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DO ESTROGEN RECEPTORS PLAY A ROLE IN THE SEXUAL DIFFERENTIATION OF THE RAT BRAIN?

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SUMMARY

Several lines of evidence suggest that the conversion of testosterone to estradiol may play a decisive role in the sexual differentiation of the rat brain. This evidence includes the effectiveness of estrogens and aromatizable androgens on this process and the ineffectiveness of A-ring reduced androgens, which are not readily aromatized; and indications that anti-estrogens such as MER-25 may attenuate the actions of testosterone. Our own work has established the following points. 1. Testosterone-derived [^{3}H]-estradiol is retained by cell nuclear receptor sites in hypothalamus, preoptic area, and amygdala of 5-day-old male and female rats. 2. Estrogen receptor sites resembling those in adult brain and pituitary are present in these brain regions of neonatal rats and in cerebral cortex as well, where there is no aromatizing activity. (The pre- and postnatal ontogeny of these estrogen receptors is described.) 3. An extracellular protein, identical with the α-fetoprotein, acts to retard natural estrogens from reaching these receptor sites during the first 2–3 weeks of postnatal life. 4. An anti-estrogen, CI 628, given to intact neonatal male rats, or to neonatal female rats prior to treatment with testosterone-propionate (TP) attenuates the inhibition of lordosis behavior which would otherwise occur. CI 628 does not, under the conditions of our study, block the defeminizing effects of TP on ovarian cyclicity.

INTRODUCTION

Steroid hormones have a stimulatory action on the differentiation of a number of animal systems. Ecdysone, the moulting hormone of invertebrates, stimulates the differentiation of the imaginal discs to form portions of the exoskeleton [1]. Testosterone (T), a major androgenic steroid of vertebrates, is believed to be responsible for initiating the sexual differentiation not only of the reproductive tract and the gonads in certain cases, but of the neuroendocrine system as well. The extent and reversibility of this differentiation differs widely among vertebrate species, and there is considerable variation in the relative importance of T itself or of some of its metabolites as causal agents. Among mammals, gonadal differentiation is not reversed by hormones, and differentiation of the reproductive tract and brain occur during a limited sensitive period of early development occurring either *in utero* or in early postnatal life. These events of early development are caused by the secretion of T by the testes during the sensitive period and can be imitated by administration of T or its propionate during this period to females or to castrated males. In at least two species, rat and hamster, estrogens are equally if not more effective compared to T [2, 3], and the purpose of this paper is to summarize the present evidence regarding the role of the estrogens as mediators of T effects on the sexual differentiation of the brain.

CONVERSION OF TESTOSTERONE TO ESTRADIOL

The discovery by Naftolin, Ryan and coworkers of brain enzymes capable of converting androstenedione or T *in vitro* to estrone or estradiol (E_2), respectively, raises the possibility that the 'aromatization' process may be obligatory, or at least significantly involved, in sexual differentiation [4]. The demonstration of aromatization *in vitro* established that this process is intrinsic to brain tissue, besides occurring in steroid producing glands and the placenta. Some indication that this process occurs *in vivo* in the neonatal rat brain may be found in the work of Weisz and Gibbs[5]. This study was replicated and extended in our laboratory to include an analysis of androgen-derived estradiol in the cell nuclear fraction. Indeed, 30–50% of the [^{3}H]-radioactivity in the cell nuclear fraction from a pooled sample of limbic areas (preoptic area, amygdala) and hypothalamus of 5 day old male or female rats was identified by extraction, chromatography, and crystallization as [^{3}H]-E_2, whereas cerebral cortex cell nuclei contain very little [^{3}H]-E_2. This enrichment is indicative of the presence of cell nuclear estrogen receptor sites in the neonatal rat brain. This result is consistent, moreover, with autoradiographic evidence for neuronal retention in neonatal rat hypothalamus, preoptic area, and amygdala of radioactivity injected as [^{3}H]-T and [^{3}H]-E_2, and with the observation that unlabeled T and E_2

both compete for this retention of both labeled steroids [7, 8].

TOPOGRAPHY AND PROPERTIES OF NEONATAL ESTROGEN RECEPTOR SITES

The above-mentioned success in identifying cell nuclear estrogen receptor sites in neonatal rat brains stands in contrast to earlier failures to demonstrate such sites with doses of [^{3}H]-E_2 capable of revealing such sites in adult rat brains [9, 10]. In these studies, regional (*i.e.*, hypothalamic *vs.* cortex) differences in tissue retention of low doses of [^{3}H]-E_2 and cell nuclear retention of [^{3}H]-E_2 were not evident until the fourth postnatal week of life. It has become apparent that there are at least two explanations for these earlier results. First, as will be mentioned in the next section, the fetoneonatal estrogen binding protein (f EBP) is apparently able to sequester small to moderate doses of [^{3}H]-E_2 and to prevent this steroid by mass action from interacting significantly with its intracellular receptor sites. Second, the cerebral cortex contains some estrogen receptor sites during the first two weeks of postnatal life (see below) and the presence of these sites tends to obscure the regional differences in binding of [^{3}H]-E_2 which are seen in the adult rat brain.

Regional cell nuclear binding at postnatal day 3 of high doses (100 nm/kg or greater) of [^{3}H]-E_2 or [^{3}H]-diethylstilbestrol (DES), is highest in pituitary, followed by hypothalamus, amygdala, cerebral cortex, and preoptic area. Cell nuclear binding by pooled hypothalamus, preoptic area, and amygdala (HPA) as well as by cerebral cortex cell nuclei is blocked by prior injection of a non-steroidal anti-estrogen, CI 628 [11], as well as by concurrent injection of unlabeled estradiol 17α and 17β (unpublished). 5α Dihydrotestosterone (DHT), 3β 5α androstanediol, 19 hydroxy 5α dihydrotestosterone, and progesterone at molar ratios up to 220 times, are without significant competitive effect against [^{3}H]-DES.

Otherwise, the distribution and specificity of cell nuclear estrogen retention in the neonatal rat brain are similar to those of a soluble (cytosol) binding protein identified by Barley *et al.*[12] in the neonatal rat brain and by Fox[13] in the neonatal mouse brain. This class of macromolecules, unlike f EBP [14, 15] binds to DNA attached to cellulose [13]. Such binding to DNA is a characteristic of intracellular steroid receptor proteins. Moreover, these macromolecules, unlike f EBP, exhibit high affinity for the synthetic estrogen RU 2858 [12] and sediment in low ionic strength media at approximately 8 Svedberg units [13, 15], properties shared by cytoplasmic estrogen receptors from the adult rat brain. Furthermore, following *in vivo* administration of [^{3}H]-RU 2858 to neonatal animals, the levels of this cytosol protein are markedly reduced in cortex as well as HPA, concomitant to maximal cell nuclear uptake of the labeled hormone [16]. Thus, it seems likely that this class of macromolecules functions as a cytoplasmic receptor for the nuclear binding mechanism.

Estrogen retention by neurons in the cerebral cortex has been detected by autoradiography [17, 18]. As noted above, the cortical estrogen receptors appear to be identical to those in HPA with respect to sedimentation coefficient, binding specificity for various steroids, ability to bind the anti-estrogen CI 628, and their role as a precursor for the nuclear receptor. Their function remains for the moment obscure. But, owing to the absence of aromatizing activity in the cortex [6], its function does not involve a response to testosterone-derived estradiol. It is possible that the cortical estrogen receptors may be responsive to estradiol secreted during the second postnatal week in both sexes [19, 20].

Cell nuclear binding in cerebral cortex declines to low adult levels during the third postnatal week, whereas binding in other brain regions and in pituitary changes only slightly during this period and then increases again as the animal approaches adulthood. The total cell nuclear estrogen binding capacity of HPA on postnatal day 3 is estimated to be one-half of that observed on postnatal day 26 and only one-third of that observed in the adult female rat (unpublished). Cytosol receptor content follows a developmental time course similar to that of cell nuclear binding *in vivo* [18].

THE FETONEONATAL ESTROGEN BINDING PROTEIN (f EBP) AND ITS POSSIBLE PROTECTIVE ROLE

Fetal and neonatal rat blood contains, in abundance, an estrogen binding protein produced by the yolk sac and embryonic liver [21, 22]. This protein appears to be identical to the alphafetoprotein [23, 24]. This f EBP has a sedimentation coefficient in sucrose density gradients of $\approx$ 4*s* [15, 22] and shows a marked preference for E_2 over synthetic estrogens such as DES or RU 2858 [11, 22, 25] in contrast to the neonatal tissue receptor. Thus whereas low doses of [^{3}H]-E_2 are largely sequestered by f EBP and do not reach the cell nuclear receptor sites in significant amounts, comparable doses of the synthetic estrogen [^{3}H]-RU 2858 do not bind to f EBP and do bind to brain cell nuclear receptor sites [25].

The f EBP is also found in cerebrospinal fluid and in washes of neonatal brain tissue and can therefore also be detected in cytosol from neonatal rat brains perfused at sacrifice to remove blood contamination [15]. This protein reacts immunochemically like f EBP from blood [26] and its presence in brain interferes with the detection of the true cytosol receptor sites unless one uses special methodological precautions with [^{3}H]-E_2 as ligand [16] or uses a synthetic estrogen such as [^{3}H]-RU 2858. It remains to be determined whether the presence of f EBP in brain has any significance besides the protective function

ascribed to the blood f EBP [11, 15, 25, 27, 28]. It is interesting to note, however, that mouse alphafetoprotein, believed to be identical to f EBP, binds to T lymphocytes [29] and exerts an immunosuppressive action [30]. The involvement of E_2 in this action is unknown.

It may be predicted from the differential binding of [^{3}H]-E_2 and [^{3}H]-RU 2858 by f EBP and by brain receptors that the latter would be more effective than E_2 in promoting sexual differentiation of the brain. Indeed this appears to be the case, and dramatically so, from recent studies of Doughty *et al.*[3]. RU 2858 is also more effective than E_2 in promoting uterine growth in rats during the first two weeks of postnatal life when titers of f EBP are high; and it is equal to E_2 in effectiveness when f EBP titers are low or undetectable at the end of the third postnatal week of life [25]. DES, which is also a poor ligand for f EBP and which therefore binds better than E_2 to neonatal brain receptors, is also known to induce brain sexual differentiation [31–33].

Deleterious effects of estrogens are not confined to the brain. DES has been shown to induce reproductive tract abnormalities—masculine development of the reproductive tract and increased incidence of primary vaginal carcinoma—in female human children exposed to DES in fetal life [34, 35].

FUNCTIONAL ASPECTS

There are now several kinds of evidence pointing to the involvement of aromatization in the sexual differentiation of the rat brain. First, as described above, there is for the rat the demonstrated conversion of testosterone-7-[^{3}H] to [^{3}H]-estradiol, and the retention of this steroid by cell nuclei in the hypothalamus and limbic areas of the neonatal rat brain. In this connection, it is noteworthy that intrahypothalamic implants of estradiol [36] as well as testosterone [37–39] into neonatal female rats leads to anovulatory sterility in adult life. Second, there is the demonstration of cell nuclear and soluble estrogen receptors in neonatal rat brains. These receptors have a specificity which parallels the action spectrum of steroids, insofar as it is presently known, in inducing brain sexual differentiation. Proof that aromatization actually plays an important role in sexual differentiation of the brain would require either the use of agents which block aromatization or agents which prevent access of estrogen to its intracellular receptor sites. With regard to the former approach, Clemens[33] has reported some success in blocking the masculinizing effects of T given to castrated, new born hamsters by pentobarbital and SKF 525A, agents which may cause decreased conversion of the T to E_2. Recently in a pilot study we have been successful in 'feminizing' the lordosis behavior of male rats by giving them an inhibitor of aromatization, androst-1, 4, 6-triene 3, 17 dione (Lieberburg, Wallach and McEwen, unpublished).

With respect to receptor antagonists, there have been reports of success in preventing 'defeminization' of female rats by giving a non-steroidal anti-estrogen, MER-25, together with testosterone propionate or RU 2858 [40, 41]. There have also been three reports of failures to obtain such a blocking effect by MER-25 [42–44]. The reasons for these discrepancies are not clear, but it should be noted that MER-25 is not a very strong antagonist compared to a number of other antiestrogens [45]. Recently we have had success in blocking the 'defeminization' of lordosis responding by neonatal TP using CI 628, a more potent estrogen antagonist (McEwen, Chaptal, Wallach, unpublished). Under the conditions of our experiments CI 628 did not prevent TP-action in inducing anovulatory sterility.

In keeping with the postulated role for E_2 receptors in the sexual differentiation of the rat brain is the observation that estrogens as well as T are effective 'defeminizing' agents toward lordosis behavior and ovulation whereas non-aromatizable androgens such as DHT are ineffective when given in sesame oil or in silastic implants [10, 46]. This is not so clearly the case in other species, however. In hamsters, neonatally-administered DHT and androsterone (also non-aromatizable) decrease the duration of lordosis elicitable by estrogen but are much less effective in doing so than T [47, 48]. Unlike T, DHT or androsterone do not, when given neonatally, increase aggression or male sexual behavior [2, 48]. Moreover, an estrogen, DES, is more effective than T or TP in masculinizing mounting behavior of female hamsters and is without effect on peripheral sex characteristics [2]. In guinea pigs, on the other hand, aromatizable androgens and not DHT are able to defeminize lordosis behavior, but DHT and other non-aromatizable androgens may have a role in the development of masculine copulatory behavior [49]. In rhesus monkeys, *in utero* exposure to DHT as well as T appears capable of behaviorally masculinizing females (Goy, personal communication).

These findings are reminders once again that male and female sexual behavior are subject to independent hormonal determination during development [50]. They also suggest that there is nothing unique about the ability of one steroid such as estradiol to facilitate sexual differentiation. It is already clear for the reproductive tract of male mammals that T is the primary steroid involved in triggering the differentiation of the seminal vesicles, vas deferens, and epididymis, whereas DHT is involved in initiating differentiation of the external genitalia and prostate [51].

We must therefore look deeper into the phenomenon of sexual differentiation to find a unifying concept for all mammals. One possibility centers around the permanence of the process of sexual differentiation. The permanence of the gonadal steroid effects on brain sexual differentiation imply that the consequences of the hormone–receptor interaction preceding this event are different from the interaction pre-

ceding the reversible 'activation' of adult sexual behavior or ovulation. Indeed, permanent structural alterations in the preoptic area appear to be one of the consequences of sexual differentiation in rat and hamster. These alterations involve changes in the pattern of dendrites in this brain region [52] and alterations in the ratio of synaptic contacts on dendritic spines to those on dendritic shafts [53]. Studies of T and E_2 effects on explants of fetal mouse hypothalamus in culture indicate that these structural changes may arise in part from an altered rate of neurite outgrowth stimulated by these two steroids [54]. It remains to be seen whether such cellular growth and differentiation occur under the influence of gonadal steroids in fetal neural tissue of other mammals besides rat and hamster.

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DISCUSSION

De Moor. Dr. McEwen, you tell us that androgens and estrogens can androgenize and masculinize the female rat, but this is not true for all parameters used. You cannot androgenize the liver enzymes with estrogens; you cannot androgenize the transcortin levels with estrogens and this is also taking place via the brain. So in the brain there must be two different mechanisms: one acting through the conversion of androgens to estrogens and another one acting directly.

McEwen. That is a very good point. There may indeed be other sexually dimorphic traits in the rat determined either by aromatizable or non-aromatizable androgens, or both. And if one goes beyond the rat to other species there is evidence that dihydrotestosterone, or at least non-aromatizable androgens, may be involved in the defeminization or masculinization of rhesus monkeys and guinea pigs.

Pasqualini. You checked the receptors in the brain 2 h after administration of estradiol, is this time the maximal level of uptake? Did you look for the curve of the time course of the radioactivity?

McEwen. The time course? Yes, we did study the time course of estrogen retention in the neonatal rat brain and found that, as in the adult, one or two hours is the optimal time for cell nuclear retention of radioactivity.

Pasqualini. When we looked for the estradiol receptors in the fetal brain of guinea pig we found that in the total radioactivity in cell, a great portion was converted to estrone. Have you looked for this conversion? Also, do you have differences in the metabolism of estradiol in the brain between fetal male and females?

McEwen. In those particular experiments we were looking at females.

Pasqualini. Is there some estrone?

McEwen. Yes in the tissue there is estrone. In the cell nuclei, however, the radioactivity is only estradiol.

Naftolin. The cortical binding is troublesome. Are you sure that it is still estradiol? Did you recrystallize it for identification?

McEwen. Yes.

Naftolin. Because other estrogens are made by cortex.

McEwen. There is no doubt about it. If I could speculate

a little bit there is a peak of estrogen secretion in rats and other rodents in the second postnatal week of life. Also at this time the α fetoprotein levels are declining. It is therefore quite likely that estrogen secreted between days 10–14 interacts with cortical estrogen receptors and exerts some kind of influence on cortical development in both sexes.

Naftolin. What part of the cortex was it?

McEwen. As far as we can tell, estrogen receptors exist throughout the entire cerebral cortex. We are working on an autoradiographic map in an effort to answer your question more precisely.

Grumbach. Have you done any work in other species, the reason I ask is that in the human fetus both the males and females are literally bathed in estrogen beginning relatively early in gestation through the middle part of gestation. Towards term it reaches nanogram per ml quantities and from the work of the Paris group in the human the α fetoprotein does not appear to bind estradiol. This has been very troublesome to me in trying to apply evidence from rodents to what happens in men, do you have other species that you've looked.

McEwen. Dr. Plapinger in my laboratory, Dr. Pasqualini's group, and Dr. Nunez have all tried and failed to find estrogen binding to α fetoprotein in guinea pig. Perhaps the occurrence of an estrogen-binding α fetoprotein is limited to certain species like the rat and mouse. Nevertheless, there are the deleterious effects of synthetic estrogens like DES on sexual differentiation in the human as well as in the rat and mouse. So there may be some kind of protective mechanism capable of distinguishing between natural and synthetic estrogens, and yet I don't know what it might be.

Solomon. One of the protective mechanisms in human fetuses is sulfation. I'm hesitant in bringing this up as I can't give you quantitative numbers the type that recently published for maternal circulation to tell you how much in the fetus of estrone and estradiol is unconjugated but my guess would be it would be largely sulfate and glucuronide conjugated so this may be the way the fetus protects itself from the catastrophic amount of estrogens that are circulating.

Grumbach. The estradiol that has been done we have not done them, they've been done by Shoot and Sherman and coworkers and they measured unconjugated estradiol in the fetal circulation and not conjugated estrogen. There is no information that I am aware of on the amount of free, that is unbound, estrogen circulating in the human fetus. That may be as you say there are other serum factors that may affect the amount of estradiol that's available.

McEwen. The work of Robert Goy at Madison, Wisconsin, has indicated that non-aromatizable androgens are capable of masculinizing rhesus monkeys with respect to male sexual behavior. In other words, estrogens may not be as crucial to sexual differentiation of the primate brain as they appear to be for the rat brain. And on that point I would like to say that the crucial feature of a developmental effect of a steroid is not what steroid does the job but, rather, that developing target cells acquire a receptor which makes them sensitive and susceptible to the steroid during a critical period of their development.