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The Effect of Chronic Estrogen Treatment on Immunoreactive β-Endorphin Levels in Intact Female Rats[1] (42110)

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Abstract. Intact female rats were treated chronically with estradiol benzoate (EB) until a state of constant estrus (CE) was achieved and maintained. When compared to female rats on the day of estrus, estrogen-treated rats in constant estrus demonstrated a 33% decrease in the concentration of immunoreactive β-endorphin (IR-BE) in the plasma, and a 45–50% decrease in the content and concentration of IR-BE in the anterior pituitary and hypothalamus. The content and concentration of IR-BE in the neurointermediate lobe of the pituitary were similar in each group. Column chromatography revealed that the reduction in IR-BE in the plasma and anterior pituitary of EB-treated CE female rats appeared to be due to a reduction in peptides coeluting with β-endorphin and β-lipotropin, whereas the reduction in IR-BE in the hypothalamus represented a decrease in a peptide which coeluted with β-endorphin. These data suggest that constant estrus, induced by prolonged treatment of intact female rats with estrogen, resulted in a reduction in central and peripheral levels of IR-BE in these animals as compared to female rats on the day of estrus.

β-Endorphin (BE) is an endogenous opioid peptide synthesized and released by the anterior pituitary (AP), neurointermediate lobe of the pituitary (NIL) (1), and the arcuate nucleus of the hypothalamus (2). Previous reports indicate that BE levels in the pituitary and plasma are mediated by hypothalamic neurotransmitters such as norepinephrine (3, 4), dopamine (5–7), and serotonin (8–10), as well as, hypothalamic neuropeptides such as corticotropin releasing factor (11–13). Recent evidence suggests that estrogen may also mediate the synthesis and release of BE from the pituitary and hypothalamus.

The effects of estrogen on central and peripheral levels of BE are controversial. Acute castration of female rats was reported to produce an increase on BE levels in the plasma and NIL without affecting BE levels in the AP (14). Estrogen replacement diminished BE levels in the plasma and NIL and also produced a reduction in BE levels in the AP. In contrast, chronic castration of female rats was observed to decrease BE levels in the plasma, AP, and NIL (15). Treatment of chronically castrated animals with estrogen only partially reversed the effect of castration on BE levels in the plasma and NIL, while completely reversing the castration-induced decrease in BE in the AP. Hypothalamic BE in chronically castrated female rats remained unchanged (15–17). However, in response to estrogen, hypothalamic BE was reported to increase (15), and to decrease (17). These differences in BE levels in response to castration and subsequent estrogen replacement may be attributable to the length of time the animals were castrated or the duration of estrogen replacement.

In order to avoid some of the problems involved in using a paradigm involving castration and estrogen replacement, a model was used in the present study in which intact female rats were treated chronically with estrogen until a state of constant estrus (CE) was achieved and maintained. Upon sacrifice, immunoreactive β-endorphin (IR-BE) levels in the plasma, AP, NIL, and hypothalamus were measured and compared to IR-BE levels in intact female rats on the day of estrus.

Material and Methods. *Animals.* Female Sprague–Dawley rats (225–275 g) were obtained from Harlan Industries (Indianapolis, Ind.). Animals were maintained in a temperature controlled room (22 ± 2°C) and kept

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on a 14:10 light/dark cycle (lights on at 0500 hr). Food (Ziegler Diet, Buckshire Farms, Landsdale, Pa.) and water were available *ad libitum*.

Assessment of reproductive status. Vaginal smears were made daily for 4 weeks. Animals demonstrating 4- to 5-day vaginal estrous cycles were used in the present study. Ten cycling female rats were injected with estradiol benzoate (EB; Sigma Chemical Co., St. Louis, Mo.) at a dose of 5 μg/rat/day (in 200 μl of corn oil). Additionally, 10 cycling female rats were injected with vehicle (corn oil) and served as controls. All animals were injected and smeared daily for a period of 8 weeks. Animals demonstrating persistent vaginal cornification were categorized as being in constant estrus. Constant estrous female rats were sacrificed with a matching control rat that was in estrus. All animals appeared to be free of pituitary tumors at the time of sacrifice.

Collection of plasma. Plasma was obtained by decapitation and exsanguination between 1000 and 1200 hr. Trunk blood was collected into heparinized 30 × 105-mm plastic centrifuge tubes to which 150 μl of 200 IU heparin (Sigma Chemical Co., St. Louis, Mo.) in sterile saline was added. The blood was centrifuged at 1000*g* for 10 min at 4°C. The resulting plasma was removed and frozen at −20°C until assayed for immunoreactive β-endorphin.

Collection of pituitaries. Pituitaries were removed and separated into the anterior pituitary (AP) and neurointermediate lobe of the pituitary (NIL). Each pituitary portion was placed in a 12 × 75-mm polystyrene test tube, to which 2 ml of phosphate buffer (0.02 *M* $NaPO_4$, 0.15 *M* NaCl, 10 m*M* EDTA, and 0.01% sodium azide, pH 7.5) containing 1 m*M* *N*-ethylmaleimide had been added. Pituitary portions were then homogenized and frozen at −20°C. Pituitary protein content was measured by the technique of Lowry *et al.* (18).

Collection of hypothalami. At the time of decapitation, the whole brain was removed and quickly frozen on dry ice. Each brain was then partially thawed and the hypothalamus dissected. Cuts were made 3 mm anterior to the optic chiasm and rostral to the mammillary bodies. Sections were bordered laterally by the lateral hypothalamic sulci and dorsally by a cut at the top of the third ventricle. The sections included the organuum vasculosum of the laminae terminalis, the preoptic area, the supraoptic nucleus, the suprachiasmatic nucleus, the anterior hypothalamic area, the median eminence, the infundibular stalk, the arcuate nucleus, the ventromedial nucleus and the dorsomedial nucleus. Tissues were homogenized in buffer containing 1 m*M* *N*-ethylmaleimide and stored in 12 × 75-mm polystyrene tubes at −20°C. Hypothalamic protein content was measured by the technique of Lowry *et al.* (18).

Radioimmunoassay of β-endorphin. The radioimmunoassay for immunoreactive β-endorphin (IR-BE) has been described in detail elsewhere, along with its suitability to measure IR-BE in the rat (19). In our hands, the antibody to β-endorphin (generously supplied by Dr. S. S. C. Yen, University of California, San Diego) demonstrated a complete cross-reactivity with β-lipotropin on a molar basis. Increasing volumes of sample (plasma, and pituitary and hypothalamic lysates) were found to be parallel to the standard curve for camel (ovine, bovine, rat) β-endorphin (Peninsula Laboratories, San Carlos, Calif.). The intra- and interassay coefficients of variation were 8 and 13%, respectively. All samples of a given type (i.e., plasma, pituitary and hypothalamic lysates, or column eluates) were assayed together.

Gel chromatography. Samples were chromatographed in a 1.6 × 70-cm glass column packed with Sephadex G-50 medium. Three milliliters of either pooled plasma, or pituitary or hypothalamic lysate (diluted in assay buffer), were applied to the column. The column was eluted with 0.1 *N* acetic acid, 0.05% bovine serum albumen, and 0.02% sodium azide. Fractions of 1.5 ml were collected. Elution profiles were obtained by determining the immunoreactivity of each fraction using the β-endorphin radioimmunoassay, which recognized β-endorphin and β-lipotropin equally. The elution profiles of the samples were compared to those obtained for either camel (ovine, bovine, rat) β-endorphin or human β-lipotropin (generously provided by the National Pituitary Agency, NIAMDD). Immunoreactivity producing an

elution profile similar to that of camel β-endorphin was considered to be β-endorphin. Similarly, immunoreactivity producing an elution profile similar to that of β-lipotropin was considered to be β-lipotropin.

Statistical analysis. Statistical analyses of the data were performed using Student's *t* analysis. A significance level of $P < 0.05$ was chosen.

Results. *Induction of constant estrus in cycling female rats.* All of the animals receiving EB demonstrated vaginal smears consisting of nucleated and cornified epithelia by the end of the second week of treatment. Following the fourth week, 50% of the EB-treated rats were considered to be in constant estrus, with only cornified epithelium appearing in the vaginal smears. During the seventh and eighth weeks, all animals being treated with EB appeared to be in constant estrus. Throughout the 8-week period, all of the animals receiving vehicle continued to demonstrate 4- to 5-day vaginal estrous cycles.

Plasma, pituitary, and hypothalamic IR-BE. Plasma levels of IR-BE were reduced by 33% in EB-treated CE female rats as compared to female rats on the day of estrus. Also reduced were the content and concentration of IR-BE in the AP (48 and 45%, respectively), and the content and concentration of IR-BE in the hypothalamus (49 and 50%, respectively) (Table I). The reductions in plasma, AP, and hypothalamic IR-BE levels in EB-treated CE female rats versus female rats on the day of estrus, were statistically significant ($P < 0.05$).

Chromatographic analysis. Column chromatography revealed that the decrease in IR-BE in the plasma (Fig. 1) and the AP (Fig. 2) of EB-treated CE female rats was represented by a decrease in both β-endorphin and β-lipotropin. β-Endorphin represented 69 and 71% of the IR-BE in the plasma of the treated and the control rats, respectively. In the AP, β-endorphin was 71% of the total IR-BE in the CE female rats and 64% of the total IR-BE in the control female rats on the day of estrus. The decrease in IR-BE in the hypothalamus was represented completely by β-endorphin (data not shown).

Weights of reproductive organs. As an added verification of the effect of the treatment with EB, the uteri and ovaries from each animal were dissected and weighed at the time of sacrifice. When expressed on a milligram organ per gram body weight basis, the uteri from the animals receiving EB were of significantly greater weight ($P < 0.05$) than the uteri from animals on the day of estrus. Conversely, ovarian weight was reduced significantly ($P < 0.05$) in the EB-treated as compared to the nontreated animals (Table II). There was no significant difference in body weights between the two groups of animals.

Discussion. The findings of the present study indicate that the chronic treatment of intact female rats with estrogen until a state

TABLE I. PLASMA, PITUITARY, AND HYPOTHALAMIC IR-BE LEVEL IN EB-TREATED INTACT FEMALE RATS IN CONSTANT ESTRUS AND INTACT FEMALE RATS ON THE DAY OF ESTRUS

	n	Day of estrus[a]	Constant estrus[a]
Plasma (ng/ml)	10	1.10 ± 0.10	0.74 ± 0.08[b]
AP			
Content (μg/gland)	10	3.52 ± 0.63	1.83 ± 0.22[b]
Concentration (μg/mg protein)	10	0.15 ± 0.025	0.083 ± 0.013[b]
NIL			
Content (μg/gland)	10	22.50 ± 2.97	23.16 ± 3.17
Concentration (μg/mg protein)	10	10.32 ± 1.35	9.74 ± 1.29
Hypothalamus			
Content (ng/section)	10	39.13 ± 2.47	20.00 ± 1.09[b]
Concentration (ng/mg protein)	10	0.525 ± 0.036	0.262 ± 0.028[b]

[a] Each value represents the mean ± standard error of 10 determinations.
[b] Significantly different ($P < 0.05$) than female rats on the day of estrus.

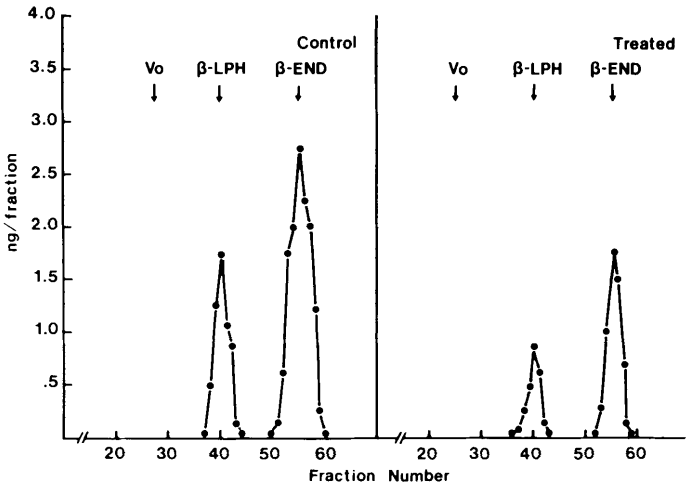

FIG. 1. Chromatogram of the plasma from control female rats on the day of estrus (left) and female rats treated chronically with estradiol benzoate until a state of constant estrus was reached and maintained (right). V_0, void volume; β-LPH, the position in which human β-lipotropin eluted; β-END, the position in which camel β-endorphin eluted.

of constant estrus was reached resulted in a significant reduction in IR-BE in levels in the plasma, AP, and hypothalmus when compared to cycling female rats on the day of estrus. IR-BE levels in the NIL were unaffected. Column chromatography indicated that the reduction in IR-BE in the plasma, AP, and hypothalamus of EB-treated CE female rats represented a decrease in a peptide which coeluted with β-endorphin.

In order to study the mediation by estrogen of hypothalamic, pituitary, and plasma levels

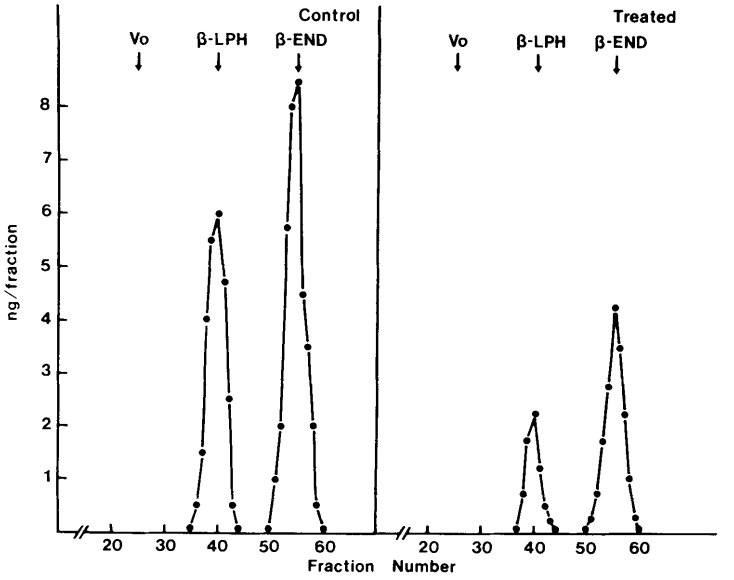

FIG. 2. Chromatogram of the AP from control female rats on the day of estrus (left) and female rats treated chronically with estradiol benzoate until a state of constant estrus was reached and maintained (right). V_0, void volume; β-LPH, the position in which human β-lipotropin eluted; β-END, the position in which camel β-endorphin eluted.

TABLE II. REPRODUCTIVE ORGAN WEIGHTS IN EB-TREATED INTACT FEMALE RATS IN CONSTANT ESTRUS AND INTACT FEMALE RATS ON THE DAY OF ESTRUS

	n	Day of estrus[a]	Constant estrus[a]
Uteri (mg/g body wt)	10	197 ± 16	284 ± 14[b]
Ovaries (mg/g body wt)	10	29 ± 2	13 ± 2[b]
Body weight (g)	10	273 ± 6	262 ± 4

[a] Each value represents the mean ± standard error of 10 determinations.

[b] Significantly different ($P < 0.05$) than female rats of the day of estrus.

of BE, other laboratories have utilized a model which involved the castration of female rats and subsequent replacement of estrogen (14, 15, 17). In these studies comparisons were made among castrated female rats, castrated female rats treated with estrogen and sham-operated control female rats in either all stages of the estrous cycle (14) or all stages of the estrous cycle except estrus (15). The results of these investigations have been contradictory. It appears that the data obtained were dependent on the length of time the animals were castrated, the duration of estrogen replacement prior to sacrifice, and possibly the control group used for comparisons. For these reasons, the present study utilized a model in which the estrogen mediation of BE levels was assessed by making comparisons between intact animals treated with estrogen for a prolonged period of time, and intact female rats on the day of estrus. It is believed that the model used in the present study is relevant physiologically, since it compares female rats on the day of estrus, which follows the natural exposure to estrogen as part of the 4-day estrous cycle, to animals that are in constant estrus due to a chronic exposure to estrogen. In addition, constant estrus is also a naturally occurring phenomenon observed in aging female rats in which circulating levels of estrogen are chronically elevated (20).

It has been reported that female rats castrated for 6 days demonstrated an increase in BE levels in the plasma, whereas castration and estrogen treatment for 6 days resulted in plasma levels of β-endorphin which were not different from those observed in intact control animals which were sacrificed randomly during the estrous cycle (14). By contrast, in comparison to a control group of intact female rats that were in pro-, met-, and diestrus, BE levels in the plasma were reduced 3 weeks after castration, and were only partially restored to control levels by administration of estrogen for 2 weeks (15). In the present study, treatment of intact female rats for a period of 8 weeks resulted in BE levels in the plasma which were significantly lower than those observed in intact female rats on the day of estrus. These findings are in agreement with those of Mueller (21), who observed that treatment of intact male rats with estrogen also resulted in a decrease in BE in the plasma. The decrease in plasma BE in rats treated chronically with estrogen may result from a decreased release of BE from the AP. This hypothesis is supported by the observation that BE levels in the AP were also reduced in estrogen-treated rats.

Noradrenergic agents have been demonstrated to increase plasma levels of IR-BE when administered peripherally (4, 22) and to stimulate directly the release of IR-BE from the AP *in vitro* (3). It has been reported that gonadal steroids reduce the synthesis of norepinephrine in the hypothalamus (24, 25). It is possible that chronic treatment of intact female rats with estrogen may chronically deplete hypothalamic norepinephrine thereby decreasing any stimulation of BE release from the pituitary which may be produced by the noradrenergic system. Such a reduction in the noradrenergic activity might contribute to the decrease in BE levels in the plasma of EB-treated CE female rats, observed in the present study.

Short-term castration and estrogen replacement (6 days) (14) and long-term castration (3 weeks) and estrogen replacement (2 weeks) (15) resulted in BE levels in the AP similar to those observed in intact sham-operated control rats. By contrast, chronic treatment (8 weeks) of intact animals with estrogen resulted in a significant reduction in AP BE levels. Such observations suggest that a suppressive effect of estrogen on AP levels of BE may only be observed when estrogen is administered to intact animals for a period greater than 2 weeks. The exact nature of the role played by the *in situ* ovary in mediating

how exogenously administered estrogen influences BE levels in the AP remains to be determined. However, it may be necessary to consider the effect of ovarian progesterone on estrogen-induced alterations in BE levels in the AP. It has already been reported that progesterone may influence BE turnover in the brain (17, 23).

Chronic treatment with estrogen for 8 weeks did not result in an alteration in BE levels in the NIL of intact female rats. By contrast, removal of the ovaries for 6 days resulted in an increase in BE levels in the NIL which could be reversed by estrogen replacement (14), whereas animals castrated for 3 weeks demonstrated a reduction in BE levels in the NIL which was only partially reversed by treatment with estrogen (15). These findings suggest that in intact female rats, the NIL is either unaffected by the chronic presence of estrogen or that the effects of estrogen on the NIL are transient, and are no longer detectable by 8 weeks of treatment. It is also possible that the effects of estrogen on the NIL are not observed until the ovaries have been removed. Changes in BE levels in the NIL also appear to be dependent on the length of time the animals were castrated and the duration of estrogen replacement.

It has been reported that castration does not influence levels of BE in the hypothalamus, but that treatment with estrogen will either increase (15), or decrease (17) hypothalamic BE. The findings of the present investigation are in agreement with those of Wardlaw *et al.* (17) who also found that treatment with estrogen resulted in a decrease in BE levels in the hypothalamus. Such a decrease in hypothalamic levels of a hormone are often difficult to interpret since it may reflect a decrease in BE synthesis or an increase in BE turnover.

Column chromatography demonstrated that the decrease in IR-BE in the AP and the plasma of the EB-treated CE female rats represented a decrease in peptides which coeluted with β-endorphin and β-lipotropin. These findings suggest that long-term treatment of intact female rats with a gonadal steroid results in a reduction in the synthesis and release of these peptides by the AP. Adrenal steroids appear to have a similar effect on the synthesis and release of β-endorphin and β-lipotropin by the pituitary. Administration of the synthetic corticoid, dexamethasone, was found to decrease AP levels of mRNA for the proopiomelanocortin prohormone (26, 27), which is enzymatically processed to form β-lipotropin and β-endorphin (1). Dexamethasone treatment also decreased immunoreactive β-endorphin in the AP (28), and the release of β-endorphin and β-lipotropin from the AP *in vivo* (29) and *in vitro* (1). Thus it seems that gonadal steroids, as well as adrenal steroids, may inhibit the synthesis and release of β-endorphin from the AP.

In light of reports that estrogen increased pituitary and plasma levels of BE in castrated female rats [(15); Forman, unpublished observation], it is possible that pituitary and plasma levels of BE would be increased in aged CE female rats, in which plasma levels of estrogen are chronically elevated (20). It has been shown that chronic treatment of prepubertal rats with estradiol benzoate hastens the onset of the constant estrous state as these female rats age (30). It was observed that in old CE female rats, IR-BE in the plasma and the NIL was significantly elevated as compared to young female rats of the day of estrus, and was not diminished by chronic castration. It was concluded that increased circulating levels of estrogen most likely did not contribute to the increase in plasma and NIL IR-BE levels in old CE female rats (31). The present observations would support that conclusion since estrogen-induced constant estrus resulted in a decrease in IR-BE levels.

The effect of chronic treatment with estrogen was evidenced by the occurrence of a sustained state of constant vaginal estrus in all of the treated rats. This is in agreement with the earlier report of Kawashima (32), who observed that chronic treatment of intact female rats with estrogen resulted in persistant vaginal estrus. In addition, upon sacrifice, the weight of the ovaries of the treated animals was significantly diminished, whereas the weight of the uterus was significantly increased, when compared to control female rats on the day of estrus. Both of these observations are typical of the effects of the chronic presence of estrogen on reproductive organ weight (32, 33).

In summary, chronic administration of estrogen to intact female rats which produced a state of constant estrus, resulted in a decrease in β-endorphin in the plasma, AP, and hypothalamus. These results suggest that estrogen mediates central and peripheral BE levels, and that the effects of estrogen may differ in intact as compared to castrated female rats.

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