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# Estrogen and Androgen Receptor Proteins in Embryonic and Neonatal Brain: Hypotheses for Roles in Sexual Differentiation and Behavior

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SYNOPSIS. We have demonstrated and partially characterized putative estrogen and androgen receptors from mouse hypothalamus for a range of perinatal ages. For the first time, estrogen and androgen receptors from embryonic mouse and rat hypothalamus are described and characterized; they display similar parameters as the receptor proteins of adult mice and rats. The ontogeny of these proteins is discussed in the context of models for the control of the "critical period" of sexual differentiation of the brain.

The androgen-binding proteins, presumed to be receptors, are compared for hypothalamus and kidney and for the androgen-resistant mutant mouse, testicular feminization (*Tfm*). The putative receptor forms that are observed help to define the possible function of brain androgen receptors during sexual differentiation.

Development and modification of DNA-cellulose chromatography for the affinity separation of steroid receptors of brain is described. The methods allow complete separations of receptor proteins from non-receptor, steroid-binding proteins and subsequent analysis of the resultant receptors.

## INTRODUCTION

Sex steroids, including androgens and estrogens, help direct the sexual differentiation of the brain. Mediation of these developmental events and subsequent adult functions may require several macromolecules. Elucidation of possible mechanisms for these processes depends in part on descriptions of putative hormone receptors within the "critical period" of steroid responsiveness. By using DNA-cellulose affinity chromatography, we have been able to analyze the very low levels of sex steroid receptors that exist in developing mouse brain. We now apply this methodology to embryonic tissues and consequently can propose possible mechanisms for steroid function throughout the perinatal period.

Information on the putative androgen receptor has been strengthened by the availability of a mutant with deficient receptor (Fox, 1975*c*). Since the mutant animal possesses some residual receptor, we report on the nature of this activity for two tissues in the mouse.

## ESTROGEN AND ANDROGEN RECEPTORS IN EMBRYONIC AND NEONATAL RODENT BRAIN

### *Chromatography of receptors on DNA-cellulose*

We have fractionated and characterized mammalian DNA-binding proteins by affinity chromatography with columns of DNA-cellulose (Fox and Pardee, 1971; Herrick and Alberts, 1976). Taking advantage of the affinity for DNA of several steroid receptor proteins (Yamamoto and Alberts, 1972; Yamamoto *et al.*, 1974), we adapted this method for the analysis and comparison of uterine and hypothalamic estradiol receptors (Fox and Johnston, 1974). Not only has DNA-cellulose chromatography yielded qualitative and quantitative data and concentration of steroid receptors, but it also provides a

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matrix for studying the dynamic processing of receptor forms (Fox, 1977*a,b*, 1978). As some of these *in vitro* parameters correlate (Fox, 1977*b*) with *in vivo* characteristics (Linkie, 1977), it is significant that *in vitro* DNA-binding of glucocorticoid receptors correlates genetically with nuclear accumulation which occurs *in vivo* (Yamamoto *et al.*, 1974).

With this technology, we previously identified and characterized both estrogen and androgen receptors in cytosol extracts of hypothalamus/preoptic area of three to four week old mice (Fox and Johnston, 1974; Fox, 1975*a,c;* Wieland and Fox, 1976; Fox, 1977*a,b,c*, 1978; Wieland *et al.*, 1978). The fractionation of androgen and estrogen receptors in hypothalamic cytosol extracts is illustrated in Figure 1a. To demonstrate these receptors, $^3$H-labelled extracts are chromatographed sequentially on columns of Sephadex G-25 (not shown) and then DNA-cellulose (Fig. 1a). Steps of higher ionic strength elute the $^3$H-testosterone- and $^3$H-estradiol-labelled receptor proteins, respectively, from the DNA-cellulose. While approximately 90% of the androgen-labelled material elutes with 130 mM NaCl, the estrogen-labelled material elutes with concentration steps of NaCl at and above 210 mM. Thus, both putative receptors can be demonstrated and separated from one another on these columns.

The $^3$H-labelled material that flows directly through the columns in the 50 mM NaCl wash includes two components (Fig. 1a). The first is radioactivity that is bound

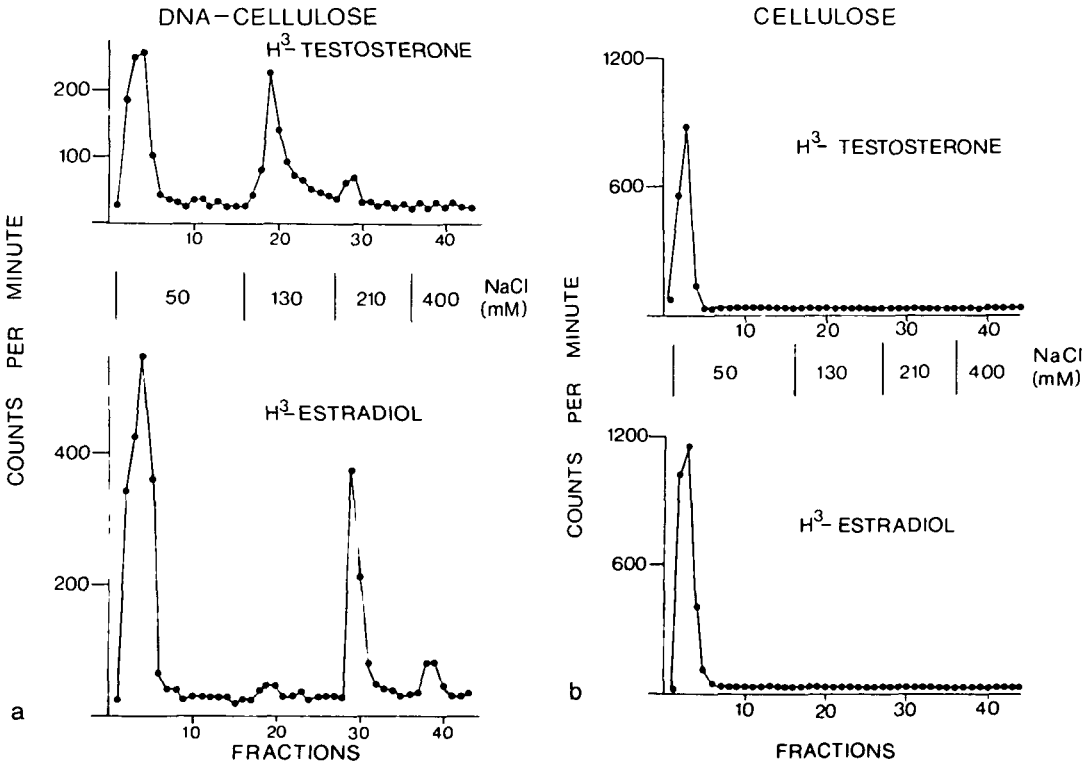


FIG. 1. Adherence of androgen and estrogen receptors to DNA-cellulose. Two extracts, each containing ten mouse hypothalamus-preoptic areas, were prepared as in Wieland *et al.* (1978). One was labelled with 10 nM $^3$H-testosterone, the other with 10 nM $^3$H-estradiol. The extracts were freed of unbound hormone by passage through Sephadex G-25 and each was then split into two equal portions. One portion was applied to DNA-cellulose (a), the other to cellulose columns (b). After a washing with low salt buffer, receptors were eluted from the DNA-cellulose columns by increasing the salt concentration in the indicated step-wise manner (Fig. 1a).

non-specifically or with low affinity to non-receptor macromolecules and does not adhere to DNA-cellulose. The second is a fraction of receptors that does not bind. Greater than 90% of the hypothalamic androgen receptor does bind typically to DNA-cellulose (Wieland *et al.*, 1978) while 50% of the estrogen receptor from hypothalamus binds to DNA-cellulose (Fox and Johnston, 1974; Fox, 1977*a,b*). Furthermore, the binding of these receptors to DNA-cellulose is DNA-dependent. Virtually none of the $^3$H-labelled material adheres to the control columns of cellulose (Fig. 1b).

*Demonstration and characterization of embryonic estradiol receptor.*

We achieved the first direct demonstration and fractionation of neonatal estradiol receptors by fractionation on DNA-cellulose (Fox, 1975*a,b*). This procedure separates the $^3$H-estradiol-bound receptors from a competing perinatal estradiol-binding protein, called $\alpha$-fetoprotein (AFP). Although AFP is present in high amounts and effectively competes with specific estradiol receptor for available $^3$H-estradiol in crude cytosol extracts, this low affinity estradiol-binding protein does not adhere to DNA-cellulose. When crude cytosol extracts, containing both specific estradiol receptor and AFP, are labelled with $^3$H-estradiol and fractionated on DNA-cellulose, only the estradiol receptor complex is selectively retained by the DNA-cellulose. Following elution, this receptor characteristically sediments at approximately 4S on sucrose density gradients.

Experimental data obtained in this fashion, along with hormone-binding (Barley *et al.*, 1974) and autoradiographic studies (Sheridan *et al.*, 1974), suggest the presence of typical adult-like estradiol receptors in brain tissue of neonatal mice. Furthermore, our data show that in the presence of AFP the receptor's apparent affinity for estradiol is reduced relative to that of the adult, and explains why demonstration of such receptors from young rodents had not been achieved earlier (Fox, 1975*b*).

Extending the application of DNA-cellulose affinity chromatography, we have demonstrated for the first time that adult-like estradiol receptor proteins exist in embryonic rat and mouse hypothalamus and other brain tissue (Vito and Fox, 1977; and manuscript in preparation). Two technically different approaches have been used to demonstrate, fractionate, and characterize embryonic estradiol receptors.

One approach involves labelling unfractionated cytosol extracts with near-saturating concentrations of non-radioactive estradiol. This insures that even in the presence of AFP, specific estradiol receptor also binds hormone. These extracts are then fractionated on DNA-cellulose, to which estradiol receptor adheres, while unbound estradiol and estradiol bound to AFP wash through. Columns containing specific estradiol receptor are now filled with a ten nano-molar solution of $^3$H-estradiol and incubated at an elevated temperature (30°C). This allows the receptor-bound, non-radioactive estradiol to "exchange" with the $^3$H-estradiol. The now radioactively-labelled estradiol receptor is eluted and recovered in the standard fashion. Subsequent analysis of these receptor preparations by sucrose density gradient sedimentation reveals a macromolecular complex typical of adult estradiol receptor. Therefore, "affinity-exchange" chromatography (Vito and Fox, 1977) has successfully demonstrated typical estradiol receptor in embryonic rodent brain.

A second and more quantitative approach has also been used to characterize embryonic brain estradiol receptor. It is similar to the previous one in that both methods exploit the affinity of DNA-cellulose for embryonic estradiol receptor to fractionate it from AFP. In this method, however, the receptor is labelled directly with $^3$H-estradiol in the absence of competing AFP and without the necessity of an exchange reaction. Under conditions of low ionic strength, unlabelled cytosols are directly chromatographed onto DNA-cellulose, the AFP is washed away, and then the estradiol receptor retained by the

column is labelled with $^3$H-estradiol at 4°C.

Using this technique, estradiol receptor from embryonic (2 days prior to birth) and adult hypothalamus/preoptic area (30 days after birth) are qualitatively very similar when eluted from DNA-cellulose by a linear concentration gradient of NaCl (Fig. 2a). Furthermore, embryonic estradiol receptor prepared in this manner sediments as a 4S species during sucrose density gradient analysis (Fig. 2b), typical of estradiol receptor from adult hypothalamus and of the embryonic receptor demonstrated by "affinity-exchange" (above). Additional experiments (Vito and Fox, manuscript in preparation) reveal that embryonic and adult estradiol receptors exhibit virtually identical hormone affinities and specificities.

*Ontogeny of estrogen and androgen receptors*

DNA-cellulose has provided the first direct demonstration and partial fractionation of estradiol receptors in both embryonic and neonatal hypothalamus. We also report for the first time the presence of low levels of androgen receptor in embryonic as well as neonatal (Fox, 1975*a,b,c*) mouse hypothalamus. Clearly the quantitative detection of both these receptors is highly dependent on the technical approaches that are available. For estradiol receptor, account has to be made for interference by AFP or perhaps other proteins in perinatal tissue (Fox, 1975*b*; Vito and Fox, 1977). For androgen receptors, extra care must be taken to obtain conditions for maintaining optimal hormone binding

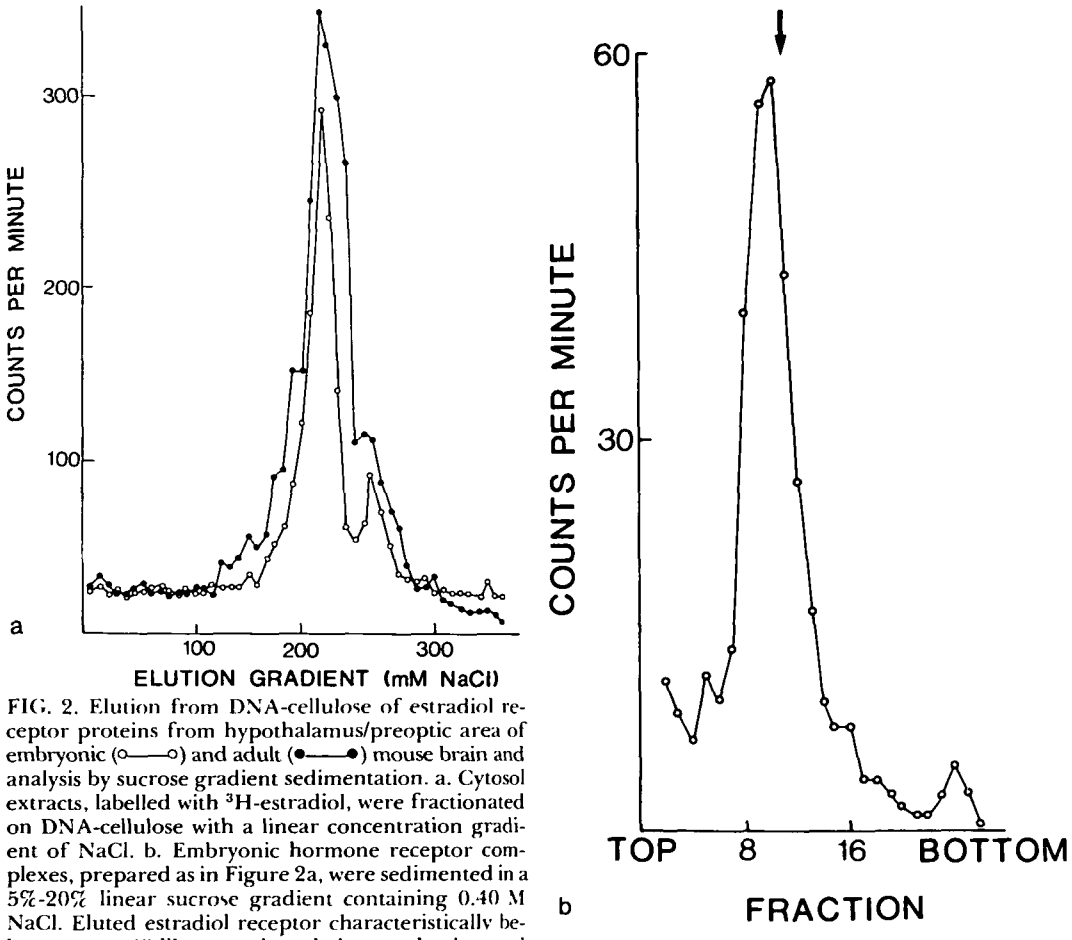


FIG. 2. Elution from DNA-cellulose of estradiol receptor proteins from hypothalamus/preoptic area of embryonic (o——o) and adult (•——•) mouse brain and analysis by sucrose gradient sedimentation. a. Cytosol extracts, labelled with $^3$H-estradiol, were fractionated on DNA-cellulose with a linear concentration gradient of NaCl. b. Embryonic hormone receptor complexes, prepared as in Figure 2a, were sedimented in a 5%-20% linear sucrose gradient containing 0.40 M NaCl. Eluted estradiol receptor characteristically behaves as a 4S-like protein relative to the internal fluorescent marker, dansyl-BSA.

(Fox, 1975*c*). DNA-cellulose chromatography helps to achieve both these goals.

Based on the data summarized here, we tentatively depict the ontogenic pattern of androgen and estrogen receptors for perinatal mouse hypothalamus (Fig. 3). It is now evident that both types of receptors are present in hypothalamus before birth. Increases in levels of receptor relative to tissue weights and protein contents appear to differ for the two types. It may prove, as this study now suggests, that the androgen receptor is present at rather low levels prior to birth and increases throughout the perinatal period at a significant rate. In contrast, the estrogen receptor may be present at significant levels quite early in the embryo and gradually increase in concentration. Additional data for young postnatal mice (Attardi and Ohno, 1976) is consistent with this quantitative difference in ontogenic patterns for the two receptors.

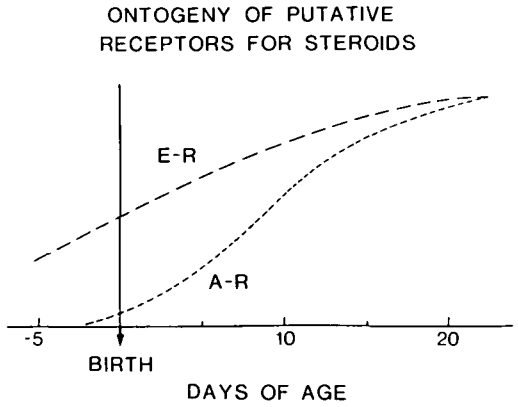


FIG. 3. Appearance in embryonic and neonatal mouse hypothalamic cytosol extracts of receptor macromolecules specific for estrogens (E-R) and androgens (A-R). This current approximation summarizes data based in large part on DNA-cellulose chromatography of extracts labeled with either 17$\beta$-estradiol or with the androgens, testosterone or 5$\alpha$-dihydrotestosterone. The profiles are arbitrarily normalized to respective pre-pubertal (three weeks old) levels for emphasis of the perinatal period and are not meant to depict the exact shapes of the curves. This figure illustrates the relative rates of increase for these two specific receptor types. Previous studies (Fox, 1977*c*) indicate that at three weeks of age and older, the absolute level of estrogen receptor in hypothalamus is about double the level of androgen receptor. To date, the cytosol levels of binding for estrogens and androgens appear the same for both females and males.

## CHARACTERIZATIONS OF PUTATIVE ANDROGEN RECEPTORS

Having demonstrated the existence of the presumed receptor proteins for both estrogens and androgens in embryonic and neonatal hypothalamus of mouse, the next task is to determine the functions of these macromolecules. Specifically, *are* they true hormone receptors, and do they mediate the effects of specific steroids? If one or both of them do function in this manner, at what ages and times are they functional? For example, does the existence of either the estrogen or the androgen receptor in embryonic brain indicate that cellular responses to these specific sex steroids occur, or might these macromolecules appear in tissues prior to other components required for responsiveness to steroids?

To gain insights into possible mechanisms for steroid action, we have compared steroid-binding macromolecules in separate tissues, in a specific mouse mutant, and in a primate. Previously, we noted differences in DNA-binding properties of estrogen receptors from hypothalamus and uterus (Fox and Johnston, 1974). Subsequently, these differences were shown to correlate with reported *in vivo* differences (Linkie, 1977) for the behavior of estrogen receptors in those two tissues (Fox, 1977*a,b*). It is hoped, therefore, that the biochemical descriptions of estrogen and androgen receptors from diverse sources will lead to understanding their diverse processes *in vivo*.

### *Androgen receptors from normal hypothalamus and kidney*

When eluted from DNA-cellulose with a linear concentration gradient of NaCl, the androgen receptors from hypothalamus and kidney exhibit quite different profiles (Fig. 4). The patterns indicate that all of the receptor from hypothalamus is represented in the kidney by a corresponding species. The resolution obtained is sufficient to suggest that kidney contains an additional species of androgen receptor (eluting between 180 and 260 mM NaCl)

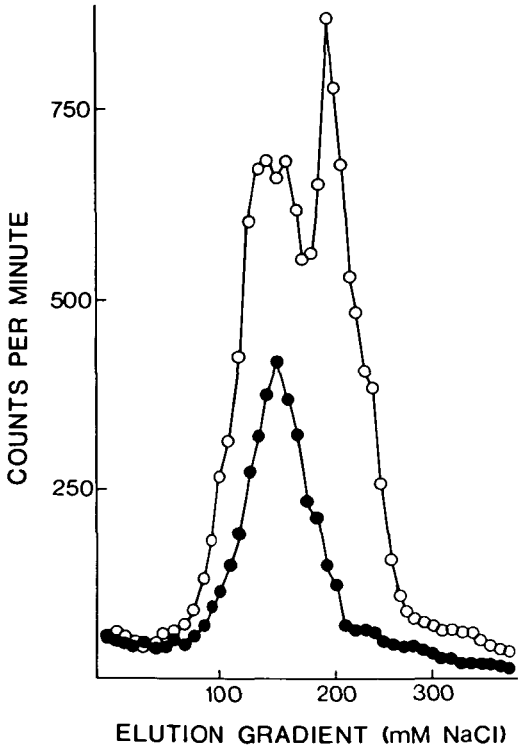


FIG. 4. Chromatography of hypothalamic and kidney androgen receptors on DNA-cellulose. Extracts of mouse hypothalamus (●——●) or kidney (○——○) were incubated with 10 nM $^3$H-dihydrotestosterone, applied to columns of denatured DNA-cellulose containing 50 mM NaCl, and washed with at least 12 column volumes of 50 mM NaCl buffer. Columns were then eluted with a linear concentration gradient of NaCl ranging from 50 to 400 mM.

that may be, at most, a minor component of hypothalamus.

This difference recalls a comparable contrast in patterns for the estrogen receptor from hypothalamus and the peripheral tissue, uterus. Under these same conditions of DNA-cellulose chromatography, one major species is found in hypothalamus, whereas a different species is prominent in uterus, as determined by sedimentation analysis (Fox and Johnston, 1974). These two species correspond to the major and minor DNA-elution peaks depicted for hypothalamic estrogen receptor in Figure 2a. In contrast, the higher salt eluting peak (Fig. 2a) is the major component of uterine estrogen receptor.

We have discovered conditions for converting estrogen receptors from the hypothalamic form to the uterine form (Fox, 1977*a*,*b*; 1978). Experiments are progressing in our laboratory to determine whether an analogous relationship exists for the major species of androgen receptors from hypothalamus and kidney.

### *Residual Tfm receptor in hypothalamus and kidney*

For our earliest studies of androgen-binding macromolecules in mouse hypothalamus (Fox, 1975*a*) a mutant was analyzed as a control for the genetic specificity of this activity. High affinity androgen binding is deficient in cytosol extracts of the androgen-resistant mouse mutant, testicular feminization (*Tfm*) (Fox, 1975*c*, 1977*c*; Wieland *et al.*, 1978). The deficiency of this protein in animals that are resistant to androgen is consistent with its functioning in the mediation of androgen responses.

Those studies revealed, however, that a detectable level of residual binding activity (10 to 15% of normal levels) exists for *Tfm*. This residual activity also adheres to DNA-cellulose and resembles the normal receptor in both sedimentation parameters and affinity for androgens (Wieland and Fox, 1976).

Some studies of this androgen-resistant mutant suggest that it may manifest certain responses to androgens. Given the possibility that the residual androgen receptors in the mutant may mediate these responses, we have undertaken to define this binding activity in detail (Wieland *et al.*, 1978). In turn, this analysis may help to determine definitively whether the *Tfm* lesion causes a complete androgen-insensitivity or an incomplete "resistance" to certain androgen responses. Among the possible descriptions of this residual mutant activity are the following: 1) The *Tfm* activity may be a low amount of the normal androgen receptor. 2) Normal animals may contain a minor androgen receptor species, which is "unmasked" in this mutant by the absence of the major component. 3) The *Tfm* activity may be an altered protein.

To compare androgen receptors from

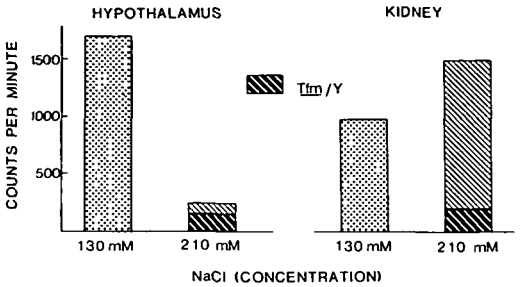


FIG. 5. Comparison of DNA-binding for normal and *Tfm*/Y androgen receptors. Extracts of normal and *Tfm*/Y hypothalamus and kidney were labelled with $^3$H-DHT, applied to DNA-cellulose columns, and washed as in Figure 4. Receptors were then eluted by sequentially increasing the NaCl concentration in the elution buffer to 130 mM and 210 mM. Heights of bars represent net activity recovered with each step.

normal and *Tfm* mice, we examined their DNA-binding properties (Fig. 5). In both tissues, a major fraction of the normal receptors elutes with 130 mM NaCl and the remainder elutes with 210 mM NaCl. These step-wise elutions conform to the pattern expected from Figure 4. The behavior of the *Tfm* activity differs from normal for both hypothalamus and kidney; all of the *Tfm* activity elutes with 210 mM NaCl. It remains to be determined conclusively whether the *Tfm* activity is a normal or an altered receptor protein and whether this different behavior is a characteristic intrinsic to the hormone-binding protein.

*Androgen-binding macromolecules in extracts from the rhesus monkey,* Macaca mulatta

Characterizations of the putative androgen receptors from the mouse and rat have been broadened by comparing their properties in different tissues such as hypothalamus and kidney with those of the residual activity for the androgen-resistant mutant, testicular feminization (*Tfm*). The androgen-binding activity from all tissues studied has similar high affinities (within two- to three-fold) for both testosterone and dihydrotestosterone and is easily blocked by estradiol (Bullock and Bardin, 1974; Fox, 1975*a,c*). For estradiol the apparent inhibitory constant is within ten-fold of the androgen affinity (Attardi *et al.*, 1976). To try to determine the significance of these affinities we have examined androgen-binding macromolecules in extracts from the rhesus monkey.

Judging by DNA-cellulose chromatography and sedimentation criteria, we have not yet convincingly identified androgen receptors for extracts of rhesus brain tissues. We have, however, obtained evidence for significant quantities of high-affinity androgen-binding macromolecules (Table 1). This activity binds both dihydrotestosterone and testosterone and, furthermore, this binding can be blocked by steroidal estrogens. Similar activities have been detected in hypothalamus, cerebellum and

TABLE 1. *Specificity of androgen binding in rhesus hypothalamus.*

| Labeled steroid | Competitor | % Binding* |
|---|---|---|
| $^3$H-Dihydrotestosterone | ---- | 100 |
| | Dihydrotestosterone | 0 |
| | Testosterone | 1 |
| | Estradiol | 10 |
| $^3$H-Testosterone | ---- | 100 |
| | Testosterone | 0 |
| | Dihydrotestosterone | 7 |
| | Estradiol | 24 |

* Extracts of hypothalamus were labelled with 5 nM $^3$H-androgen. Competing non-radioactive steroids were included at 1000 nM (× 200). The % binding (Fox, 1975*c*) is normalized to 100 for the non-competed sample. The 100% values represent $8.2 \times 10^{-15}$ moles bound dihydrotestosterone per mg protein (714cpm) and $7.9 \times 10^{-15}$ moles bound testosterone per mg protein (409cpm), respectively. These specificities, obtained for old rhesus females (approximately 20 years old), are typical of all the experiments. However, for younger females (approximately 7 years old) we obtain $45 \times 10^{-15}$ moles bound androgen per mg protein (4640cpm).

cerebrum for dihydrotestosterone (with a $K_D$ of 0.5-0.6 $\times$ $10^{-9}$M).

A similar activity was detected for extracts of these same brain regions (and other tissues) by analysis with LH-20 columns (Ivan Lieberburg, Neil Maclusky, Lewis Krey and Bruce McEwen, personal communication). These investigators found virtually the same activity in samples of rhesus serum (diluted 100-fold). Since the tissue blocks contain some contaminating serum, they calculate that this activity may be fully accounted for by the serum content. This conclusion is consistent with rhesus experiments conducted at the Wisconsin Regional Primate Research Center by William Bridson (manuscript submitted for publication). Using polyacrylamide methods, Bridson has extensively studied the physiological parameters of plasma binding by dihydrotestosterone, testosterone and estradiol. He finds similar results as for sex steroid binding globulins reported for other primates (King and Mainwaring, 1974).

Given these results and the presence of blood in the tissue blocks we examined, it is probable that the androgen binding we report here for rhesus is also a plasma-binding component. In probing the molecular basis for the multiple affinities of the rodent putative androgen receptor, this protein may prove illuminating since it can also bind dihydrotestosterone and testosterone and be blocked by estradiol. More important technically, however, may be the requirement to remove this component in further searches for a possible androgen receptor for primate brain, perhaps in the way that removal of AFP facilitated fractionation of the rodent estrogen receptor from perinatal rodent extracts.

## INVOLVEMENT OF STEROID RECEPTORS IN SEXUAL DIFFERENTIATION OF BRAIN

The role of estradiol as a mediator of androgen effects during sexual differentiation of the brain continues to be documented and is strongly supported, as described in this symposium. Our descriptions of estradiol receptors, their interactions with DNA, and their conversions to "nuclear" forms, define mechanisms by which perinatal estrogens, derived from androgen, may operate. However, demonstration of androgen conversions to estrogens and subsequent binding to estrogen receptors does not preclude the involvement of direct androgen receptors in the complete process of androgen mediation. Evidence that the neonatal effects of exogenous testosterone can be blocked either by the estrogen antagonist MER-25 (McDonald and Doughty, 1973/74; Doughty and McDonald, 1974) or by the antiandrogen, cyproterone acetate (Wollman and Hamilton, 1967; Arai and Gorski, 1968) suggests that both estrogens and androgens may be necessary as ligands for neonatal response to androgen. These and other arguments (Fox, 1975*c*; Attardi and Ohno, 1976) raise the question: Do the receptor-like androgen-binding proteins detected in hypothalamus function in normal as well as experimentally elicited masculinization?

Available evidence does not definitively specify the function of the presumed hypothalamic androgen receptor. With the goal of testing their plausibilities, we consider several possible functions (Table 2).

The events of sexual differentiation may require both estrogen and androgen receptors (Table 2,A) as has been discussed

TABLE 2. *Possible functions of the androgen receptor in sexual differentiation.*

| | |
|---|---|
| A. | Participates in conjunction with the estrogen receptor |
| B. | Participates singly for certain events |
| C. | Participates only in adult responses |
| D. | Signals the end of the critical period |
| E. | Serves other functions than as a "receptor" |

above. While available data (aptly summarized in the symposium) strongly implicates the estrogen receptor, evidence for the androgen receptor is much less complete. Subtler methodologies than those available for examining the estrogen receptor may be required for detecting androgen receptor involvement. This may be especially true if effective levels of androgen binding in developing brain are very low or occur in very few cells. It is also possible, however, that androgen receptors may be required alone for some physiologic functions (Table 2,B). Such events might be revealed as investigators elucidate and detail more fully the biochemical and morphological responses to androgen during the "critical period."

Since the "critical period" may contain different points of steroid responsiveness, both A and B in Table 2 could occur. Several lines of evidence suggest that multiple responses to steroid do occur during a period extending from several days before birth until approximately ten days after birth for rats (Barraclough, 1968). Consistent with these responses to exogenous steroids are observations on the sexual behavior of normal female rats and mice. These female rodents exhibit increasing degrees of male-type behavior as a result of their proximity *in utero* to male embryos (Clemens, 1974; Gandleman *et al.*, 1977; Gerall, 1978). Presumably these graded behaviors reflect prenatal responses to exogenous androgens. In contrast, neonatal castration of male rats results in their lacking certain male characteristics while exhibiting female characteristics. If androgens were only needed at a single point during the "critical period" and if the "critical period" includes embryonic life, then castrated males ought to be already masculinized at birth. Therefore, either androgen must be present postnatally for masculinization, or the essential triggering in the "critical period" may be a multipoint process.

The appearance of androgen receptors in embryonic and neonatal hypothalamus may precede their functioning in adult behavior (Table 2,C). However, if their appearance perinatally rises as sharply as is suggested in Figure 3, it may be that they function at the interface between the "critical period" and more mature periods. A signal for the end of the "critical period" might be required for "fixing" sexual differentiation, and this signal might involve the level of androgen receptor that appears at this time (Table 1,D).

Finally, we and others use the term "steroid receptors" loosely to label a class of macromolecules with properties of high affinities for specific steroids and characteristic sedimentation behaviors. Definitive proof does not exist, however, that any of these are truly "receptors," *e.g.*, direct mediators of primary steroid response. Even if the evidence for several other steroid receptors may be increasingly convincing, we do not know that the presumed receptor for androgens, with its multiple selectivities, functions as they do. Thus the study of these proteins in diverse tissues—particularly informationally rich ones, such as in the neuroendocrine axis—may help to enlighten us in this research.

In considering the relative functions of estrogen and androgen receptors (discussion of Table 2), several notions about the critical period surface. Not only may there be several trigger points for steroid responsiveness in the critical period, but for each event of sexual differentiation, a two-point process may be required; triggering events may have to be "fixed" by a later event. Stated differently, after a decision is made between female and male states during sexual differentiation, a specific, additional signal may be necessary to prevent a change in this decision.

Several components that may comprise the "critical period" of sexual differentiation are depicted in Figure 6. Emphasizing aspects of timing, are two contrasted models for defining the end of the "critical period." Multiple points (vertical lines) for steroid responsiveness are represented in both. The earliest periods of effective response to steroid may define the beginning of the "critical period." However, the end of the "critical period" may be especially important for maintaining differentiated

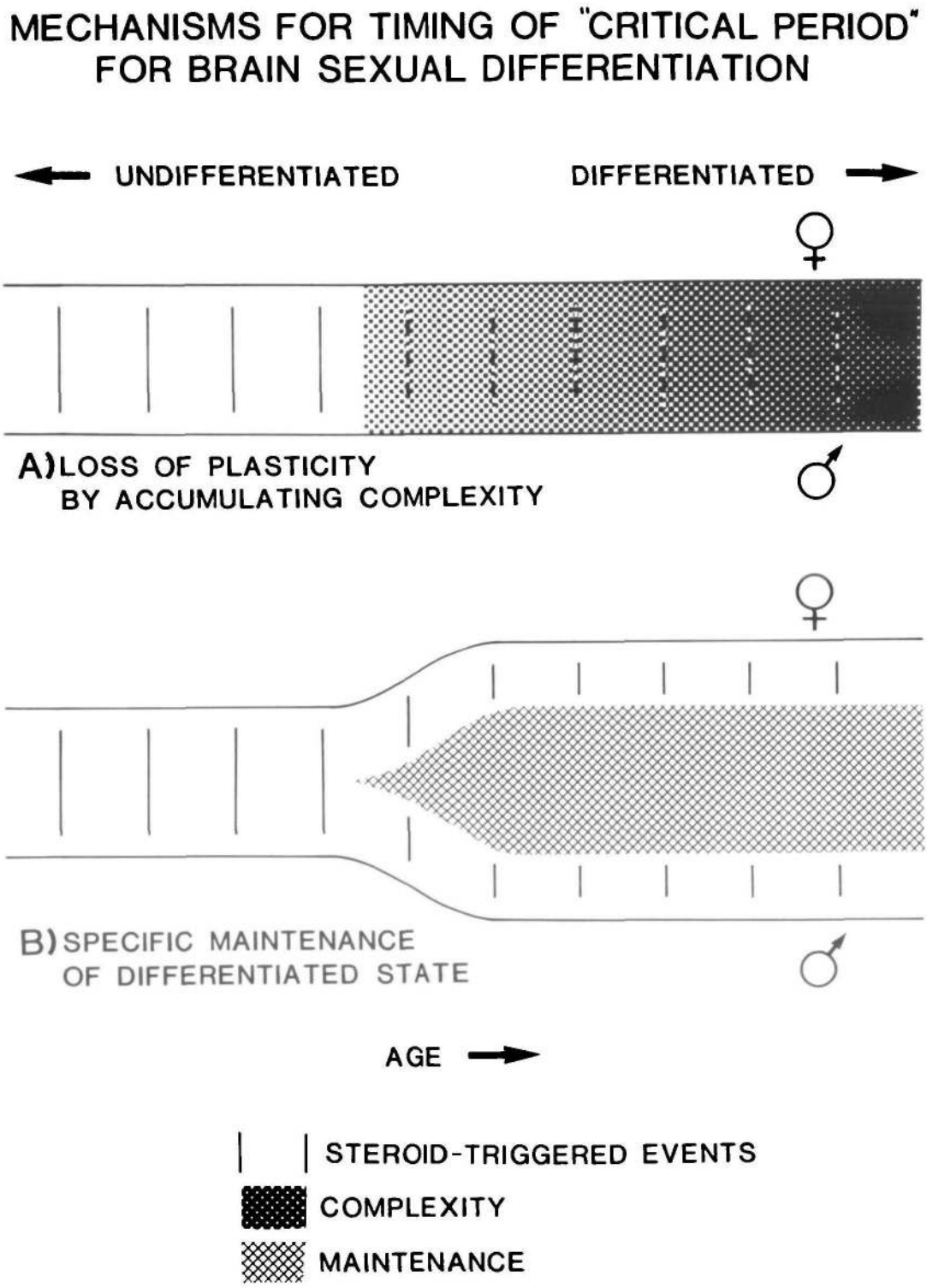


FIG. 6. Speculative models for timing of the beginning, progression, and end of the "critical period" for sexual differentiation of brain.

states. Accordingly, a specific active mechanism may exist to marshall in the "differentiated" state.

Mechanism A) in Figure 6 indicates a critical period that ends due to obstruction by competing structures and processes. Accumulating complexities of events may interfere with the potential for plasticity, even though the mechanisms for steroid responsiveness might remain. Therefore, with this mechanism, plasticity ceases merely as a consequence of other events. However, interruption of those complexities or events might allow the brain to regain plasticity. In contrast, mechanism B) invokes specific maintenance of the differentiated state; in a sense, this is directed and continuous inhibition of the potential for plasticity. As suggested in Table 2 (part D), steroids may function in such a process. Insufficient maintenance, such as the removal of the active agent, could recall a state of plasticity. The actual physiology might be described best by a combination of A) and B).

Two series of experimental observations illustrate the ideas presented in Figure 6. The first concerns inhibition of female sexual behavior (lordosis) by the intact septum and the potential for recalling the critical period in adults (Nance *et al.*, 1977*b*). Female rats receiving septal lesions as adults exhibit increased behavioral sensitivity to estrogen when tested several weeks later (Nance *et al.*, 1974, 1975*a*). However, chronic doses of testosterone propionate following these lesions attenuate the increase in sensitivity (Nance *et al.*, 1977*b*). Unlike females, adult male rats normally exhibit little lordosis behavior. However, when given septal lesions, accompanied either by extended estrogen exposure (Nance *et al.*, 1975*b*) or the imposition of a hypothyroid state (Nance *et al.*, 1977*a*), operated males subsequently tested several months later also display a full lordotic response to estrogen. Consequently, such males acquire as adults the behavior of females and neonatally castrated males. If lordosis is considered as one example of a behavior which is sexually differentiated during the "critical period," then this effect can be taken to represent a recall of that "critical period."

On the one hand, this lesion may allow plasticity by interrupting anatomical entities (Fig. 6, mechanism A). Steroid and thyroid hormones may control the expression of the lesioning effect by regulating the ability or inability of the inhibiting anatomical entities to recover function (Nance *et al.*, 1977*a*). On the other hand, androgen may prevent plasticity by a specific maintenance mechanism (Fig. 6, mechanism B); hence, the lack of facilitation of lordosis in both lesioned females given androgen and in lesioned males with no concurrent administration of hormone. Estrogen and thyroid deficiency may antagonize this androgen response, thus allowing plasticity and consequently function, thereby allowing altered differentiation.

A second line of experimentation indicates that the end of the critical period is variable. Hypothyroidism was shown to lengthen the period during which androgen could significantly induce persistent estrus in rats, whereas injections of thyroxine reversed the effect (Kikuyama, 1969). In a separate study (Phelps and Sawyer, 1976), effects of early androgen administration on lordosis were reduced by thyroxine injection. Thyroxine may have acted by influencing the end of the critical period. Such effects could occur either by general effects on maturation (Fig. 6,A) or by a specific mechanism for fixing the differentiated state by a hormonally dependent mechanism (Fig. 6,B).

We believe that the distinction emphasized by contrasting mechanisms A and B (Fig. 6) is a useful one if it suggests specific new experiments. By providing alternate and testable processes for the control of a "critical period" it may promote considerations that the "critical period" is both alterable and perhaps, recallable. Understanding the possible roles of hormones in controlling plasticity and its potential may permit impositions of optimal physiological states for redirection and recovery of functions in mature animals.

## REFERENCES


Arai, Y. and R. A. Gorski. 1968. Critical exposure time for androgenization of the rat hypothalamus determined by anti-androgen injection. Proc. Soc. Exp. Biol. Med. 127:590-593.

Attardi, B., L. N. Geller, and S. Ohno. 1976. Androgen and estrogen receptors in brain cytosol from male, female, and testicular feminized (tfm/y) mice. Endocrinology 98:864-874.

Attardi, B. and S. Ohno. 1976. Androgen and estrogen receptors in the developing mouse brain. Endocrinology 99:1279-1290.

Barley, J., M. Ginsburg, B. D. Greenstein, N. J. Maclusky, and P. J. Thomas. 1974. A receptor mediating sexual differentiation? Nature 252: 259-260.

Barraclough, C. A. 1968. Alterations in reproductive function following prenatal and early postnatal exposure to hormones. Advan. Reprod. Physiol. 3:81-112.

Bullock, L. P. and C. W. Bardin. 1974. Androgen receptors in mouse kidney: A study of male, female and androgen-insensitive (tfm/y) mice. Endocrinology 94:746-756.

Clemens, L. G. 1974. Neurohormonal control of male sexual behavior. *In* W. Montagna and W. A. Sadler (ed.), *Reproductive behavior,* pp. 23-52. Plenum Press, New York.

Doughty, C. and P. G. McDonald. 1974. Hormonal control of sexual differentiation of the hypothalamus in the neonatal female rat. Differentiation 2:275-285.

Fox, T. O. 1975*a*. Androgen and estrogen binding macromolecules in developing mouse brain. Neurosci. Abstr. 1:770.

Fox, T. O. 1975*b*. Oestradiol receptor of neonatal mouse brain. Nature 258:441-444.

Fox, T. O. 1975*c*. Androgen- and estrogen-binding macromolecules in developing mouse brain: Biochemical and genetic evidence. Proc. Natl. Acad. Sci. U.S.A. 72:4303-4307.

Fox, T. O. 1977*a*. Nuclear estradiol receptor formation: Serum complexing activity (5S-CA) utilized to compare mouse hypothalamus and uterus. In *Proceeding of the Second Annual (1976) Maine Biochemical Science Symposium,* pp. 544-572. Univ. Maine Press, Orono.

Fox, T. O. 1977*b*. Conversion of the hypothalamic estradiol receptor to the nuclear form. Brain Res. 120:580-583.

Fox, T. O. 1977*c*. Estradiol and testosterone binding in normal and mutant mouse cerebellum: Biochemical and cellular specificity. Brain Res. 128:263-273.

Fox, T. O. 1978. Selective complexing of the "nuclear" 5S estradiol receptor by a serum component, 5S-CA. Proc. Nat. Acad. Sci. U.S.A. 75: 2664-2668.

Fox, T. O. and C. Johnston. 1974. Estradiol receptors from mouse brain and uterus: Binding to DNA. Brain Res. 77:330-336.

Fox, T. O. and A. B. Pardee. 1971. Proteins made in the mammalian cell cycle. J. Biol. Chem. 246:6159-6165.

Gandelman, R., F. S. vonSaal, and J. M. Reinisch. 1977. Contiguity to male foetuses affects morphology and behaviour of female rats. Nature 266:722-724.

Gerall, A. A. 1978. Differentiation of sexual behavior in rodents. *In* R. W. Goy and B. S. McEwen (eds.), *Sexual differentiation of the brain.* Neurosciences Research Program Bulletin, MIT Press, Cambridge.

Herrick, G. and B. Alberts. 1976. Purification and physical characterization of nucleic acid helix-unwinding proteins from calf thymus. J. Biol. Chem. 251:2124-2132.

Kikuyama, S. 1969. Alteration by neonatal hypothyroidism of the critical period for the induction of persistent estrus in the rat. Endocrinol. Jap. 16:269-273.

King, R. J. B. and W. I. P. Mainwaring. 1974. *Steroid-cell interactions,* pp. 45-50. Univ. Park Press, Baltimore.

Linkie, D. M. 1977. Estrogen receptors in different target tissues: Similarities of form—dissimilarities of transformation. Endocrinology 101:1862-1870.

McDonald, P. G. and C. Doughty. 1973/74. Androgen sterilization in the neonatal female rat and its inhibition by an estrogen antagonist. Neuroendocrinology 13:182-188.

Nance, D. M., C. Phelps, J. E. Shryne, and R. A. Gorski. 1977*a*. Alterations by estrogen and hypothyroidism in the effects of septal lesions on lordosis behavior of male rats. Brain Res. Bull. 2:49-53.

Nance, D. M., J. E. Shryne, J. H. Gordon, and R. A. Gorski. 1977*b*. Examination of some factors that control the effects of septal lesions on lordosis behavior. Pharmac. Biochem. Behav. 6:227-234.

Nance, D. M., J. Shryne, and R. A. Gorski. 1974. Septal lesions: Effects on lordosis behavior and pattern of gonadotropin release. Horm. Behav. 5:73-81.

Nance, D. M., J. Shryne, and R. A. Gorski. 1975*a*. Effects of septal lesions on behavioral sensitivity of female rats to gonadal hormones. Horm. Behav. 6:59-64.

Nance, D. M., J. Shryne, and R. A. Gorski. 1975*b*. Facilitation of female sexual behavior in male rats by septal lesions: An interaction with estrogen. Horm. Behav. 6:289-299.

Phelps, C. P. and C. H. Sawyer. 1976. Postnatal thyroxine modifies effects of early androgen on lordosis. Horm. and Behav. 7:331-340.

Sheridan, P. J., M. Sar, and W. E. Stumpf. 1974. Autoradiographic localization of $^{3}$H-estradiol or its metabolites in the central nervous system of the developing rat. Endocrinology 94:1386-1390.

Vito, C. C. and T. O. Fox. 1977. Embryonic estradiol receptors from mouse and rat brain. Neurosci. Abstr. 3:359.

Wieland, S. J. and T. O. Fox. 1976. DNA affinity separates androgen and estrogen binding activity: Study of residual androgen binding macromolecules in *Tfm* mutant mouse brain. Neurosci. Abstr. 2:661.

Wieland, S. J., T. O. Fox, and C. Savakis. 1978. DNA-binding of androgen and estrogen receptors from mouse brain: Behavior of residual androgen receptor from *Tfm* mutant. Brain Res. 140:159-164.

Wollman, A. L. and J. B. Hamilton. 1967. Prevention by cyproterone acetate of androgenic, but not gonadotrophic, elicitation of persistent estrus in rats. Endocrinology 8:350-356.

Yamamoto, K. R. and B. M. Alberts. 1972. *In vitro* conversion of estradiol-receptor protein to its nuclear form: Dependence on hormone and DNA. Proc. Natl. Acad. Sci. U.S.A. 69:2105-2109.

Yamamoto, K. R., M. R. Stampfer, and G. M. Tomkins. 1974. Receptors from glucocorticoid-sensitive lymphoma cells and two classes of insensitive clones: Physical and DNA-binding properties. Proc. Natl. Acad. Sci. U.S.A. 71: 3901-3905.