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ANDROGEN RECEPTORS IN THE BRAIN OF NEONATAL NORMAL MALE AND ANDROGEN INSENSITIVE RATS

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Summary: Androgen binding was investigated in the cytosol of brains from neonatal normal and tfm rats using sucrose gradient and charcoal assay. The neonatal normal rat brain contains androgen receptors which sediment at 8S in a sucrose gradient at low ionic strength and at 4S in the presence of 0.5M KCl. These receptors have a high affinity (Kd = 1.2×10^{-9}M) for dihydrotestosterone (DHT), with a binding capacity of 7.3×10^{-15} moles/mg cytosol protein. By contrast, there is very little, if any, high affinity androgen receptors in the neonatal tfm rat brain. Apparently, metabolism of testosterone does not affect androgen binding in the neonatal rat brain. The presence of these receptors and the occurrence of aromatization in the brain of neonatal rats are discussed in relation to their possible roles in sexual differentiation of the brain.

Testicular feminization (tfm) is an inherited form of male pseudohermaphroditism in which the affected individual is a genotypic male but phenotypic female with presence of small cryptorchid testes and absence of Wolffian and Müllerian duct derivatives. The syndrome has been described in man (1), cattle (2), dog (3), rats (4), and mice (5). In the tfm rat, the X-linked tfm mutation renders target organs insensitive to endogenous or exogenous androgens (6-8). This insensitivity to androgen has been shown to be associated with a deficiency in the cytosol androgen receptors (6,9-12). Testes of neonatal tfm rats exhibit normal ultrastructure and are capable of producing normal amounts of testosterone (13), however, no other major secondary reproductive organs ever appear as they do in normal rats. On the other hand, it is known that brain cells of normal rats only gradually become sexually mature during the first 12 or so days following birth (14). With the preceding three observations in mind, an attempt has been made to determine whether the as-yet sexually undifferentiated neonatal tfm rat brain tissue exhibits androgen receptors and how metabolism of testosterone and DHT affect the receptor activity.

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Materials and Methods:

Materials. Neonatal normal male and tfm rats were obtained from the International Foundation for the Study of Rat Genetics and Rodent Pest Control, Oklahoma City, Oklahoma. All rats were King-Holtzman hybrids. Dihydrotestosterone, (1,2,4,5,6,7-^{3}H(N)) (110-150 Ci/mmol), testosterone-1β, 2β -^{3}H (40-60 Ci/mmol), (4-^{14}C)-labelled steroids (testosterone, DHT, 3α-androstanediol) with a specific activity of 50-60 mCi/mmol, were purchased from New England Nuclear Corporation.

Preparation of cytosol: The brain tissue was obtained from neonatal normal and tfm rats, washed twice in ice cold TEDG (0.05M Tris, pH 7.4, 0.15mM EDTA, 0.25mM dithiothreitol, 10% glycerol) and homogenized in 2 volumes TEDG for 5 seconds with a Polytron homogenizer. The tissue homogenate was centrifuged at 105,000 x g for 1 hour and the resulting supernatant (cytosol fraction) was diluted to have a protein concentration of 8-10 mg/ml.

Sucrose gradient: Five hundred μl of the cytosol were incubated with ^{3}H-DHT (2.5 x 10^{-9}M) at 1°C for 2 hours. Yeast alcohol dehydrogenase (7.6S) with bovine serum bulbimin (4.6S) were added to each gradient to serve as an internal marker. Three hundred μl of the incubate were layered on the top of continuous 5 to 20% sucrose density gradient. The samples were centrifuged at 180,000 x g for 22 hours in a SW 50.1 Beckman Spinco rotor. Fractions of 8 drops were collected by puncture of perforating the bottom of the tubes and radioactivity was assessed in a liquid scintillation counter. Approximate sedimentation values were determined as described by Martin and Ames (15).

Dextran-coated charcoal assay: Two hundred μl of cytosol were incubated with increasing amounts of ^{3}H-DHT (0.5-8 x 10^{-9}M) at 1°C for 2 hours. To absorb free steroid, 0.5 ml of dextran coated charcoal (0.05M Tris, 1.5mM EDTA, 0.5% charcoal (Norit-A), 0.05% dextran, 10% glycerol) was added to each tube. Mixtures (tubes) were vortexed for 5 seconds, allowed to stand for 5 minutes, and centrifuged at 1,000 x g for 5 minutes at 4°C. Radioactivity in the supernatants was measured and the result was considered as total binding. Nonspecific binding was measured in parallel incubation mixtures containing a 500-fold excess of unlabelled DHT in addition to ^{3}H-labelled steroid. A specific binding was calculated by subtracting nonspecific binding from total binding. The results were analyzed by the method of scatchard (16). Protein was determined by the method of Lowry et al. (17) using bovine serum albumin as a standard.

Metabolism of testosterone: The brain tissues from neonatal normal and tfm rats were minced and incubated in a Dubnoff metabolic shaking incubator in 0.5 ml of Dulbecco's phosphate buffered saline, pH 7.4, containing glucose (0.01M) and testosterone-^{3}H (2 μM, 144 x 10^{5} dpm) as substrate for two hours at 37°C in an atmosphere of 5% CO_2:95% O_2. Control incubations, containing no tissue, were carried out in every instance. Reactions were stopped by the addition of 2 ml of methanol and the resulting solutions stored at -20°C until extraction. Twenty μg each of authentic testosterone, DHT, and 5α-androstane-3α, 17β-diol (3α-diol) as carrier, and ^{14}C-steroids for recovery were added to the incubate. Steroids were extracted twice with ether and evaporated to dryness. After a phenolic partition the NaOH layer was neutralized with 5N H_2SO_4 and extracted with ether. Ether fractions were washed twice with water, spotted on silica gel GF thin-layer plates and chromatographed in a chloroform-methanol system (97:3, v/v). Testosterone, DHT and 3α-diol were acetylated by the addition of pyridine and acetic anhydride and chromatographed on Whatman paper number 1 in a cyclohexane-methanol-water system (100:85:15, v/v). Steroids eluted from the paper chromatogram were counted in a Packard Tri-Carb liquid scintillation spectrometer.

Results:

It was found that if ^{3}H-DHT is incubated with brain cytosol to study the binding of DHT to macromolecules, the receptor for DHT sediments in the 8S region of sucrose gradients in the brain cytosol from neonatal normal males (Fig. 1). However, such

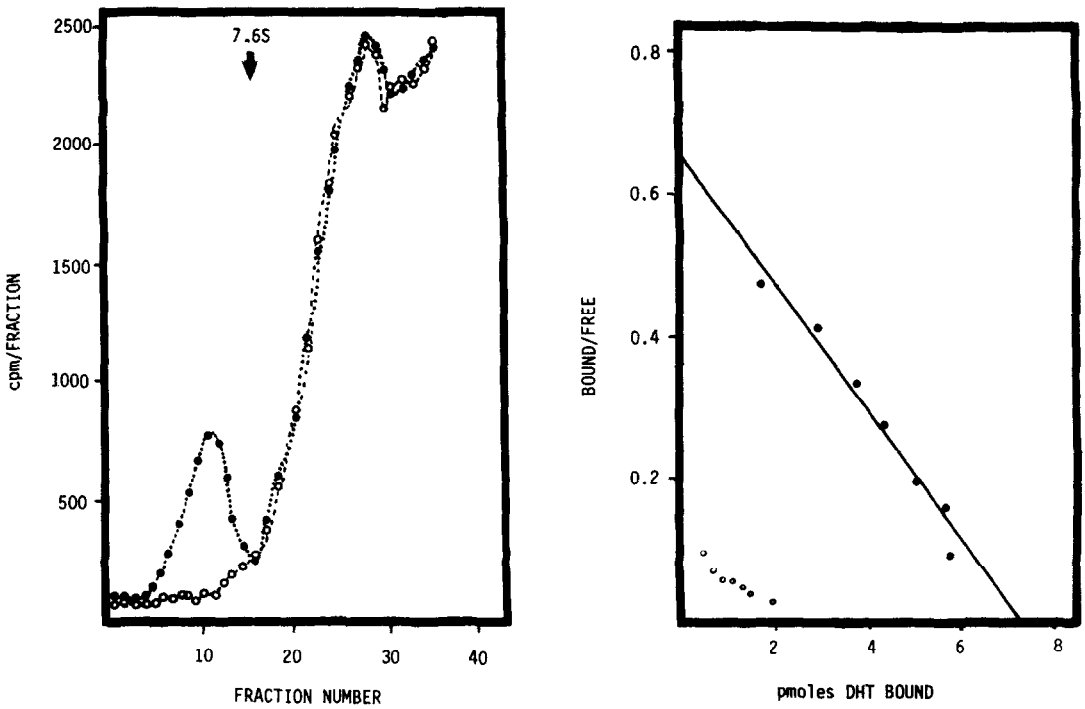

Figure 1. Brains from neonatal normal (●-●) and tfm (o-o) were homogenized in TEDG and the cytosol was obtained by centrifugation at 105,000 for one hour. Following incubation of cytosol with 2.5 x 10^{-9}M of ^{3}H-DHT at 1°C for two hours. Cytosol receptor binding was analysed by 5-20% sucrose gradients (left). The receptor for DHT sediments in the sucrose gradient at 8S in the cytosol from brains of neonatal normal males. But no 8S binding was demonstrated in the noenatal tfm rat brain cytosol. Also, cytosol was incubated with ^{3}H-DHT (0.5-8X10^{-9}M) at 1°C for two hours. Scatchard plot (right) was made from the data of the saturation analysis for ^{3}H-DHT binding in neonatal normal (●-●) and tfm (o-o) brain cytosol. The dissociation constant (Kd) for the receptor DHT complex from neonatal normal rats was estimated to be 1.2 x 10^{-9}M and the number of available binding sites (N) were 7.3 x 10^{-15}moles/mg of cytosol protein. Neonatal tfm rat brain cytosol, however, showed no obvious evidence of any high affinity and limited-capacity binding sites.

receptors sediment at 4S in the presence of 0.5M KCl. In normal rats when cytosol fractions were incubated with varying amounts of radioactive DHT for charcoal adsorption and Scatchard analysis, the apparent dissociation constant (Kd) turned out to be 1.2 x 10^{-9}M for DHT and the binding capacity was 7.3 x 10^{-15} moles/mg cytosol protein (Fig. 1). By contrast, the brain of the neonatal androgen insensitive tfm rat had no detectable cytosol androgen receptors (Fig. 1).

Since activities of 5α-reductase and 3α-hydroxysteroid dehydrogenase may be different in neonatal normal and tfm rat brains, resulting in different androgen binding, the brain cytosol prepared from neonatal animals was incubated with ^{3}H-testosterone. The formation of DHT from testosterone by neonatal tfm brain tissue is slightly greater

TABLE I

Metabolism of testosterone by the neonatal rat brain[b]

Tissues	Metabolites		Total Recovery[b]
	DHT	3α-Adiol	
	$1X10^3$ dpm	$1X10^3$ dpm	$1X10^4$ dpm
Neonatal normal	3.37 ± 0.246[c]	0.91 ± 0.062	9.05 ± 0.369
Neonatal tfm	4.29 ± 0.401[d]	0.81 ± 0.049[d]	9.06 ± 0.378

a Initially, each flask contained 50 mg of tissue and 2.0 μM testosterone (1.44×10^5 dpm)

b The sum of identified metabolites and residual testosterone.

c Mean ± S.D.

d Differences from neonatal normal ($P > 0.1$)

($P > 0.1$) than that of normal. There was also conversion of a small amount of DHT to 3α-diol by both normal and tfm brain cytosol (Table I). These suggest that the metabolism of testosterone and DHT does not affect the decrease in androgen binding in tfm animals.

Discussion:

The present study demonstrates the presence of binding macromolecules for DHT in the neonatal normal rat brain. These receptors have high affinity and limited capacity for their steroid ligands. They sediment at 8S in sucrose gradients at low ionic strength and at 4S at high strength. Some regions of the normal rat brain particularly the pituitary, hypothalamus and limbic system are androgen target organs and their cytosol receptors bind androgens (10,18). It is known that in normal tissue an androgen enters the target cells and binds to a specific, high affinity cytoplasmic receptor. The hormone-receptor complex subsequently undergoes translocation into the nucleus, where it binds to a specific acceptor site on the genome (DNA) and activates transcription of new RNA followed by stimulation of protein synthesis (14,19). However, the same androgen receptors are defective in the brain of neonatal tfm rats, as has been observed in other androgen target organs in the adult tfm animals (6,9,10). Although the level of testosterone in the circulation at the neonatal stage is comparable in tfm rats to that in normal males (13), it appears that the brain of neonatal tfm rats would not be masculinized by endogenous testosterone and the major organs of male reproduction do not develop in the affected animals due to the lack of receptor binding.

Inherently the brain of many mammals including rat and man, like the reproductive system, is female; if not exposed to male sex hormones at a critical period in its development, it remains so regardless of genetic sex (14,20). In the rat, the differentiation of the brain occurs after birth (throughout the first 12 postnatal days which are critical period for sexual differentiation). In the tfm rat the neonatal testes are normal structually and functionally (13) and thus their testicular secretion should serve to organize the central nervous system towards development in postpuberal life of behavioral and neuroendocrinological patterns typical of the male. However, present data indicate that tfm animals are unable to respond to androgens due to a defect in their androgen-receptor mechanism. Therefore, because their brain tissue cannot respond to androgens, and thus remains sexually undifferentiated, the resulting abnormal function of their neuroendocrine system leads to manifestations of the testicular feminization syndrome.

The A-ring reduction of testosterone to 5α-DHT has been known for sometime to be an important reaction for the action of androgens upon peripheral target organs (21) including the neuroendocrine system (22). The rat pituitary and certain of their brain tissues can convert testosterone to DHT and 3α-diol (23). There is no apparent correlation between the rate of production of DHT and 3α-diol and the sexual differentiation of the rat brain. The rat pituitary and other brain tissue are also capable of aromatizing testosterone to estrogens (24-26). It is believed that DHT (which is not aromatized to estrogen) is much less effective in neonatal masculinization than testosterone and other aromatizable androgens (27,28) and that aromatization occurs in the brain of the newborn rat, but not in that of adults (25). Such an aromatization of the androgen may be necessary for its neonatal action in male sexual differentiation of the rodent brain (29,30). This is further substantiated by the fact that a small dose of estradiol is as effective as testosterone in masculinizing neonatal rats (26). Thus, it would seem more likely that estradiol plays an important role in mediating the action of aromatizable androgen on the neonatal masculinization of the rat brain during the differentiation process.

References:

1. Morris, J.M. (1953) Am. J. Obstet. Gynecol. 65, 1192-1211.
2. Short, R.V. (1967(Ann. Rev. Physiol. 29, 373-400.
3. Schultz, M.G. (1962) J. Am. Vet. Med. Assoc. 140, 241-244.

4. Stanley, A.J., and Gumbreck, L.G. (1964) Program of the Endocrine Society, p. 40.
5. Lyon, M.F., and Hawkes, S.G. (1970) Nature (London) 227, 1217-1219.
6. Bardin, C.W., Bullock, L.P., Sherins, R.J., Mowszowicz, I., and Blackburn, E.R. (1973) Rec. Progr. Horm. Res. 29, 65-109.
7. Goldstein, J.L., and Wilson, J.D. (1972) J. Clin. Invest. 51, 1647-1658.
8. Goldman, A.D. (1970) in Mammalian Reproduction, Mosbach Col. 21 (Gigian, H., and Plits, E.J., Eds.) pp. 389-436, Springer-Verlag, Heidelberg.
9. Milian, B., and Roy, A.K. (1973) Nature (London) New Biol. 242, 248-250.
10. Naess, O., Haug, E., Attramadal, A., Aakvaag, A., Hanson, V., and French, F. (1976) Endocrinology 99:1295-1303.
11. Attardi, B., and Ohno, S. (1974) Cell 2, 205-212.
12. Bullock, L.P., and Bardin, C.W. (1974) Endocrinology 94, 746-756.
13. Chung, K.W., and Allison, J.E. (1979) Cell Tissue Res. 196, 213-219.
14. Gorski, R.A. (1980) Neuroendocrinology (Krieger, D., and Hughes, J., Eds.), pp. 215-222, Sinauer Associates, Inc., Sunderland, MA.
15. Martin, R.G., and Ames, B.N. (1961) J. Biol. Chem. 236, 1372-1379.
16. Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660-672.
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randal, R.J. (1951) J. Biol. Chem. 193, 265-275.
18. Naess, O., Attramadal, A., and Aakvaag, A. (1975) Endocrinology 96, 1-9.
19. Wilson, J.D. (1978) Ann. Rev. Neuroscience 2, 279-306.
20. Harris, G.W., and Levine, S. (1965) J. Physiol. 181, 379-400.
21. Liao, S., and Fang, S. (1969) Vitam. Horm. N.Y. 27, 17-90.
22. McEwen, B.S. (1978) In Hormone Receptors, Vol. I: Steroid Hormones, (O'Malley, B., and Birnbaumer, L., Eds.), pp. 353-400, L. Press, New York.
23. Denef, C., Magnus, C., and McEwen, B.S. (1973) J. Endocrinol. 59, 605-621.
24. Naftolin, F.K., Ryan, K.J., and Petro, Z. (1972) Endocrinology 90, 295-298.
25. Naftolin, F.K., Ryan, K.J., Davies, I.J., Reddy, V.V., Flores, F., Petro, Z., Kuhn, M., White, R.J., Takaoka, Y., and Wolin, L. (1975) Rec. Progr. Horm. Res. 31, 295-319.
26. Gorski, R. (1966) J. Reprod. Fertil. (Suppl) 1, 67-75.
27. Luttge, W.G., and Whalen, R.E. (1970) Horm. Behav 1, 265-272.
28. McDonald, P.G., and Doughty, C. (1974) J. Endocrinol. 61, 95-103.
29. Attardi, B., and Ohno, S. (1976) Endocrinology 99, 1279-1290.
30. Korenbrot, C.C., Paup, D.C., and Gorski, R.A. (1975) Endocrinology 97, 709-717.