The Effect of Neonatal Exposure to Diethylstilbestrol, Coumestrol, and β-Sitosterol on Pituitary Responsiveness and Sexually Dimorphic Nucleus Volume in the Castrated Adult Rat (43834)

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Abstract. **The neonatal hormone enviroment influences the sexually differentiated patterns of development. Estrogens, derived from intracerebral aromatization, promote male pattern development of the central nervous system. The purpose of this study was to determine the effects of neonatal exposure to environmental estrogens on luteinizing hormone (LH) secretion and development of the sexually dimorphic nucleus of the medial preoptic area (SDN-POA) in castrated adult rats. Neonatal rats of both sexes received injections of either corn oil, 0.1 μg diethylstilbestrol (DES), 3 μg β-sitosterol (B1), 30 μg β-sitosterol (B2), 0.1 μg coumestrol (C1), 1 μg coumestrol (C2), or 10 μg coumestrol (C3) on Day 1–10 of life and were castrated on Day 21. Right heart catheters were placed on Day 42, and GnRH (50 ng/kg) was administered. Blood was sampled for LH at 0-, 5-, 10-, 15-, and 30-min intervals. All doses of β-sitosterol and coumestrol elicited increased basal levels of LH in females. In males, B1, B2, C2, and C3 increased basal levels of LH. The GnRH-induced LH increase was prevented in females treated with diethylstilbestrol and 10 μg of coumestrol. Males in all treatment groups exhibited GnRH-induced LH surges. The animals were sacrificed by decapitation on Day 49. Volumes of the SDN-POA of the groups were compared. Treatment with the agents did not result in significantly increased SDN volume in females; nor was there a difference in SDN size among the male groups. These data show that exposure to environmental estrogens early in development alters both postpubertal pituitary response to GnRH and basal LH secretion in females and alters only basal LH secretion in males. No significant enlargement (i.e., masculinization) of the SDN-POA was exhibited.** [P.S.E.B.M. 1995, Vol 208]

The exposure of the central nervous system (CNS) to sex steroid hormones during development influences the formation of a sexually dimorphic brain. Males and females show developmental differences in neuroanatomy, changes in reproductive physiology, and changes in sexual behavior. A well-studied sex difference is the differential release of gonadotropins from the anterior pituitary. Males have a tonic release pattern while females exhibit cyclical release (1). Males also exhibit structural CNS differences that seem to impact male typical behavior. The sexually dimorphic nucleus (SDN) is enlarged in the male and seems to have a role in promoting male mating activities (2). Lesions of this area in adult male gerbils interfere with open field scent marking and are associated with decreased mating behavior (3). The SNB, a nucleus in the caudal spinal cord which innervates the muscle responsible for penile reflexes, is also significantly enlarged in the male (4). In rats, each of these sexually dimorphic characteristics differentiate

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0037-9727/95/2081–0072$10.50/0

during the critical period, lasting from the final third of gestation through the first 10 days of life. These changes in the CNS, determined during development, influence patterns of behavior seen in adult animals.

The default pattern for development is female. Masculinization is accomplished by the male sex hormone testosterone. Exposure to testosterone affects both physiology and CNS structure. In the brain, testosterone is aromatized to estradiol, which acts locally to promote male pattern development. Females are apparently protected from estrogenic effects in the CNS by α-fetoprotein binding of endogenous steroidal estrogens. However, females may be masculinized by exposure to testosterone, which can cross the blood-brain barrier and undergo aromatization, or to forms of estrogen which do not bind to α-fetoprotein.

Phoenix *et al.* explored the organizational effects of the neonatal hormonal milieu on masculine and feminine behaviors exhibited by adult guinea pigs. Their study showed that administration of testosterone during the critical period of development could permanently masculinize the behavior of females as measured by lordosis and mounting frequencies (5). Later work by Thornton *et al.* showed that prenatal administration of flutamide, an antiandrogen, increased the lordosis response of females, indicating that they are at least partly defeminized *in utero* by testosterone from their male siblings (6).

The early hormonal environment is not entirely determined by endogenous sources of estrogens. Developing animals may also encounter exogenous estrogens from plant, fungal, and xenobiotic sources. Exposure to DES (a synthetic estrogen), genistein (a plant estrogen), and zearalenone (a fungal estrogen) has been shown to enhance masculine patterns of CNS development (7, 8). Our previous studies have shown that females exposed neonatally to selected doses of DES, genistein, and zearalenone showed significantly decreased pituitary responsiveness to GnRH and increased SDN volume (7). Males exposed similarly showed increased pituitary responsiveness to low-dose genistein and no difference in SDN size among groups (7, 8). Essentially, treatment with estrogenic substances suppresses GnRH-induced LH secretion in both males and females, but females show more sensitivity regarding changes in SDN volume. Since pituitary responsiveness to GnRH presumably reflects hypothalamic activity and pituitary status, "masculinization" of the hypothalamus and/or pituitary manifests as decreased responsiveness to GnRH.

The current study examines the effect of neonatal exposure to other exogenous estrogens on development of the hypothalamus and pituitary. The animals were treated with various doses of DES, coumestrol (a coumestan phytoestrogen), and β-sitosterol (a sterol phytoestrogen). We hypothesized that these estrogenic compounds would masculinize the CNS and decrease pituitary responsiveness to GnRH. Furthermore, as previous studies have shown that the volume of the SDN of the medial preoptic area (SDN-POA) may be used as an anatomic marker of CNS masculinization in rats exposed neonatally to environmental estrogens (7, 8), we measured the SDN-POA volume in all animals.

Materials and Methods

Pregnant rats of the Charles River CD strain were purchased at Day 15–17 of gestation and maintained in air-conditioned quarters with Purina Laboratory Chow (Ralston-Purina, St. Louis, MO), water available *ad libitum,* and a 14:10-hr light:dark cycle, with lights on 0500–1900 hr EST.

Day of delivery was defined as Day 1 if delivery was observed to occur before 1200 hr. Pups were treated from Day 1–10 with 0.05-ml sc injections of one of the following compounds: V1, corn oil; D1, DES (0.1 μg in corn oil); C1, coumestrol (0.1 μg in corn oil); C2, coumestrol (1 μg in corn oil); C3, coumestrol (10 μg in corn oil); B1, β-sitosterol (3 μg in corn oil); and B2, β-sitosterol (30 μg in corn oil). All pups within the same litter received the same treatment.

On Day 21 of life, the animals were castrated under ketamine anesthesia (100 mg/kg) and weaned. On Day 42, right heart catheterization under ketamine anesthesia (100 mg/kg) was performed. To control for nonspecific effects of cannulation and fluid injection, the animals were randomized to receive either saline alone (1 ml/kg) iv or GnRH dissolved in saline (50 ng/kg; 1 ml/kg) iv 4 hr after cannulation. Blood samples (0.3 ml) were collected via the catheter immediately before (time = 0 min; $t = 0$) and 5, 10, 15, and 30 min after injection of GnRH or saline. Blood volume was replaced with an equal volume of 10 U/ml heparinized saline each time. Fifteen minutes following the last blood collection, the sampling procedure was repeated with saline injection in those animals that received GnRH or with GnRH injection in those that received saline.

Blood samples were allowed to clot at room temperature and were then centrifuged at approximately 1500*g* for 10 min. Sera were then aspirated and frozen at −20°C for later radioimmunoassay (RIA).

Serum LH concentrations were measured by double-antibody RIA with a rat LH RIA kit supplied by NIDDKD and the National Hormone and Pituitary Program (University of Maryland School of Medicine, Baltimore, MD). Second antibody (sheep, antirabbit) was graciously supplied by Dr. L. Tyrey, Duke University Medical Center, Durham, NC. Aliquots of se-

rum (50 μl or less) were assayed in duplicate and the means expressed in terms of NIDDKD-rLH-RP-3.

Intra- and interassay coefficients of variation for the measurement of LH in three serum pools were 1.8% and 8.2%, respectively. The assay sensitivity was 0.48 ng/ml of serum, and values falling below that level were taken to be 0.48, their maximum possible value.

On Day 49, the animals were decapitated, and the brains were promptly removed from the heads and blocked in the DeGroot plane (9). A block including the optic chiasm and hypothalamus was placed in formalin for a minimum of 2 weeks. The brains were placed in 30% sucrose-formalin solution for 48–72 hr, prior to frozen sectioning. Sections were taken at 20-μm thickness and stained with cresyl violet acetate. The slides were coded so that the microscopist did not know their identity, and the SDN-POA as described by Gorski (10), with confirmation of surrounding structures as illustrated by Pelligrino *et al.* (11) was identified. The area of SDN-POA in each section was measured using a computer-assisted image analysis system (Olympus Corporation, Lake Success, New York). This analysis was performed under 40× magnification with the computer calibrated to measure area in square microns. Volume was calculated by adding the area × thickness of each section. Only the left-sided SDN was compared among the groups.

LH concentrations were compared among the groups by one-way analysis of variance and Student's t test for normally distributed data. LH values falling below the assay sensitivity were compared using the Kruskal-Wallis one-way analysis of variance and the Wilcoxon sign-rank test. Volumes of the SDN-POA from the several treatment groups were analyzed by one-way analysis of variance. In all statistical procedures, analysis was conducted with the Statgraphics statistical software (STSC, Inc., Rockville, MD).

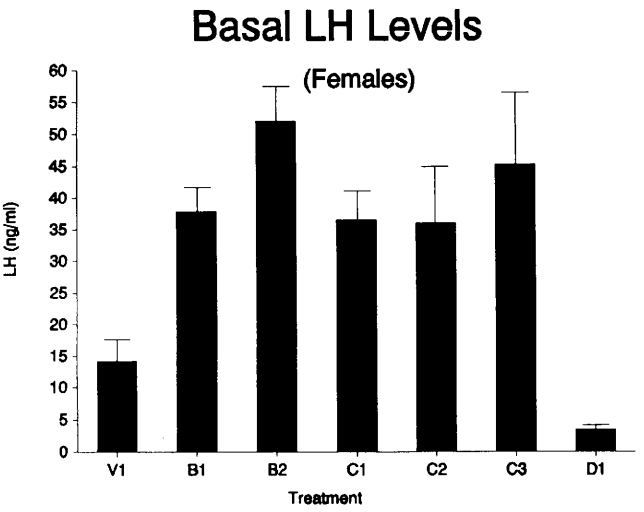

Figure 1. Serum LH (ng/ml) in castrated adult female rats exposed on Day 1–10 to corn oil (n = 11), B1 (n = 5), B2 (n = 14), C1 (n = 11, C2 (n = 8), C3 (n = 4), or D1 (n = 8).

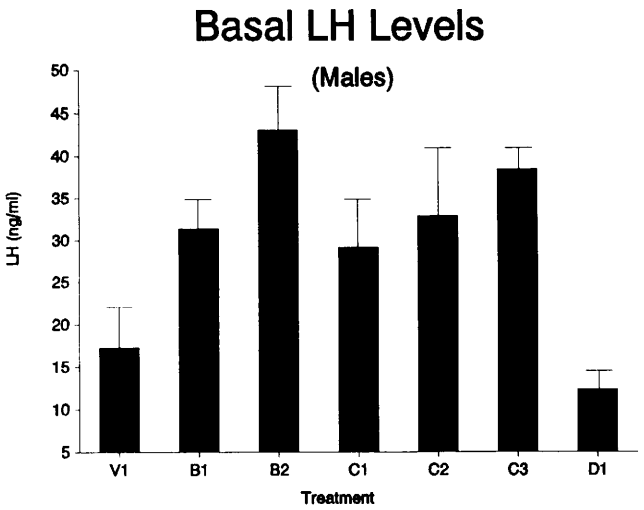

Figure 2. Serum LH (ng/ml) in castrated adult male rats exposed on Day 1–10 to corn oil (n = 11), B1 (n = 13), B2 (n = 7), C1 (n = 8), C2 (n = 7), C3 (n = 7), or D1 (n = 6).

Results

Basal LH Levels. All doses of coumestrol and all doses of β-sitosterol significantly increased basal LH levels in females (Fig. 1). Treatment with DES significantly decreased basal LH.

In males, all doses of β-sitosterol also increased basal LH levels (Fig. 2). However, only the medium (C2) and the high (C3) doses of coumestrol significantly increased basal LH levels. DES did not significantly affect basal LH levels in males.

GnRH-Stimulated LH Release in Females. The effect of neonatal exposure to DES, B1, B2, C1, C2, C3, and corn oil is shown in Figure 3. GnRH induced a significant LH increase in all treatment groups except DES and C3. Animals treated with B1 had a greater initial response to GnRH, represented by the change in LH from 0–5 min (LH2-LH1), than corn oil-treated litters.

GnRH-Stimulated LH Release in Males. The effect of neonatal exposure to DES, B1, B2, C1, C2, C3, and corn oil is shown in Figure 4. GnRH caused a significant LH increase in all treatment groups. The net rise in LH from 0–5 min was not significantly different among treatment groups.

SDN-POA Volume in Males and Females. The volume of the SDN-POA in control and estrogen-treated animals is presented in Table I and II. In control groups, as previous studies have shown, castrated adult males had approximately 2-fold higher SDN volumes than castrated adult females. In females, treatment with the agents did not result in significantly increased SDN volume when compared with the control

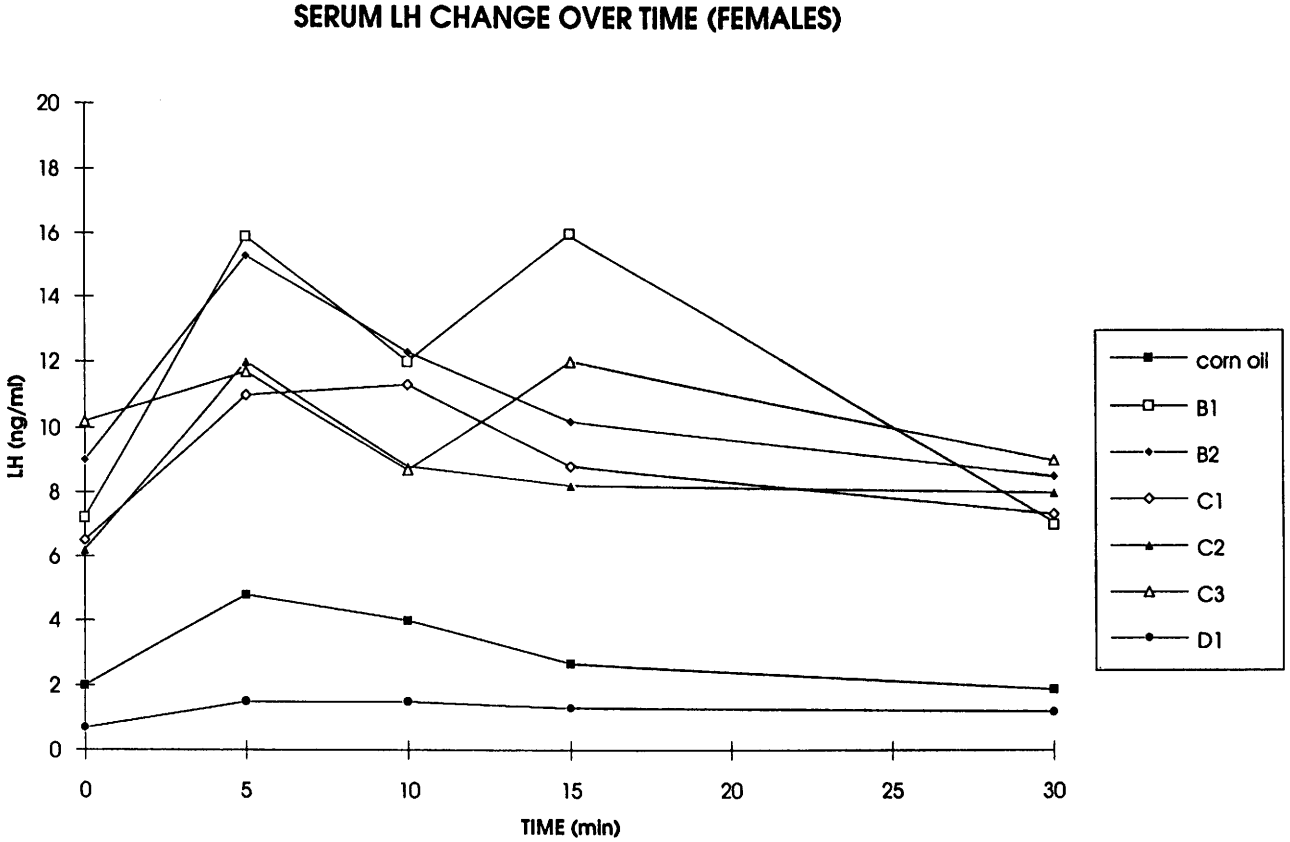

Figure 3. Serum LH (ng/ml) of castrated adult female rats exposed on Day 1–10 to corn oil (n = 11), B1 (n = 5), B2 (n = 14), C1 (n = 11), C2 (n = 8), C3 (n = 4), or D1 (n = 8), and given GnRH iv at t = 0.

group. Volume of the SDN in the C3 group, however, was greater than that of the C1 or C2 group ($P < 0.05$). Treatment of male pups had no significant effect on SDN-POA volume.

Discussion

The presence of specific gonadal steroids during critical periods of development directly influences the sexual differentation of the CNS. Although neonatal exposure to environmental estrogens is known to alter normal patterns of sexual differentation, the specific effects of these compounds on CNS anatomy and neuroendocrine function are largely unknown. The present experiment studied the dose-response relationship of a pharmaceutical estrogen (DES), a coumestan phytoestrogen (coumestrol), and a sterol phytoestrogen (β-sitosterol) on the responsiveness of the pituitary to GnRH challenge. In an effort to characterize the actions of β-sitosterol and coumestrol, several dosages were employed. The effects of DES at the 0.1-μg dose has previously been characterized in our laboratory (7), and was used as a positive control.

β-Sitosterol affected basal levels of LH secretion in males and females. All doses of β-sitosterol increased tonic LH secretion in both males and females. β-Sitosterol is a plant sterol structurally similar to cholesterol. Dietary intake of β-sitosterol affects the metabolism of cholestrol in the liver (12). β-Sitosterol may compete with cholestrol and interfere with the synthesis of gonadal steroidal hormones. Because cholesterol is the precursor for steroidal hormones in males and females, it is not surprising that treatment with β-sitosterol affects both groups similarly. Thus, β-sitosterol may create a neonatal enviroment with low endogenous levels of estrogens. Biphasic actions of estrogens are known to occur in adults in certain physiological circumstances. Low doses sensitize the pituitary and hypothalamus, causing enhancement of LH release, while high doses decrease pituitary sensitivity to GnRH (13, 14, 15). Thus, in the present experiments, the enhanced basal levels of LH in groups treated with β-sitosterol may be explained by the sensitization of the pituitary and hypothalamus to GnRH due to low level neonatal exposure to estrogens.

C1 enhanced basal LH secretion only in females. C2 and C3 enhanced basal LH secretion in both males and females. The disparity between males and females in their reaction to coumestrol can be explained by the current understanding of the compound's mechanism of action. Coumestrol, a coumestan phytoestrogen, is

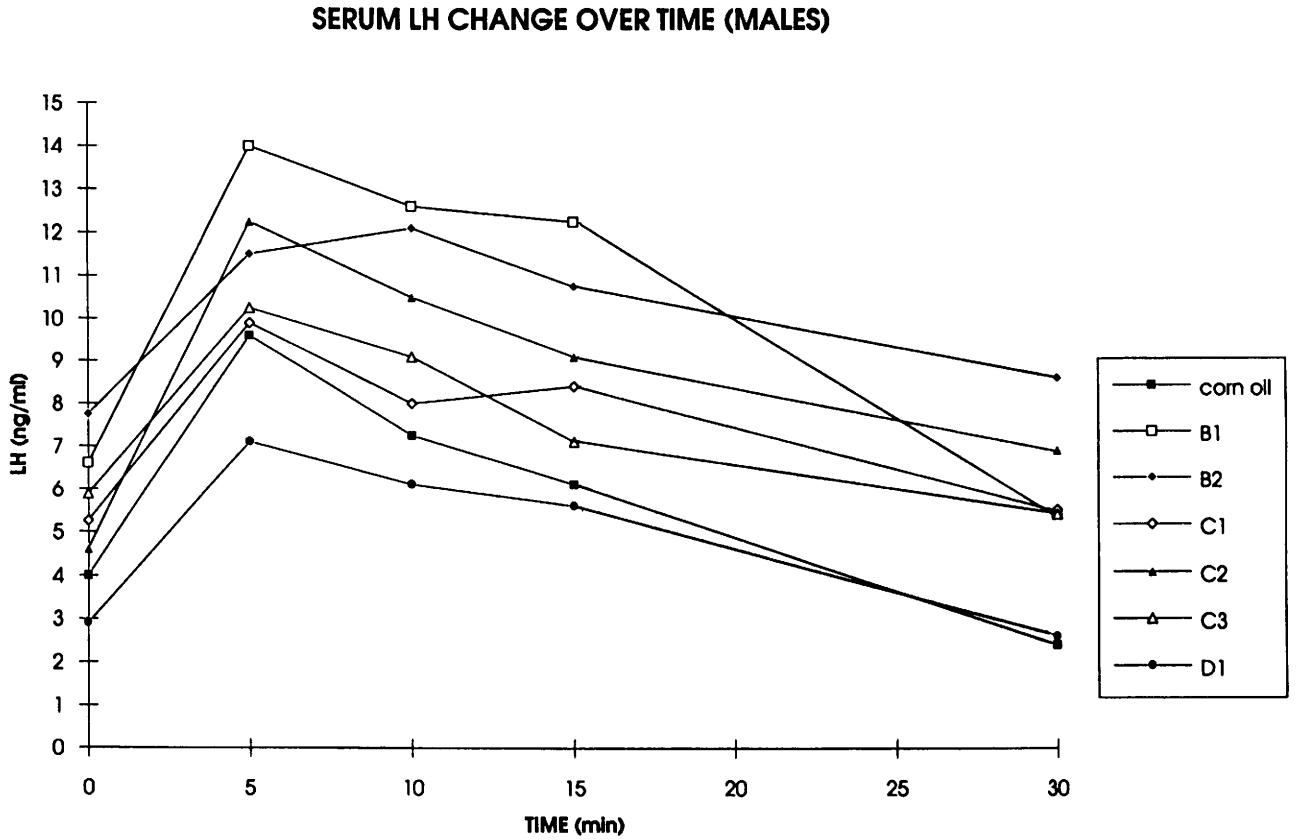

Figure 4. Serum LH (ng/ml) in castrated adult male rats exposed on Day 1–10 to corn oil (n = 11), B1 (n = 13), B2 (n = 7), C1 (n = 8), C2 (n = 7), C3 (n = 7), or D1 (n = 6), and given GnRH iv at t = 0.

Table I. SDN-POA Volume in Females[a]

Treatment group	SDN-POA volume in females (mm^3) × 10^{-3}		
Corn oil	2.99	n = 9	SD = 1.51
B1	2.98	n = 9	SD = 1.19
B2	3.78	n = 9	SD = 1.18
C1	2.25	n = 10	SD = 0.99
C2	2.59	n = 14	SD = 2.22
C3[b]	3.83	n = 10	SD = 1.37
D1	3.46	n = 11	SD = 1.38

[a] Castrated adult female rats exposed on Day 1–10 to corn oil, B1, B2, C1, C2, C3, or D1.
[b] C3 > C1, C2 ($P < 0.05$).

Table II. SDN-POA Volume in Males[a]

Treatment group	SDN-POA volume in females (mm^3) × 10^{-3}		
Corn oil	6.44	n = 9	SD = 2.34
B1	5.84	n = 16	SD = 2.39
B2	6.55	n = 4	SD = 5.73
C1	6.09	n = 9	SD = 3.32
C2	5.98	n = 9	SD = 3.85
C3	6.31	n = 14	SD = 2.51
D1	7.10	n = 6	SD = 2.87

[a] Castrated adult male rats exposed on Day 1–10 to corn oil, B1, B2, C1, C2, C3, or D1.

presumably simply an estrogen mimic. Females are more susceptible to the masculinizing effects of exogenous estrogens; hence, C1 mimics the effects of low dose estrogens and enhances pituitary response in females while having no significant effect on males.

The suppression of the GnRH-induced LH surge is seen only in females treated with C3 or DES. This is also consistent with the evidence that enviromental estrogens cause androgenization of the brain, a phenomenen that should be physiologically redundant in males. DES and the highest dose of coumestrol (C3) appear to masculinize the pituitary response to GnRH and blunt the LH surge. Females are more susceptible to masculinization than are males who have already been exposed to estrogens in the brain (via aromatization). This does not imply, however, that males cannot be affected by enviromental estrogens, since basal LH secretion and GnRH-induced secretion are affected differently (Fig. 2 and 4). In males, pituitary sensitivity to GnRH is not changed while, except for the C1 group, basal LH secretion is augmented by exposure to enviromental estrogens.

The present study extends our observations that neonatal estrogenization is associated with decreased pituitary responsiveness. Although in males only basal

LH secretion is affected by exposure to enviromental estrogens, both pituitary responsiveness to GnRH challenge and basal LH secretion is affected in females. These data indicate that enviromental estrogens have sexually distinct effects on pituitary physiology. In this model of estrogen exposure, β-sitosterol had no significant effect on GnRH-induced LH secretion but enhanced basal LH secretion. Higher doses of coumestrol diminished pituitary responsiveness while a wide range of doses of coumestrol increased basal LH secretion. These changes in pituitary and hypothalamic response indicate differences in CNS function influenced by exogenous estrogens.

As in previous studies, the SDN-POA volume was used as a morphologic marker of sexual differentation. Females exposed during the critical period of development to DES, zearalenone, and genistein have been shown to have SDN-POA volumes significantly greater than control females (7). In this study, there was no significant difference among groups when compared with control females. Notably, however, the C3-treated group had higher SDN volumes in comparison to the C1 and C2 groups ($P < 0.05$). It is conceivable that the C3 does (10 μg) simply may not have been sufficient to increase SDN volume significantly when compared with control females. The fact that C3 caused a reduction in the GnRH-induced LH surge while C1 and C2 did not suggests a possible physiologic-morphologic correlation with regard to masculinization of the female CNS. The fact that DES exposure did not result in an almost 90% increase in SDN volume in females relative to controls in this experiment as opposed to previous ones, may be at least in part due to differences in methodology. In previous experiments, SDN areas were measured according to inspection of brain sections under a microscope. It was observed that the fringes of the SDN-POA were profoundly more difficult to discern than the central portion, due presumably to the SDN-POA being less dense at its borders. In this experiment, the magnified images of the SDN were measured after the sections were displayed on a video monitor. Thus, the use of the video display, dependent on the resolution of the monitor, could have resulted in the fringes of the SDN-POA remaining unmeasured. As in previous studies, however, males in the control group had SDN-POA volumes about 2-fold greater than those of females in the control group, and there were no differences in SDN-POA volume among treated or control males (Table I and II).

The hormonal environment during the critical period exerts permanent organizational effects that may affect the behavior in adult animals. Examinations of both neuroanatomical and behavioral consequences of exposure to estrogenic substances may help to discern the mechanisms of these compounds.

The authors thank Sherry Dixon for her invaluable technical assistance and Beverly Eccles for assistance with software.

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