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Free Estradiol in Serum and Brain Uptake of Estradiol during Fetal and Neonatal Sexual Differentiation in Female Rats[1]

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ABSTRACT

Circulating estradiol is assumed not to contribute to sexual differentiation of the brain or other estrogen target tissues. The only estradiol available for binding to estrogen receptors is thought to be produced within brain cells by the aromatization of testosterone to estradiol as part of the action of androgen in the brain. However, we report that the concentration of free, biologically active serum estradiol (the concentration not bound to plasma proteins) was 0.54–2.17 pg/ml during the fetal and early neonatal period of sexual differentiation. These values were within the same concentration range for free estradiol observed in adult female rats throughout the estrous cycle (diestrus = 0.53 pg/ml; proestrus = 2.26 pg/ml), and estradiol clearly has physiological effects during diestrus as well as proestrus in adult females. When a stable, physiological blood concentration of [^{3}H]estradiol of 49 pg/ml total (0.61 pg/ml free) was achieved with Silastic capsules in 2-day-old female pups, [^{3}H]estradiol was recovered specifically bound to brain cell nuclei at approximately 2.7 fmol per pup brain or 12.4 fmol/mg DNA. The finding of brain uptake of circulating estradiol is contrary to current hypotheses. These findings suggest that estradiol in the fetal and neonatal circulation may be able to interact with testosterone and its metabolites to regulate sexual differentiation of the brain and other estrogen target tissues.

INTRODUCTION

The process of sexual differentiation occurs in rats during the last third of fetal life and continues through the first week after birth [1, 2]. While the secretion of testosterone by the fetal testes is necessary for complete masculinization, estradiol also mediates the differentiation of some, but not all, masculine characteristics [3–8]. The aromatization hypothesis proposed that testosterone secreted by the testes in male fetuses and newborns acts to masculinize (induce male traits) and defeminize (inhibit female traits) areas of the developing brain after the intracellular metabolism of testosterone to estradiol by the enzyme aromatase [9]. A corollary of this hypothesis is that estrogens in the circulation (most importantly, 17β-estradiol) do not play any role in the process of sexual differentiation [10], since it has been assumed that estradiol, by itself, mediated most aspects of brain masculinization and defeminization, and that if all circulating estradiol were not inhibited from entering brain cells, females would be masculinized and defeminized.

The effects of circulating estradiol on masculinization and defeminization of the brain (and on other developmental processes; [1]) were proposed to be prevented by the presence of very high concentrations of a plasma glycoprotein, alpha-fetoprotein, which binds estradiol with high affinity in rats and mice [11]. The prediction was thus that any estrogen bound to intracellular estrogen receptors in the brain (and other tissues) during sexual differentiation in rats was formed intracellularly via aromatization of testosterone, and therefore represented a product of androgen action. It is generally assumed that the high blood concentration of alpha-fetoprotein [12, 13] restricts the passage of estradiol into tissues during sexual differentiation by reducing the free, biologically active fraction of plasma estradiol to a concentration below which it is able to exert a physiological response [14–16]. For example it has been proposed that alpha-fetoprotein (AFP) "effectively sequesters the circulating estrogen in a biologically inactive form" ([10], p. 249), and "theoretically, therefore, no estradiol is available to reach the brain of the female fetus" ([17], p. 441). Numerous studies have been conducted showing that exposure to the synthetic estrogen diethylstilbestrol (DES) can alter sexual differentiation of the brain [5] and other estrogen-target tissues [1], but DES does not bind to AFP in rats or plasma sex steroid binding protein in humans [18] and is thus, unlike estradiol, not subjected to inhibition of uptake into target tissues.

Our experiments were designed to test the hypothesis that the concentration of free estradiol in blood is too low for a sufficient amount of estradiol to enter cells in the brain and bind specifically in brain cell nuclei during perinatal life in rats. To determine the concentration of free estradiol in fetal blood we modified the technique of centrifugal ultrafiltration [19] to measure the free fraction, and we developed a sensitive RIA for estradiol in rodent serum to measure the total concentration of estradiol in fetal blood, which was then used with the free fraction to calculate the low concentration of free estradiol in fetal blood. To determine whether estradiol from the fetal circulation could enter the

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brain and bind specifically to estrogen receptors in the nuclei of brain cells, we implanted capsules of [^{3}H]estradiol into 2-day-old female rats to create a blood concentration of free [^{3}H]estradiol that was similar to the levels of endogenous free estradiol in blood that we measured during late fetal life and at the end of the day of birth [13].

We examined brain uptake of [^{3}H]estradiol at 48 h after birth to take advantage of the virtual absence of circulating estradiol in female pups at this time. Because brain estrogen receptors [20] and plasma AFP [1] are constant during the first two days after birth, we were able to manipulate estradiol blood levels on Day 2 but relate our findings to the capacity for endogenous estradiol to enter brain cells and bind to estrogen receptors during late fetal life and the day of birth, a time during which sexual differentiation of the brain occurs [21, 22] but when the much higher levels of circulating estradiol would interfere with our experiments.

Our findings here were based on experiments in females to reduce interference due to intracellular estradiol derived from the aromatization of testosterone in males. However, these findings should also be applicable with regard to the hormonal regulation of sexual differentiation in males, since we know of no evidence to suggest that during sexual differentiation, male and female rats differ with regard to any aspect of estrogen physiology: total or free estradiol, plasma estrogen binding proteins, brain uptake, or binding to intracellular receptors [1].

MATERIALS AND METHODS

Animals and Housing

A breeding stock of Sprague-Dawley rats was purchased from Harlan Laboratories (Birmingham, AL) and bred in our laboratory. Rats were housed in 45 × 25 × 20 cm polyethylene cages on corn cob bedding. Food (Purina Breeder Chow 5008; Ralston-Purina, St. Louis, MO) and water were available ad libitum. Animals were maintained at 25 ± 2°C on a 12L:12D cycle with lights on at 0800 h. All animal care procedures were as specified by the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources for the National Institute of Health. Experimental protocols were approved by the University of Missouri Animal Care and Use Committee.

Mating Procedure

Virgin, 70- to 90-day-old female rats were time-mated by being paired with males. Females were examined by vaginal lavage each day at 0800 h for the presence of sperm (Day 0 of pregnancy). Mated females were housed three per cage until Day 21 of pregnancy, at which time they were singly housed. Only offspring of mothers with a 22-day gestation period were used for these studies (75% of females delivered during the morning of Day 22). Pregnant females were examined for delivery of pups (which lasts about 1–2 h, depending on the number of pups) by quietly entering the animal room every 15 min. At the time of detection of the first pup, time of onset of delivery was recorded. Mothers were allowed to care for the pups until the pups were taken for capsule implantation, which occurred 24 h after the recorded time of delivery.

Centrifugal Ultrafiltration

Centrifugal ultrafiltration was conducted with a commercially available MPS-1 device (Amicon Corp., Lexington, MA), and a modification of procedures that have been previously described was used [19]. Briefly, serum (200 µl) was subjected to ultrafiltration with use of the MPS-1 device, which consisted of a single YMT membrane and O-ring sealed between a sample reservoir and support base. Radiolabeled estradiol ([2,4,6,7-^{3}H]estradiol, specific activity: 104.4 Ci/mmol; NEN, Boston, MA) was purified by HPLC, and approximately 474 000 dpm (2.1 pmole, 0.58 ng) of [^{3}H]estradiol (in methanol) was transferred into glass test tubes and dried under nitrogen. A 450-µl aliquot of each serum pool was then added, and the sample tubes were vortexed. Samples were incubated at 37°C for 30 min and then at room temperature for an additional 30 min. A 20-µl aliquot (reference) was transferred to a scintillation vial for counting, while two 200-µl aliquots of the sample were transferred to duplicate sample reservoirs and centrifuged (1000 × *g*) for 2 min at 37°C. After the 2-min pre-spin, the filtrate cup was removed, and the initial ultrafiltrate was discarded. A new filtrate cup was placed under the sample reservoir, and the sample was centrifuged for another 10 min. A 20-µl aliquot of the ultrafiltrate was then transferred into a scintillation vial. Scintillation fluid was added to the ultrafiltrate (from the 10-min spin) and reference vials, and the samples were counted in a liquid scintillation counter. The percentage of free estradiol was calculated from the relative concentrations of [^{3}H]estradiol in aliquots of the ultrafiltrate and the unfiltered reference sample.

The method was validated by serial dilution (undiluted, 1:1, 1:3, 1:7) of a pool of serum from fetal and neonatal rats with buffer and measuring percentage of free estradiol. The serial dilution of serum led to 0.8%, 1.7%, 3.8%, and 7.8% free estradiol, replicating findings of Hammond et al. [23]. With the addition of increasing amounts of BSA to buffer (0, 50, 200, and 1000 µg/ml), we observed a corresponding decrease in percentage of free estradiol (90%, 71%, 61%, and 23%, respectively); each value represents the mean from duplicate runs.

Since accurate determination of low percentage values of free steroid involved the addition of a relatively high mass of the tritiated steroid to the sample, a concern was that the unbound fraction measured by ultrafiltration might vary as a function of alteration in the concentration of either radioactive or nonradioactive hormone because of saturation of

any binding components of the serum. To address this possibility, pooled serum samples from 1–2-day-old rat pups were incubated with varying masses of unlabeled steroid and a fixed mass of the tritiated steroid. Addition of nonradioactive estradiol to concentrations well above our working range did not produce an increase in the percent of unbound hormone; specifically, addition of different masses (0, 1, 10, 100 ng/200 μl serum) of nonradioactive estradiol resulted in 1.36%, 1.31%, 1.34%, and 1.32% free estradiol, respectively.

Estradiol Radioimmunoassay

Estradiol radioimmunoassay was performed as previously described by vom Saal et al. [24]. Serum volumes ranged from 10–200 μl due to the wide range of estradiol concentrations in serum at different times in life. ^{125}I-Estradiol and antisera were obtained from ICN Biomedicals (Costa Mesa, CA), and unlabeled estradiol was obtained from Steraloids (Wilton, NH). Sensitivity of the assay was 0.5 pg. Intra- and interassay coefficients of variation were 3% and 11%, respectively. We determined the percent cross-reactivity of the estradiol antiserum with estrone to be 0.6%. With or without implantation of varying numbers of Silastic capsules containing nonradioactive estradiol (experiment 2) or prior injections of competitor diethylstilbestrol (experiment 3), the ratio of estrone to estradiol in serum of 2-day-old female rats was about 20:1.

Silastic Implants

Female pups were implanted at 24 h after birth with Silastic capsules (0.04 in i.d., 0.85 in o.d.; Dow Corning, Midland, MI). Each capsule was 7 mm long, with 5 mm between the capped ends (sealed with Silicon Type A adhesive; Dow Corning). Each capsule held a volume of 4.1 μl. For [^{3}H]estradiol, each capsule contained 4.1 μCi of [2,4,6,7,16,17-^{3}H]estradiol (specific activity: 136.2 Ci/mmol; 8 ng/4.1 μl sesame oil; 2.9 nmole/kg body weight). Cholesterol (770 ng/4.1 μl) was also added to the solution. For nonradioactive estradiol, each capsule contained the same concentration used for [^{3}H]estradiol: 8 ng/4.1 μl sesame oil (2.9 nmole/kg BW). All capsules were preincubated for 24 h in PBS containing gelatin. At 24 h after birth, pups were anesthetized with Metofane (methoxyflurane; Pitman Moore, Mundelein, IL), and capsules were implanted s.c. via an incision in the back of the neck. The wound was closed with a liquid adhesive, and the pups were kept warm prior to being returned to the dam when fully awake. Pups were killed within 5 min of being taken from their mother for blood and tissue collection 24 h later (when 48 h old).

Isolation of Brain-Cell Nuclei

Twenty-four hours after implantation of capsules that contained radiolabeled estradiol (experiment 3), pups were anesthetized with Metofane, and the brain was perfused for 20 sec by an injection of 2 ml cold Ringer's into the left ventricle of the heart. Brains were removed from the skull, and brain tissue rostral to the medulla was saved for analysis. Brains from three pups were pooled, and nuclei were isolated using the hexylene glycol technique [25]. Phase contrast microscopy indicated the pellet consisted of intact nuclei with very few apparent cytoplasmic organelles visible at the light microscopic level. Labeled steroids were extracted from the nuclei or 200 μl of serum with 2 ml of an 80:20 mixture of ethylacetate:chloroform. Extracted steroids were reconstituted in HPLC-grade methanol, and appropriate aliquots were taken for RIA, HPLC separation, and reference counts. DNA in isolated nuclei was assayed with Hoechst dye 33258 (Sigma, St. Louis, MO) by using the method of Labarca and Paigen [26]. Calf thymus DNA (Type I; Sigma) was used as the standard, calibrated assuming 20 A_{254} U/mg DNA/ml.

Separation of Estrogen Metabolites by HPLC

Steroids extracted from 200 μl serum and cell nuclei from 3 brains were reconstituted in 130 μl HPLC-grade methanol, which was spiked with authentic nonradioactive standards (estrone, estradiol, and estriol, 3 μg/100 μl) purchased from Steraloids. Duplicate aliquots (10 μl) were removed for reference counting, and 100 μl was injected into a Perkin-Elmer Series 4 HPLC System (Irvine, CA) with a 5 μm Rainin C-18 (Rainin Instrument Co., Woburn, MA) reverse-phase column using a gradient of acetonitrile and water. A Gilson model 202 (Gilson Inc., Middleton, WI) fraction collector was used to collect fractions (in scintillation vials) containing estrone, estradiol and estriol, based on visualization of corresponding nonradioactive standards with a Perkin Elmer LC-95 UV detector. The samples were dried under nitrogen and counted in the collection vials after addition of liquid scintillation fluid. Based on the total amount of radioactivity in all fractions collected 1–20 min after injection of the sample, recovery was 97% of the total amount of radioactivity injected.

RESULTS

Experiment 1

The objective of this experiment was to compare the concentration of free, and thus clearly bioavailable, estradiol in blood during late prenatal and early neonatal life (during sexual differentiation) to the concentration of free estradiol in adult female rats. We examined free estradiol concentrations in adult females on the first day of diestrus (the day after estrus) and on proestrus (just prior to the preovulatory surge in luteinizing hormone), when circulating estradiol is at its nadir and peak, respectively [27], and compared these values with levels of free estradiol in fetuses and neonates.

TABLE 1. The mean (± SEM) total and free concentrations (pg/ml) of estradiol (E_2), as well as the percent of total E_2 in the free fraction, in serum of female rats at different times in life.

	Prenatal life	Hours after birth		Adulthood	
E_2	Day 19	4	48	Diestrus	Proestrus
Total concentration (pg/ml)	155 ± 4.0	126 ± 6.0	5.6 ± 0.6	13.7 ± 1.7	59.7 ± 7.7
% Free	0.35 ± 0.01	1.77 ± 0.20	1.12 ± 0.09	4.03 ± 0.17	3.9 ± 0.12
Free concentration (pg/ml)	0.54 ± 0.01	2.17 ± 0.17	0.07 ± 0.01	0.53 ± 0.05	2.26 ± 0.33

Total estradiol in the circulation was measured by RIA. To measure the low free fraction of the total (bound plus free) concentration of estradiol in blood, we modified the technique of centrifugal ultrafiltration [19]. The free serum estradiol concentration was then calculated based on the formula: total estradiol concentration × percent free estradiol = free estradiol concentration.

The concentration of free estradiol was determined in serum that was collected from female pups at a number of times during sexual differentiation: on Day 19 of fetal life (when total circulating estradiol in female fetuses reaches peak levels; n = 5 pools), at 4 h after birth (total estradiol levels are still high; n = 6 pools), and 48 h after birth (total estradiol levels are very low; n = 9 pools; [13]). Blood was also collected from adult female rats on the first day of diestrus (n = 5) and on the day of proestrus (n = 7). Blood collection was between 1200 and 1300 h, 4–5 h after the onset of the light phase of the light:dark cycle. Prior to adulthood, serum from a number of pups was pooled and then divided into two aliquots. Total (free plus bound) estradiol in one aliquot was measured by radioimmunoassay, while the percent free estradiol in the second aliquot was determined by centrifugal ultrafiltration.

The results in Table 1 show that on Day 19 of fetal life, the free concentration of estradiol in serum (0.54 pg/ml) was similar to the value observed during diestrus in adult cycling females (0.53 pg/ml). Shortly (4 h) after birth, the percentage of free estradiol was elevated, with the result that the free serum concentration of estradiol (2.17 pg/ml) was similar to that observed during proestrus in adult females (2.26 pg/ml). The means for total E_2 concentration, percentage of free E_2, and free E_2 concentration for the three time points examined during prenatal/neonatal life all differed significantly ($p < 0.001$; ANOVA). The values for free estradiol observed in adult females that we observed were similar to those reported in other studies [28].

The very high level of free estradiol (similar to peak levels during proestrus in adult females) shortly after birth was unexpected but quite interesting given the finding that sexual differentiation of the rat brain is influenced by gonadal steroids during this brief period in development [21, 22]. By 48 h after birth, the serum concentrations of total and free estradiol had dropped dramatically, revealing that only between 24 h and 48 h after birth do circulating levels of free estradiol actually become negligible; however, this was due to the absence of estradiol secretion into the blood rather than the presence of estrogen binding proteins in the blood. This latter finding was serendipitous, however, in that it allowed us to conduct the next experiment against a very low background level of endogenous estradiol.

Experiment 2

The objective of this experiment was to determine a dosage of estradiol (administered via Silastic capsules) that would result in blood concentrations of free estradiol at 48 h after birth that reproduced the levels we measured in female rats during late fetal life (determined in experiment 1) and throughout the day of birth, subsequent to the very high levels observed at 4 h after birth [13]. Since masculinization and defeminization normally begin in males during the last third of fetal life and continue into the early postnatal period [7, 21], this experiment allowed us to directly test whether within the fetal physiological range of total plasma estradiol, AFP and other estrogen-binding plasma proteins serve to maintain the free concentration of estradiol in the blood at low levels, for example below levels which exert physiological effects in adult females. We administered estradiol via Silastic capsules rather than by injection, since our goal was to maintain steady-state conditions to allow equilibrium to be established between the hormonal compartments of interest: hormone bound to plasma proteins, hormone free in plasma, and hormone specifically bound in brain cells.

Preliminary Study

Prior to conducting the experiment we first implanted (s.c.) Silastic capsules containing [^{3}H]estradiol in female rat pups at various times after birth. Pups were implanted with 1 to 3 Silastic capsules as described in *Materials and Methods*. We determined the time-course of changes in [^{3}H]estradiol in serum and brain cell nuclei by collecting blood and brain tissue from female rat pups at 8-h intervals after implantation. The time of sample collection used in the following study (24 h after implantation) was selected based on our obtaining results similar to those of Attardi and Palumbo [29] with regard to achieving stable levels of [^{3}H]estradiol in serum and brain cell nuclei. We observed stable levels of [^{3}H]estradiol in serum and brain cell nuclei between 16–40 h after capsule implantation at 24 h after birth.

The decision concerning when to collect samples from implanted animals (at 48 h after birth) was also based on

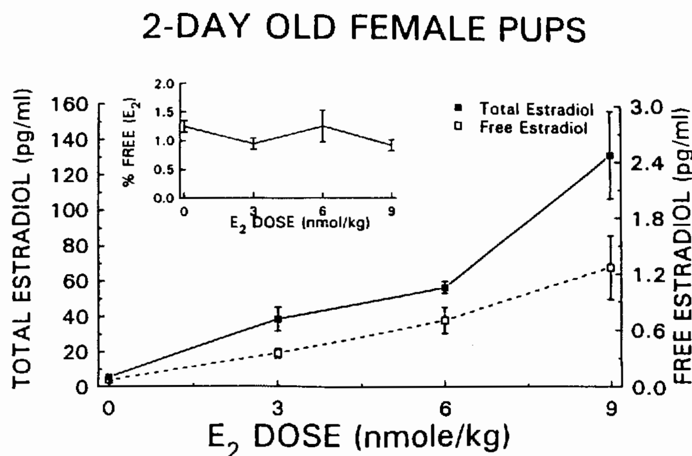

FIG. 1. Total and free serum estradiol (E_2) concentrations (mean ± SEM) in 2-day-old female pups implanted for 24 h with zero to three Silastic capsules containing E_2. The following number of samples, representing sera pooled from pups implanted with the same dosage, were assayed: 0 capsules, 0 nmole/kg, n = 9; 1 capsule, 2.9 nmole/kg, n = 5; 2 capsules, 5.9 nmole/kg, n = 5; 3 capsules, 8.8 nmole/kg, n = 4. Sample sizes are the same for total, free, and percent free E_2 at each dosage. For reference, the adult diestrus level of free estradiol was 0.53 pg/ml (from Table 1).

the very low (below 6 pg/ml) and stable serum concentration of total endogenous estradiol at 48 h after birth. In fact, at 48 h after birth, we observed the lowest blood concentration of estradiol at any time from Day 18 of fetal life (5 days prior to birth) through adulthood [13]. This finding made conducting both this and the following experiment possible. It would have been very difficult to conduct these experiments at any prior time in development due to the high and rapidly changing concentrations of endogenous estradiol during prenatal life and the day of birth.

Administration of Different Dosages of Estradiol Via Silastic Capsules

We implanted (s.c.) 24-h-old female pups with different numbers of Silastic capsules (0, 1, 2, and 3), each containing 8 ng/4.1 µl sesame oil (2.9 nmole/kg BW) of nonradioactive estradiol. We collected blood 24 h later (48 h after birth), based on the results of the preliminary study. The results in Figure 1 show a constant percentage of free estradiol with administration of increasing concentrations of estradiol via Silastic capsules, with the result that there was a linear relationship between the total and free blood concentrations of estradiol (Pearson's $r = 0.97$, $p < 0.001$). The values obtained for the free concentration of estradiol were within the range observed at the end of fetal life and the day of birth in female rats (Table 1). These results thus demonstrate that plasma estrogen-binding proteins do not serve to hold free estradiol at negligible levels when blood concentrations of total estradiol are maintained within the range observed during the prenatal/neonatal period.

Experiment 3

In this experiment we examined whether [^{3}H]estradiol (administered via Silastic capsules), maintained at physiological concentrations determined in the prior experiment, could pass out of the blood and be recovered from the brain specifically bound to nuclear receptors at 48 h after birth in female rats. We measured uptake and specific binding of [^{3}H]estradiol in the entire brain rostral to the medulla. The basis for our decision to examine the entire brain is that this experiment was designed to test the hypothesis that there is negligible estradiol available for entry into (and thus specific binding within) cells containing estrogen receptors located in any region of the brain. To test this hypothesis by attempting to detect specifically bound [^{3}H]estradiol in selected areas of the brain in 2-day-old rat pups, we would likely have had to pool tissues from an unacceptably large number of pups, each implanted with about 12 µCi of [^{3}H]estradiol.

As in the prior experiment, we collected serum and brain tissue at 48 h after birth, because blood levels of endogenous estradiol are extremely low at this time relative to the day of birth. The ability to detect binding of a radioisotope, such as [^{3}H]estradiol, to receptors is limited by its specific activity (radioactivity per unit mass), and only when the mass of unlabeled endogenous estradiol is low could a high and stable specific activity be achieved with physiological blood levels of [^{3}H]estradiol to conduct this experiment. Endogenous estradiol therefore had little effect on the specific activity of the total estradiol achieved after implantation of [^{3}H]estradiol.

Three Silastic capsules, each containing 4.1 µCi of [^{3}H]estradiol were implanted into eighteen female pups 24 h after birth as described in *Materials and Methods*. [^{3}H]Estradiol (and metabolites) were extracted from brain cell nuclei as described above, and extracts from 3 brains were pooled for HPLC analysis (3 pools/group). This treatment resulted in total circulating estradiol levels ([^{3}H] and endogenous) of 49 ± 3 pg/ml (measured by RIA). There was 1.24% of the total estradiol in the free fraction, and the concentration of free estradiol was 0.61 pg/ml, which was similar to the blood concentration of free estradiol during fetal life (Table 1) and the day of birth in both male and female rats [13]; for example, between 4 h and 24 h after birth, total serum estradiol decreased from 126 ± 6 pg/ml to 38 ± 4 pg/ml in female rat pups, corresponding to a decrease in free estradiol from 2.17 ± 0.17 to 0.54 ± 0.06 pg/ml [13].

We monitored the proportion of total radioactivity in fractions containing radiolabeled estrogens in serum and brain nuclear extracts using HPLC, since most (> 90%) estrogen binding to receptors should be accounted for by [^{3}H]estradiol rather than by other estrogen metabolites [30], such as estrone and estriol. As described above, our prelim-

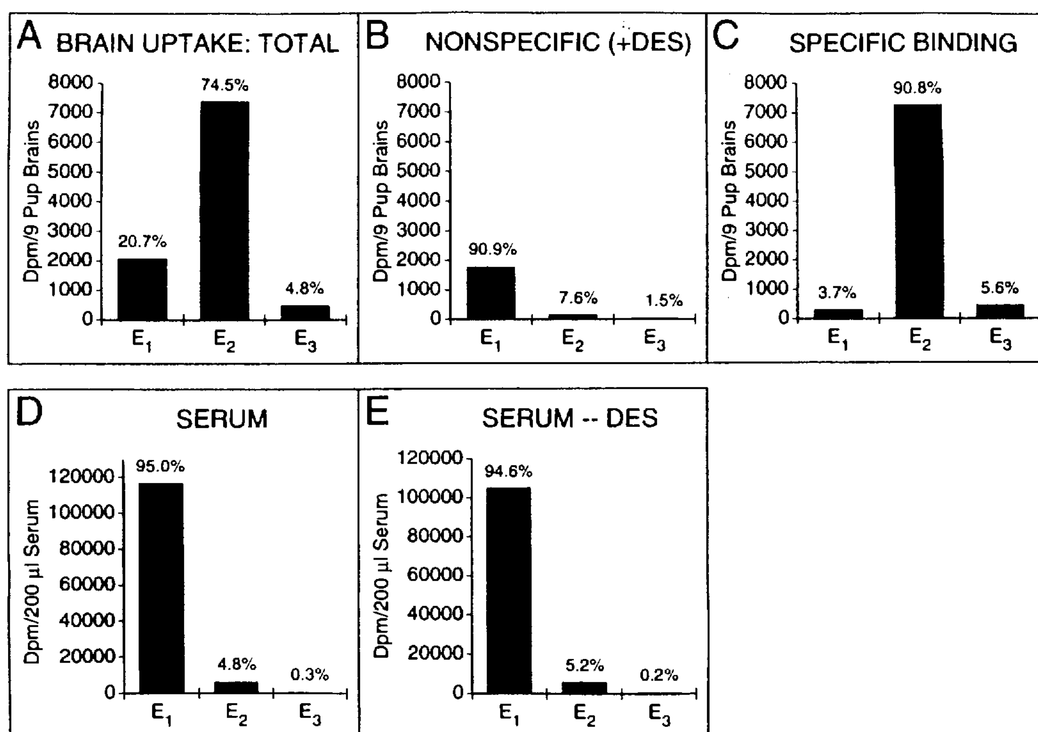

FIG. 2. [^{3}H]Estradiol was implanted via Silastic capsule in 24-h-old female pups. Radioactivity was recovered as estrone (E_1), estradiol (E_2), or estriol (E_3) after separation by HPLC of estrogens extracted from cell nuclei from 9 brains (**A**, TOTAL) and from three pools of 200 µl serum (**D**) collected at 48 h after birth, as detailed in *Materials and Methods.* Estrogen metabolites were recovered as above from a second group of 9 pups that was injected with a saturating dose of DES at both 25 h and 8 h before collection of serum (**E**) and brains for preparation of cell nuclei (**B**, NONSPECIFIC). Since DES does not bind to serum estrogen-binding glycoproteins, DES treatment could not be used to assess high-affinity binding of estrogens to alpha-fetoprotein in serum. In contrast, nuclear receptors avidly bind DES, and specific binding of E_1, E_2, and E_3 in brain cell nuclei (**C**, SPECIFIC) was calculated by subtracting dpm associated with E_1, E_2, and E_3 in DES-treated animals (**B**) from dpm associated with corresponding estrogen metabolites in non-DES-treated animals (**A**). The dpm associated with the E_1 and E_3 fractions were virtually identical in brain cell nuclei from DES (**B**) and non-DES-treated animals (**A**), whereas the dpm associated with the E_2 fraction were effectively inhibited by DES in brain cell nuclei. Thus, virtually all (91%) estrogen that was competitively displaced by DES (and thus specifically bound) was E_2.

inary study demonstrated that both the concentration of [^{3}H]estradiol in the blood and bound in brain cell nuclei was constant between 16–40 h after administration via silastic capsules (at 24 h after birth) of this dose of [^{3}H]estradiol. A system which is not stabilized (for example after injection of a bolus of [^{3}H]estradiol) is subject to estrogen-mediated changes in both receptor dynamics and steroid-metabolizing enzymes [31, 32].

Specific binding of [^{3}H]estradiol to estrogen receptors was measured by subtracting, from values for total binding of [^{3}H]estrone, [^{3}H]estradiol, and [^{3}H]estriol, the corresponding nonspecific binding value for each of these three estrogens; nonspecific binding was estimated by administering 2 saturating doses of nonradioactive diethylstilbestrol (DES) to 9 of the female pups, the first 1 h before and the second 16 h after implanting the Silastic capsules containing [^{3}H]estradiol [29]. After treatment with a high dose of DES, any estrogen specifically bound to estrogen receptors in cells should be competitively displaced by DES, and any [^{3}H]estrogen remaining in the nuclear fraction should represent nonspecific binding to other macromolecules. Specifically, all pups were implanted with [^{3}H]estradiol 24 h before sample collection and then randomly divided into two groups (n = 9/group): one group (total [^{3}H]estrogen binding) received injections of only sesame oil while the other group (nonspecific binding) received two injections of a high dose of nonradioactive DES (80 µg/50 µl sesame oil; 29.4 µmole/kg).

The results in Figures 2D and 2E show that when animals were administered [^{3}H]estradiol (with or without nonradioactive DES), most [^{3}H]estrogen recovered in the blood was found to be the metabolite [^{3}H]estrone. The value for [^{3}H]estrone/[^{3}H]estradiol of about 20:1 in the current study was within the range previously observed in adult female rats after constant infusion of [^{3}H]estradiol [33]. In our preliminary studies we observed similar [^{3}H]estrone/[^{3}H]estradiol ratios regardless of the number of capsules containing [^{3}H]estradiol which were implanted.

In pups that were treated with the nonradioactive competitor, DES, to assess nonspecific binding, the propor-

tions of tritium recovered as [^{3}H]estrone, [^{3}H]estradiol, and [^{3}H]estriol nonspecifically bound in brain cell nuclei (Fig. 2B) were nearly identical to the proportions in serum extracts (Fig. 2E). This finding provides indirect support for the hypothesis that the estrogens passed from the blood into brain cells by simple diffusion. Estrone, estradiol and estriol bind to plasma proteins, such as alpha-fetoprotein [11], with different affinities. The finding that alpha-fetoprotein has been localized within neurons has raised the possibility that alpha-fetoprotein transports estradiol into selected brain cells [34–36]. Receptor-mediated transport into target cells of albumin or alpha-fetoprotein and estrogens bound to these proteins would not result in equal proportions of these three estrogens in the blood and nonspecifically bound in cells. We thus found no basis for invoking a high level of alpha-fetoprotein-mediated active transport of estradiol rather than free diffusion of estrogen into brain cells to account for our findings, although receptor-mediated transport of the alpha-fetoprotein-estradiol complex may serve to deliver supplemental estrogen to selected brain cells.

The above hypotheses are based on evidence that the high dose of competitor DES does not alter the binding of estrogen to alpha-fetoprotein in blood but competes with specific binding of estrogen to intracellular estrogen receptors. Specifically, DES does not bind to alpha-fetoprotein [18], and DES should not lead to displacement of bound estradiol from alpha-fetoprotein in blood. Thus, the proportion of [^{3}H]estradiol recovered as [^{3}H]estrone, [^{3}H]estradiol, and [^{3}H]estriol in blood should not be altered by treatment with DES, which was exactly what we observed (Fig. 2, D and E). In contrast, differences in affinity of estrone, estradiol, and estriol for nuclear estrogen receptors [30] would lead to differences in the proportion of estrone, estradiol, and estriol recovered in cell nuclei (Fig. 2A) and blood (Fig. 2D), which was also what we observed. Specifically, there was a much higher proportion of [^{3}H] recovered from brain cell nuclei by HPLC in the estradiol fraction (Fig. 2A) when compared to serum (Fig. 2D). However, DES competed with the binding of [^{3}H]estradiol in nuclear extracts but not with the binding of [^{3}H]estrone and [^{3}H]estriol, with the result that virtually all (91%) of the estrogen specifically bound (Fig. 2C) in brain cell nuclei was estradiol.

The results of this experiment show that despite the presence of serum binding proteins in the fetal circulation, when maintained at physiological concentrations identified in experiment 1, [^{3}H]estradiol in the circulation was able to pass into brain cells and bind to brain cell nuclei. Most of the radioactivity was bound as [^{3}H]estradiol and was inhibited by nonradioactive DES. Specific, competitive binding of [^{3}H]estradiol was 7245 dpm per 9 pup brains (Fig. 2C), equivalent to 2.7 fmol per pup brain (12.4 fmol per mg DNA based on 215 μg DNA per pup brain on Day 2, or 7.1 fmol/g tissue based on an average brain weight of 0.38 g on Day 2). The total number of estrogen receptors in the hypothalamic-preoptic area of the neonatal rat brain on Day 2 has been reported at 80 fmol/g tissue [20]. The value for putative occupied estrogen receptors measured here of 7.1 fmol/g tissue from the entire brain may therefore represent substantial receptor occupancy.

DISCUSSION

The findings presented in Figure 1 show that when total serum estradiol was experimentally manipulated within the range that was observed during the prenatal and neonatal period of sexual differentiation, there was a dose-dependent, linear change in the blood concentration of free estradiol that was within the range observed in cycling adult females. This finding suggests that variation in circulating estradiol during development may be capable of dose-dependent effects on differentiation of tissues, just as variation in circulating estradiol in adult females leads to dose-dependent effects on physiology and behavior.

On Day 19 of fetal life in female rats, the concentration of free estradiol in blood was similar to the level observed during diestrus in adult females. In the adult, the levels of free estradiol observed during diestrus are sufficient to exert effects on the brain-pituitary axis, as revealed by negative feedback inhibition of LH secretion [27], and the possibility thus exists that similar levels of free estradiol in the blood of fetuses and neonates are high enough to influence the growth and differentiation of brain and other estrogen target tissues, although our studies did not directly address this possibility. While studies to directly compare the sensitivity of the fetal and adult brain to estradiol have not been conducted, Bern [37] has coined the term "fragile fetus" with regard to findings suggesting a heightened sensitivity to estrogen of fetuses relative to adults. The finding of physiological levels of free estradiol in fetal blood does not support the generally accepted hypothesis that there is negligible estradiol available in the circulation for entry into estrogen target cells in the brain and other tissues throughout the perinatal period of sexual differentiation in rats ([10], p. 249, [17], p. 441). When we created a concentration of free [^{3}H]estradiol (administered via Silastic capsules) in 2-day-old female pups (when endogenous estradiol is very low) that was similar to the concentration of free estradiol observed in fetuses and neonates, the [^{3}H]estradiol entered brain cells and was recovered specifically bound to nuclear estrogen receptors.

Our findings suggest that one function of plasma steroid binding proteins in fetuses and neonates is to regulate the concentration of free estradiol in blood such that it does not exceed the adult physiological range, since total blood estradiol concentrations (as well as concentrations of many other steroids) are extremely high during the perinatal pe-

riod of sexual differentiation [1, 38]. Exposure to supraphysiological levels of estrogens during development is associated with a variety of malformations; typically, synthetic estrogens (such as DES that does not bind to alpha-fetoprotein) have been used in these studies [1, 39]. But, the possibility that physiological concentrations of estradiol might serve to mediate some aspects of tissue differentiation has not been addressed, due to the erroneous assumption that plasma estrogen binding proteins functioned to keep free estradiol at negligible levels in the blood.

Our findings here provide support for the prediction that natural variation in the blood concentrations of estradiol as well as testosterone influences the course of sexual differentiation. In particular, variation in circulating estradiol may contribute to the numerous differences among males due to their prior intrauterine position [7], as well as differences between males and females. For example, a male mouse fetus located in utero between two female fetuses (2F males) has elevated blood estradiol levels relative to males located between other male fetuses (2M males; [7]) and in adulthood, the prostate in these males is enlarged due to a permanent (imprinted) elevation in the number of androgen receptors [40]; 2F males also are more sexually active as adults than are 2M males [41]. Whereas circulating estradiol in male fetuses may modulate subsequent responsivity of tissues to androgens as well as interact in other ways with testosterone to regulate various aspects of sexual differentiation, a similar effect in females might not be observed due to the fact that throughout sexual differentiation, females have significantly lower levels of testosterone than do males. The sex difference in testosterone is particularly dramatic during the first few days after birth in rats: blood levels of testosterone range from 10 to 25 times higher in males than in females [1, 13]. A high proportion of circulating testosterone can enter cells, since testosterone is not bound to a plasma glycoprotein in fetal or newborn rats [13, 42]; a glycoprotein named androgen-binding protein can be detected in neonatal rat serum by radioimmunoassay, but the form in the circulation does not bind testosterone [42–45].

There is a great deal of species variability with respect to the extent to which estrogen (either in the blood or formed intracellularly via aromatization of testosterone) is required for various aspects of brain sexual differentiation [8]. For example, drugs that selectively block androgen binding to androgen receptors or estrogen binding to estrogen receptors, as well as inhibitors of aromatase, all interfere with aspects of the differentiation of sexual behavior in rats [46–49]. At least some aspects of behavioral masculinization in rodents (such as infant play behavior and intermale aggression) appear to be mediated primarily by the binding to androgen receptors of either testosterone or 5α-dihydrotestosterone [50, 51]. Thus, in rodents there is ample evidence supporting the hypothesis that the binding of testosterone (or its reduced metabolite, 5α-dihydrotestosterone) to androgen receptors and the binding of estradiol to estrogen receptors are both required for complete defeminization and masculinization of brain function and behavior [1, 4–6, 8, 34, 52].

Females can show variability in masculine traits, and while this may affect reproductive performance [53, 54], what is of primary concern is the degree to which females are defeminized (lose the ability to ovulate and mate as females). The absence of significant defeminization in females, even though circulating estradiol can enter the brain and other tissues, appears to be mediated by 1) the absence of concurrent high levels of testosterone (and its binding to androgen receptors), 2) the relatively low supplemental intracellular estradiol produced by aromatization of circulating testosterone after birth [55], and 3) a dramatic decrease in circulating estradiol between the first and second day of postnatal life. What is clear is that the diffusion of free estradiol from the blood into the brain or other target tissues in fetuses and neonates is not sufficient to result in the general processes of masculinization and defeminization in females, since females either have similar (Rhesus monkey) or higher (humans, mice, and cattle) blood levels of estradiol relative to males during sexual differentiation [7].

There are a number of related observations that support this hypothesis. First, there is evidence that masculinization and defeminization in developing rats are temporally separated, with masculinization occurring primarily during prenatal life and defeminization beginning after birth [56]. Thus, variability in uptake of estradiol and testosterone into the brain of female rats and mice during fetal life (due to intrauterine proximity to male vs. female fetuses) is correlated with differences in the degree to which masculine behaviors are exhibited; for example females that develop in utero between either other female fetuses (2F females) or between male fetuses (2M females) differ in a number of traits in rats, mice and gerbils, such as territorial marking and aggressiveness, that are typically considered to be masculine traits [7]. The capacity for female rats and mice to ovulate and mate (which is eliminated in males during the process of defeminization) can be modulated slightly by gonadal steroids during fetal life (for example, 2M and 2F females differ in estrous cycle length and sexual receptivity [7]. But, prenatal exposure to elevated levels of testosterone due to a natural event in 2M female fetuses (because they are situated in utero between males), or experimentally, as a result of maternal treatment with testosterone, even at levels which markedly masculinize the genitalia and subsequently inhibit vaginal canalization, does not block the capacity to ovulate and mate during later life [7, 57, 58]. The number of androgen receptors in brain is low prenatally and does not show much of an increase until after sexual differentiation of the brain is completed in rats [20].

The concentration of free estradiol in female pups was

fourfold higher at 4 h after birth than on Day 19 of fetal life and was equivalent to values observed during proestrus in adult females. A possible explanation for variability in the percent of total circulating estradiol that is free, despite the high and relatively stable concentration of immunoassayable alpha-fetoprotein, is the existence of different isoforms of alpha-fetoprotein, some of which bind estradiol very weakly [59]. Changes in the proportion of different forms of alpha-fetoprotein, which show high versus low binding of estradiol due to differences in glycosylation, during the prenatal and neonatal period of sexual differentiation could thus account for the differences in percentage of free estradiol in blood during the transition from prenatal to postnatal life (Table 1). The increase in glucocorticoid levels associated with parturition, as well as changes during perinatal life in circulating concentrations of fatty acids to which alpha-fetoprotein also binds [60], could potentially mediate changes in the proportion of the total immunoassayable alpha-fetoprotein in blood that binds estradiol [61].

The very high concentration of free estradiol at 4 h after birth is particularly interesting in that it occurs coincident with a dramatic surge in circulating testosterone in newborn male, but not female, rats [13, 21, 22, 62]; male pups also show this elevation in free estradiol at 4 h after birth [13]. While gonadal steroids have effects on sexual differentiation throughout the prenatal and neonatal period of sexual differentiation [1], the first few hours after birth in rats are particularly important with regard to organizational effects of gonadal steroids or exogenous chemicals on sexual differentiation of brain function, particularly defeminization [63], and the concentration of free estradiol is higher at 4 h after birth than at any other time during sexual differentiation that we have examined [13]. Circulating estradiol, in conjunction with the dramatic surge in testosterone that occurs at this time in neonatal male but not female rats [13, 63], may thus play a particularly important role in influencing estrogen target cells that are differentiating during this time in development.

In summary, the significance of our findings is that absence of masculinization and defeminization in fetal and newborn female rats has been viewed as being due to the inhibition of uptake of virtually any circulating estradiol into the brain and other tissues by alpha-fetoprotein. However, in female rat fetuses and neonates, the blood levels of free estradiol are within the physiologically active range observed in adult females, and at these levels, circulating estradiol can enter brain cells and bind to receptors in cell nuclei. We propose that a similar uptake into target tissues of circulating estrogen also occurs in male fetuses and neonates, and that circulating estradiol interacts with circulating testosterone in regulating the differentiation of the brain and other tissues, particularly in the reproductive tract. The absence of defeminization in female rats may thus be due, not to an absence of estrogen uptake into the brain due to the presence of high levels of alpha-fetoprotein, but to the fact that defeminization primarily occurs during postnatal life when blood testosterone levels are markedly lower than in males. Also, brain androgen receptor numbers are low during prenatal sexual differentiation when blood testosterone levels in females are relatively high. What remains to be resolved, particularly in males, is the proportion of intracellular estradiol that derives from aromatization of testosterone versus the proportion that derives from circulating estradiol in neurons (and cells in other tissues) that contain both aromatase and estrogen receptors. It is possible that there are neurons containing estrogen receptors but not aromatase, and in these neurons only circulating estradiol could influence events such as proliferation and differentiation in both males and females. A variety of roles for circulating estrogen in sexual differentiation of males and females must now be considered in light of the physiologically relevant levels of free estradiol in blood that are available to enter tissues during sexual differentiation.

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