Barfield & Chen, 1977; Mathews & Edwards, 1977) have been implicated. Implants of progesterone into the brains of estrogen-primed rats also result in sexual behavior, but there is lack of agreement as to the location of the most effective sites of action (Ross, Claybaugh, Clemens & Gorski, 1971). Some peak areas of estradiol
uptake, such as the anterior and medial basal hypothalamus, preoptic area and amygdala are involved in the regulation of gonadotropin secretion (see McEwen, Zigmund & Gerlack, 1972).

The mechanism of steroid hormone action is thought to be by binding of the steroid to a cytoplasmic receptor molecule. The receptor-hormone complex is then translocated to the cell nucleus where it binds to the chromatin and consequently affects mRNA, rRNA, and protein synthesis (see Gorski & Gannon, 1976). Implants or injections of inhibitors of RNA or protein synthesis into neural target areas result in diminished sexual behavior (Hough, Ho, Cooke & Quadagno, 1974; Quadagno & Ho, 1975) and gonadotropin release (Jackson, 1972; 1973).

Although short term insulin deprivation has been shown to decrease the sexual receptivity of diabetic female rats, little is known about the precise times and durations that insulin must be withheld during the EB-conditioning period to produce this deficit. One objective of the present research was to provide information on the time-course of the effects of acute insulin withdrawal on EB and progesterone induced sexual behavior in diabetic rats. Additionally, impairments in cell nuclear binding of estradiol by those neural tissues purportedly mediating female rat sexual behavior have been reported for insulin-withdrawn diabetic rats. Since the cell nucleus is believed to be
the major site for estradiol action, it was thought to be of some value to examine the levels of brain cell nuclear estrogen receptors in diabetic rats subject to insulin withdrawal. Thus, a second objective of this research was to examine the effects of acute insulin withdrawal during the EB-conditioning period on cell nuclear estradiol binding. Brain cell nuclear estrogen receptor levels were examined after a duration of insulin withdrawal during EB-conditioning that significantly reduced mean lordosis ratings, and at a withdrawal interval during the conditioning period that was without behavioral effect.

GENERAL METHODS

Animals and housing. Fifty-seven female CD-strain rats (Charles River Breeding Laboratories, Wilmington, MA), weighing 150-175 g, were housed individually in wire-bottomed cages. Animals were maintained on a 12-hr on (lights on at midnight), 12-hr off light/dark cycle, with ad libitum access to tap water and Purina laboratory chow. All rats were ovariectomized via a single midventral incision under methoxyflurane (Metofane; Pittman-Moore, Washington Crossing, NJ) anesthesia.

Induction of diabetes and insulin treatment. Diabetes mellitus was induced, 10 days after ovariectomy, by intraperitoneal injection of 100 mg/kg streptozotocin (Upjohn,
Kalamazoo, MI) in citrate buffer, pH 4.8. Urine glucose levels were qualitatively determined using TesTape (Eli Lilly & Co.), and animals exhibiting severe glycosuria (++++; TesTape) were considered to be diabetic.

Nonfasting serum glucose levels were determined with a Beckman Glucose Analyzer (#2) on blood withdrawn from the tip of the tail. After an insulin withdrawal period of 24 hr, samples were taken from all rats serving as subjects in the behavioral experiment and from rats used in the 24-hr insulin withdrawal condition of the exchange assay. Insulin was withheld for 12 hr from animals in the 12-hr insulin withdrawal condition of the exchange assay prior to serum glucose measurement. All animals included in this study exhibited nonfasting serum glucose levels in excess of 410 mg %.

Rats were maintained with daily injections of U-100 Lente insulin (except under experimental conditions). Dosages of 3-4 U were sufficient to prevent weight loss and reduce the occurrence of glycosuria.

Induction and testing of sexual receptivity.

Apparatus and scoring method. Tests for female sexual behavior took place in a 60-cm x 60-cm x 25-cm high testing arena with a wood chip covered floor under dim illumination provided by a 25-W red bulb during the dark phase of the light/dark cycle. Lordosis was scored after each of
10 vigorous mounts with thrusting by sexually experienced males which were previously adapted to the arena. The quality of the lordosis response was rated by a modification of the procedure of Powers and Valenstein (1972a). A score of 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, or 3.0 was given to each response, where 0.0, 1.0, 2.0, or 3.0 correspond to no, slight, moderate, or full dorsoflexion, respectively. (This seven point method allows a finer characterization of lordosis behavior, and ratings are consistent when done by one experimenter over consecutive weeks.) Other estrous-related behaviors, such as darting and hopping, ear wiggling, and various soliciting and rejecting behaviors were recorded. Behavioral tests were done with the experimenter blind as to experimental treatment.

Preliminary testing. Sexual receptivity was induced with a subcutaneous injection of 2 μg EB followed 24 hr later by an injection of 1 mg progesterone. Behavioral testing took place 6-7 hr after progesterone administration. All ovariectomized rats were screened for sexual behavior prior to induction of diabetes, and those unresponsive to EB and progesterone treatment were not used.

Insulin withdrawal. The general experimental paradigm for testing the effect of acute insulin withdrawal on sexual receptivity in ovariectomized, diabetic rats is depicted in Figure 1. Rats received Lente insulin once every 24 hr until one day prior (Day 0) to EB injections
(Day 1), when they instead were maintained on short-acting Regular insulin (duration of action approximately 6 hr). Subcutaneous injections of .75 U Regular insulin were given at 6-hr intervals (0600, 1200, 1800 and 2400 on Day 0), so that a total of 3 U was given over a 24-hr period. On Day 1, all animals received EB at 0600 and control animals were administered Regular insulin at times and doses identical to Day 0. At 0600 on Day 2, all rats were injected with progesterone and approximately 3 U Lente insulin. (Behavioral testing took place at 1200-1300 on Day 2.) Insulin was withheld from the experimental groups for the first 24, 18 or 12 hr, the last 18 or 12 hr, or the middle 12 hr of the EB-conditioning period (Day 2). Each animal was tested under both conditions in a counterbalanced design (12 tests/animal). Behavioral tests were separated by one week. Differences between pairs of treatment were tested by the Wilcoxon matched-pairs, sign-ranks test.

Exchange assay for measurement of estrogen receptors in nuclei. Thirty-two ovariectomized, diabetic rats were randomly assigned to one of the two following groups. Sixteen animals had insulin withheld for 24 hr prior to serum glucose measurement; insulin was withdrawn for 12 hr prior to blood glucose determination for the other 16 rats. Within each group, animals were matched according to the level
Figure 1. Experimental paradigm for testing the effect of acute insulin withdrawal on sexual receptivity in ovariectomized, diabetic rats. Rats received 2 μg estradiol benzoate (EB) at 0600 (Day 1) and 1 mg progesterone at 0600 (Day 2). Rats were tested for sexual receptivity 6-7 hr after progesterone injection. Insulin injections were given daily every 6 hr (beginning at 0600) or were omitted at various times during the EB-conditioning period (refer to text for details). Open bars correspond to lights-on; dark bars refer to lights-out.
of hyperglycemia displayed after insulin withdrawal (such that eight animals per group served in the insulin withdrawal condition and eight per group served as controls).

Rats were maintained on insulin or had insulin withheld for 24 hr or the middle 12 hr of the EB-conditioning period. All animals received a 2 μg injection of EB, followed 24 hr later by injections of 1 mg progesterone and 3 U Lente insulin as in the behavioral experiments.

Rats were anesthetized with sodium pentobarbital (Nembutal) two hr after progesterone and insulin injections, and were perfused through the heart with cold, .9% saline. Hypothalamic and preoptic areas were dissected according to the method of Gray (1977) and pooled prior to homogenization. After homogenization, cell nuclei were isolated by the method of Zigmond and McEwen (1970). The levels of estrogen receptors in brain cell nuclei were determined using the exchange assay of Roy and McEwen (1978). Cell nuclear estrogen receptors were solubilized by first dispersing the nuclei in TBD buffer (10 mM Tris-HCl, 1 mM dithiothreitol, 0.5 mM bacitracin; pH 7.6) and then adding equal volume of TSDK.8 (TBD, 0.8 M KCl; pH 7.6). Samples were incubated at 0° C for 30 minutes and then centrifuged at 16,000 x g for 10 minutes. Aliquots of the supernatant were incubated with 5 nM (3H)estradiol (specific activity: 91.8 Ci/mmol; New England Nuclear) with or without 1 μM radioinert estradiol for 4 hr at 25° C. Bound and free
(3H)estradiol were separated by gel filtration on Sephadex LH-20 columns. Nuclear pellets were dissolved in .3 N KOH, and DNA contents were determined by the method of Burton (1956). Specific binding was calculated as total binding minus nonspecific binding. Results are presented as femtomoles bound/mg DNA. Student's $t$-test was used to determine statistical significance.

RESULTS

**Estrous behavior.** Figure 2 shows the effects of various durations of insulin withdrawal during EB-conditioning on sexual receptivity in ovariectomized, diabetic rats. The data are presented in the order by which testing occurred (over successive weeks) according to the period of insulin withdrawal. The decrease in sample sizes over the course of this experiment was due to the death of five rats (rats that appeared sick on the morning when EB was to be given were not included in the study); and two rats consistently rejected male mount attempts (one after week six of testing and the other after week eight) and thus could not be rated.

Withdrawal of exogenous insulin for 18-24 hr during the 24-hr estrogen-conditioning period resulted in significant reductions in mean lordosis ratings in ovariectomized, diabetic rats. Insulin withdrawal during this period resulted in a 30% decrease in mean lordosis ratings compared
to those of the insulin treated controls (p< 0.01 Wilcoxon matched-pairs, sign-ranks test). When insulin was withheld the first 18 or last 18 hr of the estrogen-conditioning period lordosis scores declined 19% (p< 0.01) and 22% (p< 0.02), respectively. Withdrawal of insulin during the first, last or middle 12 hr during conditioning had no significant effect on mean lordosis ratings. Because of the poor mounting performance of some of the male studs during testing in the middle 12-hr withdrawal condition a second separate set of tests was conducted, and the lordosis ratings for this condition (depicted in Figure 2) represent the mean of the combined scores obtained from both sets of tests (mean lordosis ratings did not differ significantly from controls).

No differences were observed in other estrous-related behaviors (i.e. darting and hopping, ear wiggling, rejecting and soliciting behaviors) during any period of insulin withdrawal compared to control conditions. In general, such behaviors were consistent for individual animals over testing conditions. Six females displayed high levels of rejecting behaviors to male mount attempts (greater than 10 rejections per test session) and the number of rejections exhibited appeared uninfluenced by insulin withdrawal (although mean lordosis ratings declined for these animals when insulin was withdrawn for 24 or 18 hr of the estrogen-conditioning period).
EFFECT OF ACUTE INSULIN WITHDRAWAL ON ESTROUS BEHAVIOR

Figure 2. Effect of acute insulin withdrawal on sexual receptivity in ovariectomized, diabetic rats. The duration of insulin withdrawal during the estradiol benzoate-conditioning period is indicated for each group. (See Figure 1 for experimental paradigm.) Control animals received insulin every 6 hr.

* p < 0.02; ** p < 0.01
All rats appeared healthy at the time of testing. Qualitative determination of urine glucose levels (TesTape) confirmed that insulin injections given 6 hr prior to testing were sufficient to prevent glycosuria at the time of testing.

**Brain cell nuclear estrogen receptor levels.** The effects of acute insulin withdrawal during the EB-conditioning period on cell nuclear estrogen receptor levels in pre-optic area-hypothalamus are presented in Figure 3. Withdrawal of insulin for the first 24 hr of the conditioning period resulted in a 38% reduction in the mean quantity of nuclear estrogen receptors (49.73 $\pm$ 4.43 fmoles/mg DNA with insulin versus 30.59 $\pm$ 3.23 fmoles/mg DNA without insulin; $t(14)=3.47, p<0.01$). When insulin was withdrawn during the middle 12 hr of the EB-conditioning period, mean nuclear receptor levels were lower for the insulin withdrawn group (43.23 $\pm$ 2.94 fmoles/mg DNA) than for the controls (53.02 $\pm$ 3.87 fmoles/mg DNA) but this reduction was not statistically significant. (Brain tissue samples of two insulin withdrawn and two control animals in the 12-hr withdrawal condition were accidently destroyed, reducing the sample size for this condition to six.)

**DISCUSSION**

The results of these time-course studies confirm and
Figure 3. Effect of acute insulin withdrawal on cell nuclear estradiol receptor levels in hypothalamus-preoptic area of ovariectomized, diabetic rats injected with 2 \( \mu \)g estradiol benzoate (EB) 26 hr prior to sacrifice (mean ± S.E.). Insulin was withdrawn for the first 24 hr or the middle 12 hr of the EB-conditioning period. Control animals were given an insulin injection every 6 hr. All rats received injections of progesterone and insulin 2 hr prior to sacrifice.

*** \( p < 0.004 \)
extend the findings of Gentry et al. (1977) who have shown that acute insulin withdrawal during the EB-conditioning period significantly impairs sexual receptivity and brain cell nuclear concentration of \((^3\text{H})\text{estradiol-17B}\) in diabetic, ovariectomized rats. The present data are in agreement with the hypothesis that some of the reproductive dysfunctions often manifest in diabetes mellitus are the result of altered cell nuclear estrogen binding. Furthermore, these data not only provide strong support for the concept that estradiol acts at the cell nucleus to initiate the lordosis reflex, but they also provide information concerning the temporal aspects of nuclear retention of estradiol in relation to female sexual receptivity.

Withdrawal of insulin for 18-24 hr during the EB-conditioning period significantly reduced mean lordosis ratings in diabetic female rats given insulin replacement six hr prior to testing. It is doubtful that this diminished sexual receptivity was the result of any overall debilitation caused by insulin withdrawal, since animals were not glycosuric and appeared fit at the time of testing. Similarly, Gentry et al. (1977) have reported that withdrawal of insulin six hr prior to testing was without significant effect on lordosis ratings, though animals were glycosuric at testing. That other estrous-related behaviors appeared undiminished by the prior lack of insulin, lends further evidence against the notion of
nonspecific illness effects. In addition, the finding that preceptive behaviors (see Beach, 1976) remained unchanged under these conditions is in agreement with the hypothesis that lordotic and soliciting behaviors may be controlled by separate neuroanatomical (and perhaps neurochemically separate) systems (Ward, Crowley, Zemlan & Margules, 1975). An alternative explanation of these data is that insulin withdrawal for 18-24 hr disrupted estrogen conditioning of the neural sites believed to be involved in the mediation of the lordosis response, thus resulting in an attenuation of sexual receptivity.

Interestingly, the results of the in vivo portion of this study suggest a mechanism for the altered action of estradiol on the neural target tissues involved in female rat sexual receptivity. It was found that a period of 24 hr insulin withdrawal, which led to a 30% reduction in mean lordosis scores, resulted in a 38% reduction in the quantity of nuclear estrogen receptors. Withdrawal of exogenous insulin for the middle 12-hr interval of EB-conditioning, however, had no significant effect on sexual receptivity or on the levels of estrogen receptors in the nuclear fraction of the preoptic-hypothalamic area. Thus, the diminished sexual behavior of diabetic rats following an 18-24 hr insulin omission period, was likely due to lowered levels of brain cell nuclear estrogen receptors.

Why then should insulin withdrawal for any 18-hr
period (or longer) during EB-conditioning affect lordosis scores, whereas a 12-hr withdrawal period during conditioning (a time interval during which the first and last 18-hr periods overlap) was ineffective in this respect? The answer to this question lies, perhaps, in the role played by the estrogen-receptor complex once it has entered the cell nucleus. In uterus, for example, it is well established that late uterotrophic responses are the product of long term nuclear retention of the estrogen-receptor complex (Clark, Anderson & Peck, 1973). Long term retention of (3H)estradiol has also been demonstrated in brain. Low levels of (3H)estradiol are found in hypothalamus, preoptic area and amygdala as long as 24 hr after injection of (3H)estradiol (McEwen, Pfaff, Chaptal & Luine, 1975). At present, the physiological significance of these low levels of nuclear binding are unknown, although, McEwen and colleagues (McEwen et al., 1975) argue that sexual receptivity is not dependent upon long term retention of estradiol by brain cell nuclei. Yet, synthetic antiestrogens that have been shown to block female rat sexual behavior also inhibit brain cell nuclear binding of (3H)estradiol in hypothalamus-preoptic area (Roy & Wade, 1977). A single injection of the antiestrogen CI-628, given six hr after estradiol injection, completely blocks lordosis in female rats; injection of CI-628, given up to
18 hr post-estradiol injection, partially suppresses this behavior (Whalen & Gorzalka, 1973). Although antiestrogenic compounds such as CI-628 also decrease whole homogenate uptake of \((^3\text{H})\)estradiol (Roy & Wade, 1977), work with actinomycin D, an inhibitor of RNA synthesis, supports the notion that the cell nucleus is the site of estrogen action for the induction of sexual receptivity. The observation that actinomycin D given 12 hr after estrogen treatment inhibits sexual behavior, suggests that long term nuclear retention of estradiol may be necessary in brain (as it is in uterus) for the manifestation of later estrogenic effects (Whalen, Gorzalka, DeBold, Quadagno, Ho & Hough, 1974). Hence, the present findings (that 24-hr insulin withdrawal impairs cell nuclear receptor levels while 12-hr insulin withdrawal is without effect, and that female sexual receptivity is reduced only after 18-24 hr omission of insulin) have important implications for hypotheses concerning the cellular site of estradiol action and the necessity of long term nuclear retention for the induction of lordosis in female rats. First, as previously mentioned, attenuation of receptivity was observed when nuclear receptor levels were diminished, thus supporting the hypothesis that the cell nucleus is the site of estradiol action for initiation of sexual receptivity. Second, the levels of nuclear estrogen receptors were significantly decreased
following 24-hr insulin withdrawal; after 12-hr insulin withdrawal, the quantity of receptors were lowered, although this reduction was not statistically significant (see Figure 3). This trend in the data leads me to infer that receptor levels were likely reduced in rats subject to 18 hr of insulin deprivation. Therefore, the fact that lordosis scores were reduced only after long periods of insulin omission, even when insulin withdrawal began six hr into the EB-conditioning period, lends credence to the view that the estrogen-receptor complex must be retained in the nucleus for a considerable length of time (greater than 6-12 hr) if sexual receptivity is to occur. Thus, in light of the above observations, these data are best explained in the following manner. When insulin was withheld during the middle 12 hr of EB-conditioning the (nonsignificant) reduction in receptor levels was insufficient to affect behavior. Insulin withdrawal during the first 18 hr of conditioning may have interfered with the initiation of early nuclear events that eventually result in lordosis behavior. Yet, when insulin was withdrawn during the final 18 hr of the conditioning period, the estrogen-receptor complexes likely were not retained in the nucleus for long enough to elicit the (secondary) nuclear events that are responsible for the full manifestation of sexual receptivity in streptozotocin-diabetic rats.

The mechanism by which insulin withdrawal affects
brain cell nuclear estrogen receptor levels in diabetic rats is unknown. It is known that the hypothalamus, particularly the ventromedial hypothalamus, contains insulin receptors (Debons, Krimsky & From, 1970; Storlien, Bellingham & Martin, 1975), and as early as 1937, overt changes in hypothalamic cells were noted in brains from untreated diabetic patients (Morgan, Vonderahe & Malone, 1937). Recently, insulin receptors have been found to be distributed throughout the central nervous system of the rat, especially in limbic structures, including anterior hypothalamus-preoptic area (Havrankova, Roth & Brownstein, 1978). In the hypothalamus, insulin affects glucose uptake (Debons et al., 1970) and oxidative activity (Schaffini, Marin & Foglia, 1970). However, it is doubtful that deficits in either of these functions can account for the observed decrements in nuclear estrogen receptor levels since rats received insulin prior to sacrifice, and translocation of the steroid-receptor complex is proposed to be by passive diffusion (Gorski & Gannon, 1976).

At present, no satisfactory explanation can be given for the present results, however, there are several pieces of data which deserve further consideration. First, recent studies have demonstrated the existence of specific insulin receptors on the nuclear envelope of rat liver nuclei (Vigneri, Goldfine, Wong, Smith & Pezzino, 1978). The function of these receptors is unknown, but perhaps they
play a role in the maintenance of membrane properties that are necessary for normal transport of molecules across the nuclear envelope. To date, no studies have investigated the presence of similar insulin nuclear binding sites in brain, but the discovery of these proteins in liver cells opens the possibility for their existence in neural cells and thus suggests a mechanism by which translocation of the estradiol-receptor complex is impaired under conditions of insulin withdrawal. Second, one well documented consequence of diabetes mellitus is the thickening of the capillary basement membrane. This phenomenon has been observed in all tissues studied except in adipose tissue (Bloodworth, Engerman & Davis, 1970). Bloodworth and colleagues (1970) suspect that this thickening is stimulated by elevated amounts of lipids, and propose that these lipids become trapped in the capillary wall, thus resulting in proliferation of the membrane. Again, no evidence exists as to the presence of an analogous thickening in the brain cell nuclear membrane. In the present study, the blood of many of the insulin withdrawn rats appeared lipemic, thus raising the possibility that translocation was disrupted due to altered nuclear membrane permeability that consequently resulted from this metabolic alteration caused by acute insulin withdrawal.

In conclusion, the results of these time-course studies support the concept that deficits in steroid binding by
neural tissues involved in reproductive functions may contribute to the reproductive failures of diabetics. In the diabetic rat, decreased sexual receptivity appears to be the result of lowered brain cell nuclear estrogen receptors. Further work is needed to shed light on the mechanism by which neural binding of estradiol is decreased in the streptozotocin-diabetic rat.
REFERENCES


