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Embryonic Rodent Brain Contains Estrogen Receptors

Abstract. *Estradiol-binding proteins with the properties of putative estrogen receptors are present in cytosol extracts of embryonic mouse hypothalamus and other brain regions. These embryonic estrogen receptors are adultlike in their high affinity and limited capacity for estradiol, sensitivity to diethylstilbestrol, ability to adhere to DNA, and behavior during DNA-cellulose affinity chromatography. As early as 4 days before birth, mouse hypothalamus has approximately 40 percent of the adult concentration of hypothalamic estrogen receptors with these properties. These observations raise the possibility that embryonic rodent brain has the biochemical potential to respond to sex hormones and that the critical period of brain sexual differentiation could be initiated prenatally.*

Sexual differentiation of rodent brain is influenced, in part, by steroid hormones acting during a "critical period" of brain development (*1, 2*). Although in mice and rats it is believed to occur during early postnatal development, several lines of evidence suggest that the critical period of brain sexual differentiation actually begins during late embryonic development (*1, 2*). For instance, perinatal administration of androgens and estrogens to rodents masculinizes and defeminizes both the genitalia and adult sexual behavior (*1*). Also, the in utero proximity of females to males (and, presumably, exposure to intrauterine androgens) correlates with the degree of androgenized genitalia and sexual and aggressive behavior exhibited by adult female mice and rats (*2*).

Since sex hormones play a key role in sexual differentiation, there is considerable effort to demonstrate putative receptor proteins for sex hormones in rodent brain during the critical period. Recently, several reports have demonstrated putative androgen and estrogen receptors in neonatal rodent brain (*3–8*). However, in the case of embryonic tissue, technical problems associated with the maternal circulation have prevented a similar analysis. In the absence of data concerning the existence of putative receptors in embryonic brain, understanding of the biochemical mechanisms that underlie the critical period is limited.

In the present study we report a new approach to the analysis of embryonic estrogen receptor which provides a more complete characterization of the estrogen receptor mechanism in mouse brain throughout its critical period. For the purposes of detection and subsequent analysis, we require that putative embryonic estrogen receptors from both hypothalamus and "brain" (whole brain minus hypothalamus) bind [^{3}H]estradiol and adhere to DNA-cellulose, thus distinguishing them from other estradiol-binding activities in embryonic brain extracts. We then ask whether these putative embryonic estrogen receptors are qualitatively similar to those of the adult in their affinity for estradiol, their sensitivity to diethylstilbestrol (DES), their DNA-cellulose elution characteristics, and their regional distribution within the brain.

To make these assessments of putative embryonic estrogen receptor, we developed a special protocol for DNA-cellulose affinity chromatography (legend to Fig. 1). Using this new approach, we found that cytosols of embryonic brain contain estradiol-binding macromolecules that adhere to DNA-cellulose (*5*). Under our conditions for DNA-cellulose affinity chromatography, cytosol estrogen receptors adhere to DNA-cellulose in the absence of estradiol (*9*), while estradiol-binding proteins not adhering to DNA as well as endogenous hormones are eliminated by washing (*3, 10*). Thus, by this procedure estrogen receptor can be labeled with [^{3}H]estradiol in the virtual absence of other known estradiol-binding proteins. This is important for quantitative analysis of embryonic estrogen receptor because embryonic cytosols contain high concentrations of both maternal estrogens and at least one estradiol-binding protein, the perinatal binding protein known as α-fetoprotein (AFP) (*11*). This is also true for neonatal cytosols, but to a lesser extent because these components decrease rapidly after parturition. Other receptor assays (*6, 8*), including conventional DNA-cellulose affinity chromatography (*3, 12*), are not as suitable for quantitative analysis since they utilize AFP-containing cytosols.

Cytosols of embryonic day 17 (E17) hypothalamus and "brain" (representing a total of 120 male and female embryos) were directly chromatographed on parallel DNA-cellulose columns, the columns were washed, and adhering macromolecules were then labeled with various concentrations of [^{3}H]estradiol. An estradiol-binding activity from E17 mouse hypothalamus (Fig. 1a) and "brain" (Fig. 1b) adhered to DNA-cellulose and reproducibly eluted with characteristics of estradiol receptor in a linear concentration gradient of NaCl. At all estradiol concentrations, a major estradiol-binding activity eluted with approximately 210 to 220 m*M* NaCl while a minor activity eluted with approximately 250 to 260 m*M* NaCl. These activities eluted from DNA-cellulose at the same salt concentrations as the 4*S* and 5*S* forms, respectively, of estrogen receptor from mouse uterine cytosols (*13*).

The putative estrogen receptor from both E17 hypothalamus and E17 "brain" appear to behave very similarly in that the major peak of estradiol-binding is positioned at 210 to 220 m*M* NaCl. At all embryonic and postnatal ages studied, we observe a slight qualitative difference in the overall elution patterns of hypothalamic versus "brain" receptor, although the data do not yet allow us to assess the significance of this subtle difference. Perhaps these patterns correlate with regional differences in the biological activity of these estrogen receptors. For example, estradiol induces an increase in progesterone receptor content in hypothalamus but not in other brain regions (*14*).

Comparison of the elution patterns obtained at several estradiol concentrations, as typified by Fig. 1, indicates that the DNA-adhering estradiol-binding activities in both E17 hypothalamus and "brain" saturate at estradiol concentrations between 3 and 8 n*M* (*15*). We find that putative embryonic estrogen receptors (from hypothalamus as well as "brain") at all ages tested (E16, E17, and E18) saturate in the same range of estradiol concentrations.

The data summarized in Fig. 1 also illustrate that macromolecular-bound [^{3}H]estradiol is fully competed by a 100-fold excess of nonradioactive DES. This behavior is typical of prepubertal estro-

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gen receptor from hypothalamus (*3*) as well as uterus (*16*), and serves to further distinguish putative estrogen receptor proteins from the nonreceptor estradiol-binding protein AFP (*3*), which is present in large amounts at embryonic ages and is much less sensitive to this quantity of DES. Calculations comparing these to other experiments with unfractionated embryonic cytosols indicate that we have eliminated AFP binding sites which exceed receptor by at least a factor of 10^5.

The relative concentrations of DNA-adhering putative estrogen receptor in embryonic hypothalamus versus "brain" (E16 through E18) were also determined. Embryonic hypothalamus contains $(4.5 \pm 0.3) \times 10^{-17}$ mole per milligram of tissue (mean ± standard error of mean) ($N = 6$), and embryonic "brain" contains $(1.0 \pm 0.04) \times 10^{-17}$ mole per milligram of tissue ($N = 7$). The concentration of DNA-adhering putative estrogen receptor increases relatively little throughout late embryonic development. The maximum observed increase in hypothalamic receptor concentration from E16 to E18 is 30 percent, while no observable change in "brain" receptor concentration occurs during this period.

To validate our approach and to permit direct comparison of putative embryonic estrogen receptor with estrogen receptor from older animals, we analyzed cytosols of prepubertal (postnatal day 21 to 30) mouse brain with the protocol described for Fig. 1. At all estradiol concentrations tested, estrogen receptors from both prepubertal hypothalamus and "brain" adhere to DNA-cellulose and elute in a manner very similar to that shown in Fig. 1. Moreover, under these conditions prepubertal estrogen receptor saturates at estradiol concentrations between 5 and 8 n*M*. These results match our previous saturation measurements of prepubertal estrogen receptor obtained by other methods (*4*). Furthermore, as is typical of putative estrogen receptor (*3*, *12*), the macromolecule-bound estradiol that elutes from DNA-cellulose behaves as a 4*S* species during sucrose gradient sedimentation. We have determined that the estrogen receptor concentration in prepubertal hypothalamus is $(10 \pm 0.5) \times 10^{-17}$ mole per milligram of tissue ($N = 6$) and in prepubertal "brain" is $(1.3 \pm 0.03) \times 10^{-17}$ mole per milligram of tissue ($N = 4$). Taken together, our data suggest that the concentration of DNA-adhering estrogen receptor in embryonic hypothalamus is four- to fivefold higher than that in embryonic "brain," and increases approximately 130 percent over a 4-week period (E16 through postnatal day 30) while the concentration in "brain" increases only 25 percent. Because of our strategy to eliminate AFP by chromatography, these values represent only estradiol-binding entities with a capacity for defined DNA adherence. Other forms of putative estrogen receptor may exist in embryos but are not detected by this method. For example, we have reported (*12*) that prepubertal hypothalamus contains both DNA-adhering and -nonadhering putative estrogen receptors. Other methods would be required to detect such forms in embryonic extracts.

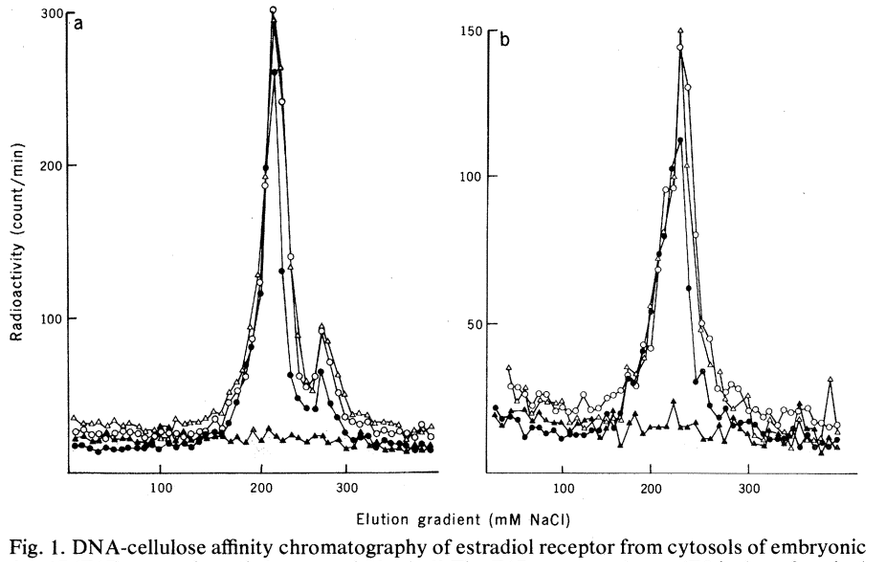

Fig. 1. DNA-cellulose affinity chromatography of estradiol receptor from cytosols of embryonic day 17 (E17) mouse hypothalamus and "brain." The E17 mouse embryos (E0 is day of vaginal plug; E19 and E20 represent day of birth) were produced in the timed-pregnancy C57BL/6J colony of our department. As the levels of cytosol estrogen receptors are equivalent for females and males of either neonatal (*3*) or prepubertal (*4*) mice, embryo sexes were not determined in this study. The whole brain was removed from each embryo and dissected rapidly. All subsequent steps were performed at 2°C. Hypothalamus and remaining "brain" were each placed in buffer containing 150 m*M* NaCl, 0.01*M* tris-HCl, *p*H 8.1 (21°C); 1 m*M* Na_3 EDTA; 1 m*M* mercaptoethanol; and 10 percent (by volume) glycerol. For E17 cytosols, the tissue to buffer ratio was eight hypothalami or four "brains" per 0.1 ml of buffer. The tissue was homogenized in a Kontes Teflon-glass homogenizer. The homogenate was combined with two 0.5-volume rinses of the homogenizer and centrifuged at 40,000 rev/min for 60 minutes at 2°C in a Beckman type 40 rotor. The supernatant fluid was stored in liquid nitrogen. On the day of the experiment, the rapidly thawed volumes were adjusted by conductivity analysis to 50 m*M* NaCl. Portions of E17 hypothalamus (equivalent to ~ 30 hypothalami or 200 mg of tissue) or E17 "brain" (equivalent to ~ 11 "brains" or 520 mg of tissue) were then directly chromatographed on 2-ml DNA-cellulose columns (*12*) equilibrated with buffer containing 50 m*M* NaCl. After it completely entered the column, each sample was allowed to remain in contact with the DNA-cellulose for 20 minutes. After they were washed with six volumes of buffer containing 50 m*M* NaCl and 1.0 mg of bovine serum albumin per milliliter for 2.5 hours, 2 ml of buffer containing [^{3}H]estradiol at the specified concentrations were layered on the columns and allowed to completely enter. The columns were incubated for 60 minutes and then washed with 12.5 volumes of buffer over 16 hours, a procedure that removes all unbound radioactivity. The [^{3}H]estradiol-bound contents of the columns were eluted with a 24-ml linear gradient of NaCl (50 to 400 m*M*). For each column, 0.5-ml fractions were collected and aliquots were removed to determine NaCl concentration with a Radiometer conductivity meter. Radioactivity was measured after the addition of 5.0 ml of Omnifluor (New England Nuclear)-toluene scintillation counting fluid. Symbols: 3.5 n*M* [^{3}H]estradiol, ●; 8.4 n*M* [^{3}H]estradiol, △; 13.0 n*M* [^{3}H]estradiol, ○; 8.0 n*M* [^{3}H]estradiol plus 100-fold excess nonradioactive DES, ▲; (a) E17 hypothalamus; (b) E17 "brain."

Collectively, our data indicate that embryonic mouse hypothalamus and "brain" contain putative estrogen receptors similar to those found in prepubertal and adult mouse brain. Our saturation measurements show that, for both embryonic and prepubertal cytosols, maximal macromolecular estradiol-binding occurs at estradiol concentrations between 3 and 8 n*M*. These data suggest that embryonic and prepubertal receptors share an affinity for estradiol. Before this study, the apparent affinity of neonatal estrogen receptor, which was considered representative of perinatal estrogen receptor, was thought to be one-half to one-fifth that of the adult (*3*, *6*, *7*). If this were the case, embryonic estrogen receptor would saturate at estradiol concentrations between 16 and 40 n*M*. Since saturation occurs instead between 3 and 8 n*M*, our embryonic data suggest that the affinity of perinatal estrogen receptor

is considerably more adultlike than was previously reported. We conclude that both the partial purification of estrogen receptor and the elimination of AFP before labeling makes more direct assessment of hormone affinity possible. In addition to the quantitative similarities, our data indicate that the estradiol-binding macromolecules from embryonic hypothalamus and "brain" are also qualitatively adultlike. Embryonic and prepubertal estrogen receptors adhere to DNA-cellulose, exhibit characteristic patterns when eluted from DNA-cellulose with a linear concentration gradient of NaCl, and are sensitive to DES.

In summary, we believe these data represent the most comprehensive analysis to date of embryonic estrogen receptors from brain of any mammalian species. This analysis was made possible by a new application of DNA-cellulose affinity chromatography which can be readily adapted (*17*) to analysis of estrogen receptors from other biological systems in which removal of nonreceptor estrogen-binding macromolecules is essential.

The masculinizing effects of prenatal exposure to sex hormones have been well documented in rodents (*1, 2*). The criticism can be raised, however, that sex hormones might be retained by embryos and act subsequently in the postnatal brain to produce these effects. However, our demonstration of putative estrogen receptors in embryonic mouse brain suggests that sex hormones might act prenatally, since estrogen receptors might mediate the actions of both estrogens and androgen metabolites (*18*). Together with our observations that embryonic mouse brain also contains putative androgen receptor (*5*) and that embryonic rat brain contains estrogen receptor (*19*), it now appears possible that embryonic rodent brain has the biochemical potential to respond to its sex steroid environment.

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Teleostean Urophysis: Urotensin II and Ion Transport Across the Isolated Skin of a Marine Teleost

Abstract. *The caudal neurosecretory peptide urotensin II rapidly inhibits by 30 percent the short-circuit current across the isolated skin of a marine teleost. The effect appears to be specific and cannot be attributed to actions of epinephrine, urotensin I, or arginine vasotocin. The results strongly suggest that urotensin II may act directly on ion-transporting cells involved in teleostean hypoosmoregulation.*

The caudal neurosecretory system, including its neurohemal organ, the urophysis, is unique to fishes and is now known to produce at least two biologically active peptides, designated urotensins I and II (UI and UII), as well as specific carrier proteins, the urophysins (*1*). Ten years ago, two effects of the urophysis were reported: stimulation of water absorption by the toad bladder (*2*) and stimulation of contraction of the trout urinary bladder (*3*). Both of these effects had possible implications for a role of the urophysis in osmoregulation. The former effect is now considered to be due to a factor indistinguishable from arginine vasotocin (AVT) found only in some teleostean urophyses (*1*) and the latter to the general spasmogenic action of UII in fishes (*1*). A relationship between the urophysis and osmoregulation in teleosts has long been suggested (*4*); however, no direct effect of urophysial peptides on transport processes has yet been shown. Here we report an unequivocal effect of UII on ion transport across the isolated skin of the long-jawed goby *Gillichthys mirabilis*, a euryhaline marine teleost.

We have observed that *Gillichthys* skin actively excretes chloride ions, when bathed on both sides with Ringer solution in an Ussing-style membrane chamber with the spontaneous transepithelial potential difference (*PD*) clamped to zero (*5*). Further, the short-circuit current (I_{sc}) at zero *PD* was found to equal the net efflux of chloride, as monitored by radioisotope fluxes, whereas there is no net flux of sodium

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