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CYTOSOL AND NUCLEAR RECEPTORS FOR 5α-DIHYDROTESTOSTERONE AND TESTOSTERONE IN THE HYPOTHALAMUS AND HYPOPHYSIS, AND TESTOSTERONE RECEPTORS ISOLATED FROM NEONATAL FEMALE RAT HYPOTHALAMUS

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SUMMARY

Characterization of cytosol receptor macromolecules for 5α-dihydrotestosterone, (DHT) and testosterone (T) was compared in the hypothalamus of 27-day-old male rats. The binding of [^{3}H]DHT increased rapidly after starting incubation, and approached a maximum at 1 h. In contrast, the [^{3}H]T binding increased slowly with a maximum at 4 h. On analysis of the saturation kinetics of the binding of androgens by the Lineweaver–Burk plot the dissociation constant (K_D) and the number of binding sites (NBS) for DHT binding were determined to be 0.3 nM and 3.8 ($n = 2$) fmol/mg protein, respectively, and the values for T binding 1.5 ± 0.28 (S.E.) nM and 2.2 ± 0.23 ($n = 3$) fmol/mg protein, respectively; indicating higher affinity of the hypothalamic cytosol for DHT than for T. Maximal binding capacities for DHT and T were 3.9 ± 0.36 [4] and 2.1 ± 0.15 [4], respectively. Relative affinity of competition of the [^{3}H]T binding by DHT was 2.8 times than by T. Hypothalamic and hypophyseal [^{3}H]DHT binding was inhibited in a competitive way by the addition of 17β-estradiol, but not by estrone, estriol, diethylstilbestrol or clomiphene citrate. The injections of estradiol even in large amount did not affect the binding of [^{3}H]DHT.

From these results it seems that the hypothalamic cytosol can bind DHT and T in a different way, indicating different characterization of cytosol receptors for DHT and T in the hypothalamus of 27-day-old male rats, and further suggesting the possibility of individual receptors for DHT and T.

Receptors macromolecules of 5–6 *S* for DHT were isolated from purified nuclei of the hypothalamus and the anterior hypophysis. From these results, together with nuclear T receptors, it is tempting to speculate that androgens can directly act on the brain through the interaction with the receptors for DHT and T in the hypothalamus and hypophysis.

The presence or absence of receptors for T was investigated in the hypothalamus of neonatal female rats at 3 and 7 days of age. On sucrose density gradients there was a small but definite peak of radioactive androgen in the 8 *S* region. The 8 *S* binding components were found to be saturable and steroid-specific. These suggest the presence of 'receptors' for T in the hypothalamus on neonatal female rats. The function of neonatal hypothalamic T 'receptor' was discussed in relation with the mechanism of induction of androgenization.

INTRODUCTION

The isolation of receptor macromolecules for testosterone (T) by Jouan *et al.*[1, 2] and Naess *et al.*[3, 4] and for 5α-dihydrotestosterone (DHT) by Kato *et al.*[5–8] and Ginsburg *et al.*[9] in the hypothalamus and hypophysis of the rat has substantiated the uptake data on specific androgen binding in the brain and hypophysis [10–20]. It has been further postulated that the interaction of androgen with the receptors in the hypothalamus and hypophysis is the basis of action of the hormone on the brain [1–9].

The importance of the conversion of T to DHT in the brain [14, 21–25] and differential action of androgens in gonadotropin suppression and sexual behavior [26, 27] caused us to investigate how characterization of cytosol receptor proteins for DHT and T are different in the hypothalamus. Receptors macromolecules of 5–6 *S* for DHT isolated from purified nuclei from the hypothalamus and the anterior hypophysis of castrated male rats were further investigated. Furthermore, in an attempt to elucidate the mechanism of androgen sterility, we determined whether putative receptors for T exist in the hypothalamus of female neonatal rat.

METHODS AND MATERIALS

Male Wistar rats were used at 3, 7 and 27 days of age. In the experiments for nuclear receptors, adult male rats were castrated 3–7 days prior to the sacrifice.

Dissection of the brain

All animals were decapitated, the whole hypothalamus including the preoptic nucleus was cut out as a block as previously described [28]. The limits of the block were 3 mm anterior to the optic chiasma, the hypothalamic fissures laterally and the mammillary body posteriorly, all at a depth of 2 mm from the basal surface. This hypothalamic section included the pars tuberalis and a portion of the pituitary stalk. All tissues were washed several times in ice-cold 0.01 M Tris–HCl buffer (pH 7.4) including 0.0015 M EDTA and 0.003 M 2-mercaptoethanol (TEm-buffer, pH 7.4), blotted on filter paper and weighed on an analytical balance.

Preparation of samples and gradient centrifugation

Hypothalami from rats were suspended in the ice-cold TEm-buffer, pH 7.4 and homogenized in a Teflon-pestle glass homogenizer for 2 min (three strokes). The homogenate was centrifuged at 105,000 *g* for 1 h in a Hitachi RP65 ultracentrifuge. The supernatant fraction cytosol (0.29 ml, except where stated) was mixed with an ethanol solution (5 μl) of 5α-dihydrotestosterone-[^{3}H] or [^{3}H]-testosterone in the absence or presence of unlabeled steroid hormones, diethylstilbestrol, cyproterone, clomiphene citrate, and incubated with gentle shaking at 0–4°C for the appropriate time.

The mixture was then layered on a centrifuge tube containing 4.8 ml sucrose gradient (5–20% linear) in TEm-buffer (pH 7.4) solution. In some cases the gradient solution contained 10% glycerol–0.01 M Tris–HCl buffer (pH 7.4), 0.001 M EDTA and 0.006 M thioglycerol to stabilize the binding proteins [29]. Centrifugation was performed in a 40 ST-2 rotor at 34,000–40,000 rev./min in a Hitachi 65P ultracentrifuge or in a SW 50.1 rotor at 35,000 to 50,000 rev./min in a Beckman L3 Model ultracentrifuge for 15–18 h at 2–4 C. Fractions (0.2 ml) were obtained from the top of the centrifuge tube with an ISCO Model 640 Density Gradient Fractionator. No more than 5 h were permitted to elapse from the time of sacrifice of the animals until the commencement of the gradient centrifugation of the cytosol.

Preparation of the nuclei and extraction

Whole hypothalami were collected from 50 male rats of 70–90 days old castrated 3 to 7 days prior to the sacrifice. Each hypothalamus were divided into 3–4 portions. They were incubated in 15 ml of Krebs–Ringer bicarbonate–glucose buffer, pH 7.4, containing 2% BSA and 30 μCi of [^{3}H]DHT (S.A. 49 Ci/mmol). Incubation was performed at 37°C with gentle shaking for 30 min. The tissues removed from the medium were rinsed with ice-cold Krebs–Ringer solution.

Nuclei were prepared by a modified method based on that of Lovtrup and McEwen[30]. The portions of hypothalamus were first homogenized in a Teflon-pestle glass homogenizer containing 10 vol. of 0.32 M sucrose–0.001 M $MgCl_2$–0.001 M K_2HPO_4, pH 6.5 (0.32 M-SMgK). The 850 *g* × 10 min pellet was washed twice with 0.32 M-SMgK. The suspended pellet in 0.8 ml of the same solution was layered on the top of 2.39 M SMgK (pH 6.5), followed by centrifugation in a SW 50.1 rotor at 50,000 rev./min (235,000 *g*) for 15 h. The pellet was washed with 0.32 M SMgK and then with 0.01 M Tris buffer (pH 7.4) by centrifugating the suspension at 50,000 rev./min for 10 min to obtain the nuclear pellet. This purified nuclear pellet was extracted with 0.4 M KCl [31], and resultant supernatant, nuclear extract, applied on sucrose density gradients (5–20% linear) in TMm containing 0.4 M KCl.

Other methods

Radioactivity was measured in an Aloka Model LSC 653 or a Nuclear Chicago Mark II model scintillation counter using a scintillation fluid containing Triton–toluene–PPO–POPOP. The counting efficiency was 38 or 50%. Protein was measured by the method of Lowry *et al.*[32]. Absorbance at 280 nm was measured in an ISCO detector (Model UA 4) attached to an ISCO Gradients Fractionator. Apparent sedimentation coefficients (*S*) were determined by the method of Martin and Ames[33] using BSA, alcohol dehydrogenase, and beef liver catalase as the standards.

Redistilled and purified solvents and reagent grade reagents were used in all experiments. 5α-dihydrotestosterone-1α,2α-[^{3}H] (S.A. 44 Ci/mmol, NEN), [1,2,6,7-^{3}H]-testosterone (S.A. 85 Ci/mmol, NEN) were obtained from New England Nuclear Corp. and purified by t.l.c. [34] before use. The steroids used were supplied by Steraloids, Ind., Merck Co., Ikapharm. Diethysilbestrol was obtained from Teikoku Zoki Co., Tokyo. Crystalline BSA, alcohol dehydrogenase from yeast and beef liver catalase 2× crystalline were obtained from Sigma Chemicals. Cyproterone and clomiphene citrate were kindly supplied by Dr. F. Neumann of A.G. Schering, Berlin and Dr. T. Miyake, the Shionogi, Osaka, respectively.

The radioactive steroids were purified by t.l.c. on silica gel, or precoated silica gel 60 TLC plates (Merck). They were developed by an acetone–benzene mixture (1:4, v/v), benzene–methanol (9:1, v/v), or benzene–ethanol (9:1, v/v), as described previously [34].

Identification of 5α-dihydrotestosterone

Radioactive materials associated with the gradient fractions were extracted three times with three vols. of methylene chloride and two times with three vols. of diethyl ether. They were then separated by t.l.c. on precoated silica gel in a system of acetone–benzene (1:4, v/v), or by paper chromatography in a system of benzene–heptane–methanol–water (33.3:66.7:80:20, v/v) [52].

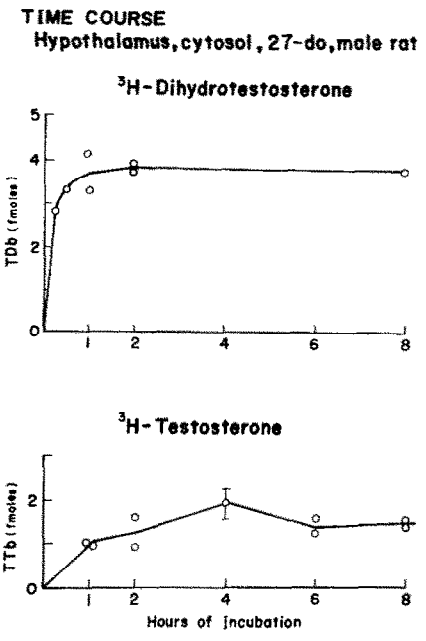

Fig. 1. The time course of [^{3}H]dihydrotesterone and [^{3}H]testosterone binding to high-affinity receptors in the cytosol fraction of the hypothalamus of 27-day-old male rats. The cytosol was incubated with 3.4 nM [3H]DHT and 3.9 nM [^{3}H]testosterone at 0°C and aliquots of 200 μl were subjected to sucrose density gradient centrifugation. Total radioactive androgens found in the 8 *S* was calculated.

RESULTS

(1) *Binding characteristics of receptors for DHT and T*

The binding of labeled DHT and T to the hypothalamic cytosols was compared by incubating cytosols from 27-day-old male rats with labeled steroids under identical conditions of equimolar and equiproteinaceous concentrations.

(a) *Equilibrium time.* The time course of the hypothalamic cytosol binding of [^{3}H]DHT or [^{3}H]T was investigated for time periods from 15 min to 8 h. Total specific binding of each steroid in the 8 *S* peak in sucrose density gradient patterns was calculated in accord with the recommendation of Shain and Barnea[35].

As can be seen in Fig. 1, there was a rapid increase in the binding of [^{3}H]DHT from 15 min to 1 h after starting the incubation, and reached a maximum at 1 h with little or no increase thereafter up to 8 h. In contrast, the [^{3}H]T binding increased more slowly to the level of a maximum at 4 h followed by remaining almost on the same level at 6 and 8 h after the addition of the labeled steroid. It appears that the DHT–receptor complexes are formed in the hypothalamic cytosols more rapidly than the T–receptor complexes. It is also noted that the [^{3}H]DHT binding is greater than the [^{3}H]T binding.

(b) *Binding affinity and capacity.* In order to determine affinity and capacity of the binding of T and DHT to the receptors, the dissociation constant (K_D)

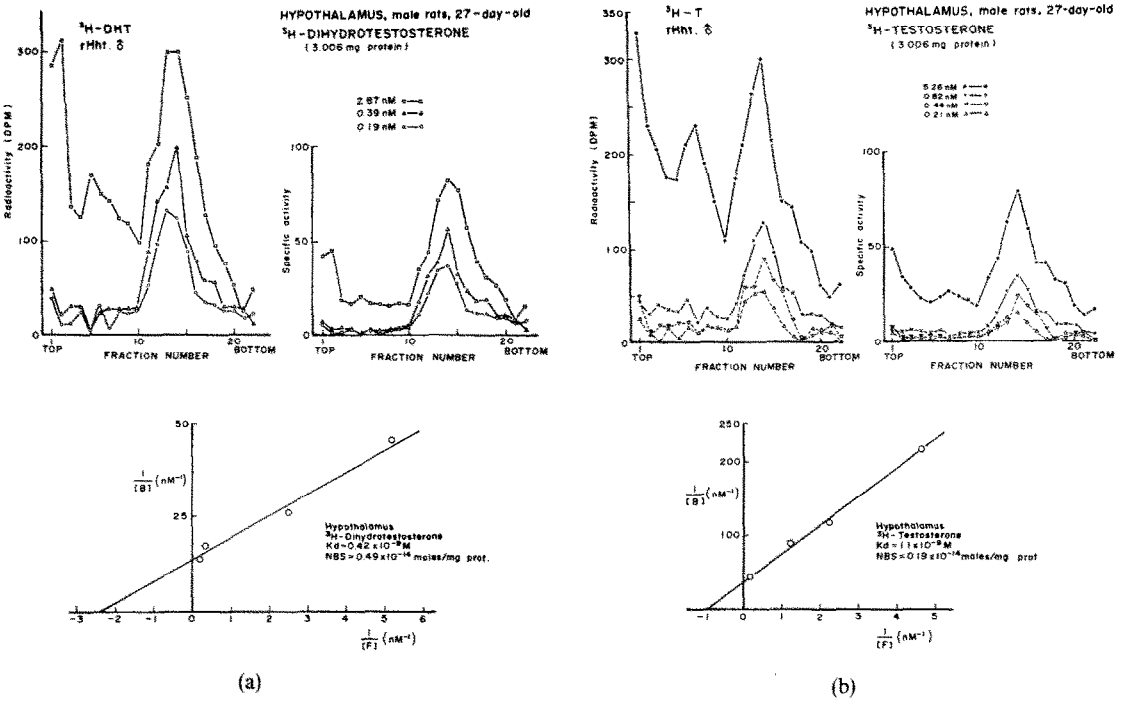

Fig. 2. Sucrose density gradient patterns of 28-day-old rat hypothalamic cytosol incubated *in vitro* with varying concentrations of [^{3}H]DHT and [^{3}H]testosterone, and the Lineweaver–Burk plot of androgens binding. The cytosol (0.29 ml) containing 3.02 mg protein and labeled estradiol was layered on 4.8 ml of 5–20% sucrose density gradient, followed by centrifugation at 40,000 rev./min for 16 h in a SW50.1 rotor in a Beckman L3 ultracentrifuge.

Table 1. Comparison of binding capacity of receptors for 5α-dihydrotestosterone and testosterone in the hypothalamus of male rats*

	Dihydrotestosterone	Testosterone
Total [^{3}H]steroid bound in respective peaks (fmol)	11.8 ± 1.08† [4]‡	6.6 ± 0.56 [4]
Relative TStb (fmol/mg prot. cytosol)	3.9 ± 0.36 [4]	2.1 ± 0.15 [4]
Ratio (St/T)	1.8	1.0
NBS§ (fmol/mg prot. cytosol)	3.8 [2]	2.2 ± 0.23 [3]
Ratio (St/T)	1.7	1.0

* Two tenth ml of hypothalamic cytosol from 27-day-old male rats was incubated with the respective [^{3}H]steroids for 8 h at 0°C, then the mixture was layered on 5 ml of 5–20% sucrose density gradients. Centrifugation: 189,000 *g*, 18 h. Total [^{3}H]steroid bound in the specific binding peak on gradient pattern was calculated in accord with the recommendation of Shain and Barnea[35]. † Mean and standard error. ‡ Number of determination. § Number of binding sites (NBS) of the cytosols was calculated on analysis of the saturation kinetics of the Lineweaver–Burk plot.

and the number of binding sites (NBS) of the receptors for the respective steroids were compared in the same hypothalamic cytosol under identical conditions of incubation for each steroid.

When the cytosols were incubated *in vitro* with increasing concentrations of [^{3}H]DHT or [^{3}H]T in a range of 0.2 to 11.1 × 10^{-9} M at 0°C for 6 h, the binding peak of radioactivity in the 8 *S* region on the sucrose density patterns became saturated (Figs. 2a and b). An analysis of the data on saturation kinetics by the Lineweaver–Burk plot is shown in Figs. 2a and b. The K_D and NBS for [^{3}H]DHT binding were determined to be 3.3 × 10^{-10} M (n = 2) and 3.8 fmol/mg cytosol protein, respectively; the values for [^{3}H]T, 1.5 ± 0.28 (S.E.) × 10^{-9} M (n = 3) and 2.2 ± 0.23 (S.E.) fmol/mg protein. Thus, the binding of [^{3}H]DHT seemed to have higher affinity for the hypothalamic cytosols than [^{3}H]T.

In the hypothalamic cytosol from 27-day-old male rats the NBS for each steroid are almost on the same level as the maximal binding capacity estimated by total radioactive androgens bound in the specific 8 *S* peak on gradient patterns (Table 1).

(c) *Specificity of hypothalamic receptors for DHT and T.* In the previous report we demonstrated that binding sites on hypothalamic receptor proteins for DHT have a structural specificity for 5α-androstane steroid skeleton and, found in this skeleton, for the 3-keto or 3 hydroxy group and 17β-hydroxy group [5–8].

Steroid specificity of the hypothalamic receptors for DHT in 27-day-old male rats was further investigated by incubating the cytosols with [^{3}H]DHT with various unlabeled compounds, and the data are summarized in Table 2.

Competitive grade of [^{3}H]DHT was in the following decreasing order; 5α-DHT > T > cyproterone. Little or no inhibition was found with 5β-DHT, and androstenedione, dehydroepiandrosterone, epiandrosterone or cortisol. Progesterone lowered the [^{3}H]DHT binding to the hypothalamic receptors to some extent. Relative affinity of competition by unla-

Table 2. Competition of *in vitro* binding of 5α-dihydrotestosterone-[^{3}H] to 5α-dihydrotestosterone binding components from hypothalamic cytosol by unlabeled steroid hormones, unlabelled diethylstilbestrol, cyproterone and clomiphene citrate

Competing substances	Concentration (M)*					
	1.0×10^{-5}	1.0×10^{-6}	1.0×10^{-7}	1.0×10^{-8}	1.0×10^{-9}	1.0×10^{-10}
5α-Dihydrotestosterone				no peak (3)†		
5β-Dihydrotestosterone				107 (97:117)‡		
Testosterone				30	78	93
Androstenedione				95 ± 5.7§ (3)		
Epiandrosterone				96 (90:101)		
Dehydroepiandrosterone				95 (95:95)		
Cyproterone		Rudimentary (3)	44 (41:46)	88 ± 9.0 (3)	92 (84:100)	
17β-Estradiol			no peak	Rudimentary (3)	57 (55:58)	75
17α-Estradiol				111 (96, 126)		
Estrone			110 (90:129)	101		
Estriol			112 (107:116)	122		
Diethylstilbestrol			109	102 (96:108)		
Clomiphene citrate	83 (86:79)	92 ± 3.6 (3)	103 (118, 87)	(116 ± 5.887) (4)	113 ± 4.8 (3)	119
Progesterone			40	63		
Cortisol				99 (104:93)		

* Final concentration. The cytosol (0.29 ml containing 3.108–3.630 mg of protein was incubated with 5α-dihydrotestosterone [^{3}H] (7.2, 8.8 or 10.0 × 10^{-10} M) and unlabeled substances for 30 min at 0–4°C. The detail of procedures was described in the text.

† Number of experiments.

‡ Average value in %. The control value (in the absence of competing substance) was taken as 100% and used for comparison with other values.

§ Mean and S.E.

beled steroids being calculated by determining the amount of 50% reduuction of [^{3}H]DHT binding in the 8 *S* on the density patterns, and taking the effect of DHT as 100%, the value for T was 14% in the hypothalamic cytosol; which is the same as that in the prostate and seminal vesicle [36].

The addition of 17β-estradiol to the incubation mixture decreased the *in vitro* binding of [^{3}H]DHT to the hypothalamic receptors in a competitive way [8], but diethylstilbestrol, estrone, and estriol did not. Clomiphene citrate, which can compete strongly for binding sites of estradiol receptors in the hypothalamus, hypophysis and uterus, also showed little effect even in the 1000-fold concentration (Table 2).

Even large amount of estradiol or estradiol benzoate, injected ip in a single dose or sc for successive 3 days or more, did not depress the hypothalamic binding of [^{3}H]DHT at all.

From these results it is indicated that 17β-hydroxy group of the estradiol skeleton can bind or attach binding sites on the androgen receptor macromolecules only under *in vitro* conditions. Since the *in vitro* binding seems unrelated to estrogenicity of the competitors, it may be of little physiological significance.

Effect of DHT on [^{3}H]T. When the binding of [^{3}H]T to the hypothalamic receptors was competed by varying concentrations of unlabeled DHT under identical conditions as the binding of [^{3}H]DHT, greater inhibitory effect of DHT was observed than that of T on [^{3}H]DHT. Relative affinity of competition of [^{3}H]T binding by DHT was 2.8 times by T. This may provide additional evidence for higher affinity of DHT binding to the hypothalamic receptors relative to T binding.

(2) *Nuclear receptors for DHT and T*

(a) *Hypophyseal nuclear receptors.* Testosterone receptor complexes have been isolated from purified nuclei of rat anterior pituitaries by Jouan *et al.*[2]. We have also demonstrated DHT receptor complexes isolated from purified nuclei of pituitaries of castrated male rats, quartered tissues of which were incubated with [^{3}H]DHT at 37°C [7, 8]. Thus, nuclear temperature-dependent translocation of T- and DHT receptors is suggested in the pituitary cell. Together with the presence of cytosol receptors for DHT and T, the results may indicate possible separate process of the action of androgens in the hypophysis.

(b) *Hypothalamic nuclear receptors.* Purified nuclei from hypothalami of male castrated rats incubated *in vitro* with [^{3}H]DHT at 37 C for appropriate hours were treated with 0.4 M KCl extract nuclear receptor complexes. Figure 3 shows representative sucrose density pattern of the nuclear extract, on which a single definite peak of [^{3}H]DHT is recognized in the 5–6 *S* region.

Steroid analysis of the radioactivity in the peak showed that virtually all the radioactivity was DHT (Fig. 3).

(3) *'Receptors' for T isolated from neonatal rat hypothalamus*

High affinity and low capacity 'classical' T receptors have been reported not to be present in the hypothalamus of the very young female rat [37–42], indicating no involvement of the brain receptors in the mechanism of androgen-induced sterility in female rats [42]. Recently we reported putative receptors for DHT in the hypothalamus of 7-day-old male rat and the ontogenetic development of the receptors [7, 8, 53]. These data were consistent with the findings of Chamberlain and Rogers[43] on the appearance of [^{3}H]DHT in the neonatal rat diencephalon following injection of [^{3}H]T. According to Sheridan *et al.*[44, 45], a specific and saturable topographic pattern of nuclear concentration of [^{3}H]steroid in neurons of the basal preoptic and amygdala has been demonstrated in the two-day rat after the injection of [^{3}H]estradiol and [^{3}H]T. These data suggest possible receptors for androgens in neonatal rat hypothalamus. Little is known, however, on the isolation of receptors for T from the hypothalamus of neonatal female rats. Whether or not cytosol receptors for T are present in the hypothalamus of neonatal female rats provides some clue to elucidate the mechanism of androgenization.

On sucrose density gradient sedimentation profiles of hypothalamic cytosols from 3-day-old rats, which were incubated *in vitro* with [^{3}H]T at 0°C for 6–8 h, there was a small but definite peak of radioactivity in the 8 *S* region. Fig. 4 shows the representative density patterns of hypothalamic [^{3}H]T binding in the presence or absence of excess (100-fold) nonradioactive competitors. The hypothalamic peak was abolished by the addition of unlabeled T. DHT also inhibited the [^{3}H]T binding, but dehydroepiandrosterone or cortisol did not. Cyproterone (1000-fold) and progesterone competed to some extent. The binding components isolated from 3-day-old rat hypothalamus seemed to be specific for androgens.

On all gradients of hypothalamic cytosols at 7 days of age, a single and more definite peak of radioactivity was also found in the 8 *S* region. As shown in Fig. 5, like the three-day hypothalamus, the addition of unlabeled T to the incubation mixture eliminated the hypothalamic peak. DHT inhibited the [^{3}H]T binding, but weaker androgen such as dehydroepiandrosterone did not. Excess (1000-fold) cyproterone also competed to some extent. Cortisol showed no competition, but 17β-estradiol depressed the [^{3}H]T binding considerably. Hypothalamic cytosols from 7-day-old male rats were found to possess T binding components of 8 *S*, possibly 'receptors', which show specificity for testosterone relative to other steroids.

'Receptors' for T in the hypothalamus of female rats evidently appear 3 days after birth in male rats and the concentration of receptors seemed to increase at 7 days of age and thereafter. The early appearance of 'receptors' for T in the hypothalamus of female

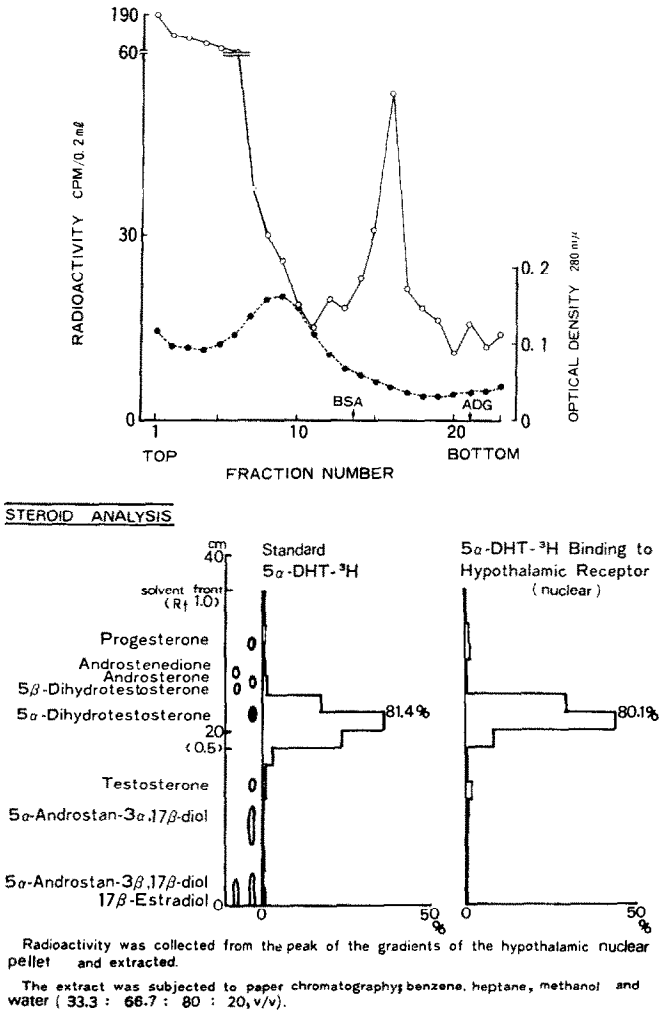

Fig. 3. A sucrose density gradient sedimentation pattern of KCl extract of purified nuclei from castrated adult rat hypothalami. Slices of the hypothalami collected from 50 rats were incubated *in vitro* with 30 μC of 5α-dihydrotestosterone-[^{3}H](49 Ci/mmol) for 30 min at 37°C. Purified nuclei were then prepared from the homogenate, followed by the extraction of DHT–receptor complexes with 0.4 M KCl from the nuclei. The extract was layered on a 5–20% sucrose density gradient, and centrifuged for 18 h at 40,000 rev./min (161,000 g_{av}). —○—, radioactivity, —●—, optical density 280 μm.

rats is compatible with our findings on that of the receptors for DHT in males [7, 8, 53].

DISCUSSION

The equilibrium time of the *in vitro* binding of [^{3}H]T to rat hypothalamic cytosol was 4 h (Fig. 1); the value for the hypothalamus was the same as that for the pituitary cytosol reported by Naess *et al.*[4]. In our previous report [5, 6, 8], little or no specific T binding was detected on density gradients of rat hypothalamic cytosols incubated with [^{3}H]T of lower S.A. (45 Ci/mmol) for shorter time of $\frac{1}{2}$ or 1 h, although a single and definite peak of [^{3}H]DHT was found in the same cytosols. This seems to be ascribable at least in part to different equilibrium time and binding capacity for the androgens (Table 1 and Fig. 1).

The dissociation constants of the hypothalamic receptors for DHT and T were 3.3×10^{-10} and 2.3×10^{-9} M, respectively (Fig. 2). Thus, it appears that in the hypothalamus DHT has higher affinity for the receptors than T (Fig. 2). This is in good agreement with different binding affinity of DHT and T for the androgens receptors in the prostate, epididymis, and seminal vesicle [36, 46, 47]. Naess *et al.*[4] have reported, however, that in the pituitary T has the same affinity for the receptors as DHT, although the latter is probably underestimated due to the metabolic 'inactivation' of DHT to 3α-diol at 0°C during the incubation of 16 h.

It is evident that hypothalamic cytosol receptors for DHT have structural specificity for 5α-androstane steroid skeleton and, found in this skeleton, for the 3-keto or 3 hydroxy groups and 17β-hydroxy group (Table 2). Relative affinity of competition of the bind-

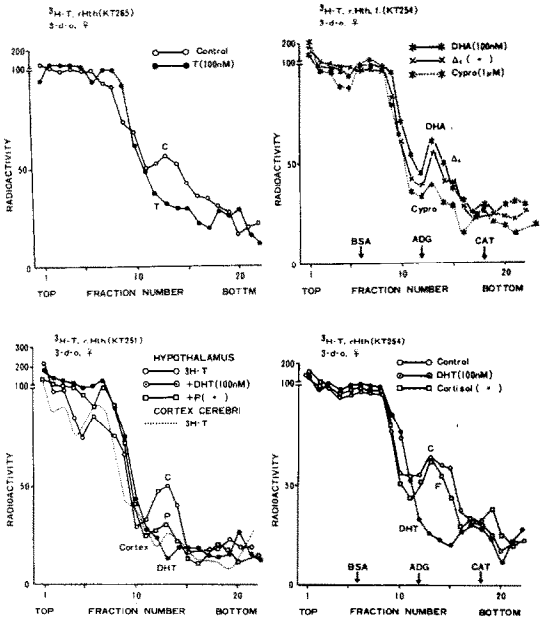

Fig. 4. The sedimentation profiles in sucrose density gradient of cytosols of the hypothalamus and cortex cerebri from female rats at 3 days of age. The cytosol was incubated with [^{3}H]testosterone (3.9 nM) in the presence or absence of excess (100-fold) unlabeled compounds. Cyproterone was added in 1000-fold excess. The details are given in the text. O——O, [^{3}H]testosterone (3.9 nM) only; ●——●, testosterone; ⊙——⊙, DHT; ×——×, androstenedione; *——*, dehydroepiandrosterone; .... cyproterone; □——□, F, cortisol; □——□, P, progesterone; – – – cortex cerebri, [^{3}H]testosterone only.

ing of [^{3}H]DHT by T seems to be greater than that of [^{3}H]T by DHT. Furthermore, inhibition by 17β-estradiol on the binding of [^{3}H]DHT in the hypothalamus and hypophysis was considerably strong. In contrast, little or no inhibition on the [^{3}H]T binding was reported in the hypophysis, this may be the case in the hypothalamus [4].

From the above-mentioned results the hypothalamic cytosol from 27-day-old male rats seems to bind DHT and T in a different way, in terms of equilibrium time, the dissociation constant, the number of binding sites, the maximal binding capacity, relative affinity for steroids. Both the androgens have been reported to differ in the central action; DHT and T are most effective in gonadotropin suppression and sexual behavior, respectively [26, 27], which may favor two cytosol receptors for DHT and T in the hypothalamus.

Although the *in vitro* effect of 17β-estradiol on the [^{3}H]DHT binding is of competitive nature [8] and considerably strong (Table 2), the estrogen effect is unlikely to have physiological significance in the central action of androgen, because no *in vivo* effect of estrogen was found.

It appears that in the nuclear fraction of the hypophysis T- and DHT receptors complexes are formed in a temperature-dependent process [2, 8]. Together with cytosol receptors for DHT and T [1–9], this may provide some evidence for possible individual process of the action of androgens in the hypophysis. Although as yet, there is little data available on nuclear T receptors, nuclear receptors for DHT in the hypothalamus have been demonstrated (Fig. 3). There is the possibility that receptors for T behave in the hypothalamus in a similar fashion to the ones for DHT. It is interesting to speculate that T and its local metabolite, DHT, can interact with the respective receptors in the hypothalamus and hypophysis, which may play a role in the mechanism of the action of androgen of the brain.

Naftolin *et al.*[48], and Weisz and Gibbs[49] have claimed that aromatized 'androgen', estrogen, can act upon the brain in the central action of androgens. Direct action of androgens in the brain may coexist with the central aromatization process because of the presence of the androgen receptors and the estrogen receptors abundant in the hypothalamus of males [7, 8]. Naess *et al.*[3, 4], however, have disproved the hypothesis of aromatization because little conversion of T to estrogen was found in the brain.

'Receptors' for T seemed to exist in the hypothalamus of 3- and 7-day-old female rats (Figs. 4 and 5), which is consistent with the early appearance of DHT binding receptors [7, 8, 53]. In view of the important role of androgen receptors in the mechanism of action of the steroids, there is the possibility that 'receptors' for T in the neonatal hypothalamus are involved in

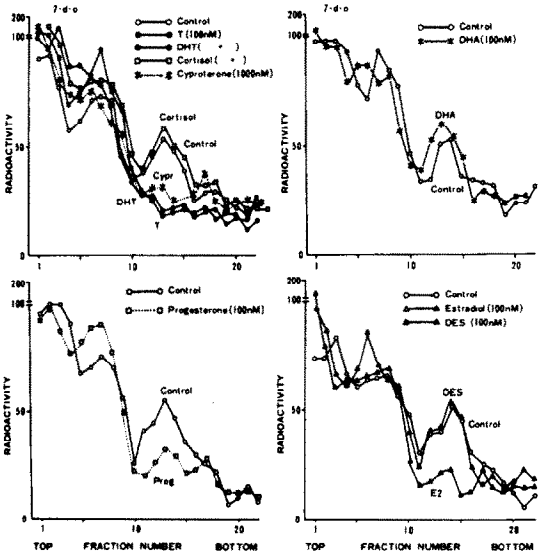

Fig. 5. Representative pattern of radioactivity in sucrose density gradient of hypothalamic cytosol from female rats at 7 days of age. The cytosol was incubated with [^{3}H]-testosterone (3.9 nM) in the presence of excess (100-fold) compounds. Cyproterone was added in 1000-fold excess. The details are given in the text. O——O, [^{3}H]testosterone (3.9 nM) only; ●——●, testosterone; ⊙——⊙, 5α-dihydrotestosterone; *——*, dehydroepiandrosterone; *———*, cyproterone; □——□, cortisol; □– – –□, progesterone; △——△, 17β-estradiol; ▲——▲, DES, diethylstilbestrol.

the mechanism of induction of androgenization in female rats, although much investigation is needed to clarify the exact role of androgen–receptor interaction.

Ontogenetically putative DHT receptors in the hypothalamus of male rats appear at earlier stages than estrogen receptors in females [7, 8, 53]. 'Receptors' for T in the hypothalamus of female rats at 3 days of age seemed to increase at 7 days of age and thereafter, suggesting the early appearance and development of 'receptors' for T in the hypothalamus of female rats. In this context, it is interesting to mention the early development of a negative feedback relation between testis and hypothalamo-pituitary system [50, 51].

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DISCUSSION

Toft. I was involved in the studies that you mentioned, Dr. Iramain *et al.* I'm very happy that you came out with the same results we did and I wonder if you had looked at other specific areas of the brain for progesterone binding. You showed the cortex I believe.

Kato. Yes, there is little progesterone binding in the cerebral cortex.

Toft. How about peri-ventricular areas?

Kato. I have not yet looked at the areas in a systematic way. However, little progesterone binding has been detected in the amygdala or reticular formation, in sharp constrast with the 7–8*S* binding in the hypothalamus and hypophysis.

Beyer. This might be a question for both you and Dr. Stumpf. There are some progesterone metabolites particularly the 5α and 5β metabolites whose action does not involve the classical interaction with intracellular receptors, but that they probably act at the membrane level. Is there the possibility of detecting very fast incorporation of these steroids into the membrane?

Kato. So far as my knowledge is concerned, there are no available data on very fast incorporation of progesterone into the membrane. It is interesting to mention that Dr. Karavolas has reported specific uptake of 5α-DHP by rat brain. However, as I showed in my slide, I have not obtained any positive results on the binding of tritiated 5a-DHP in the hypothalamic cytosol of estrogen-primed rats.

Beyer. This is not a specific binding involving a prolonged retention of the steroid by the receptors, but the interaction occurs almost immediately maybe in one second.

Stumpf. I think nobody has shown convincingly steroid receptors in the membrane, although this had been postulated for some time. We also used the 5α-dihydroprogesterone as a competitor for [^{3}H]-progesterone labeling and we found a reduction but it was a nuclear type of reduction. Otherwise we have never seen with [^{3}H]-progesterone in the rat or guinea pig any indication of radioactivity being accumulated in membranes. You talked about studies in neonates. Would you please specify the age.

Kato. Neonates at 3 and 7 days of age were used. There is a definite binding of testosterone and 5α-DHT in the hypothalamus of these animals.