The role of androgen receptors in sexual differentiation of the brain: Effects of the testicular feminization (Tfm) gene on androgen metabolism, binding, and action in the mouse

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The effects of the testicular feminization (Tfm) mutation on androgen metabolism, binding, and action were studied in the mouse. Cytosolic binding of [³H]dihydrotestosterone (DHT) was reduced by approximately 90% in the brains, pituitaries, submaxillary glands, and kidneys of Tfm/Y males, as compared to wild-type male controls. Nuclear [³H]DHT binding was abolished in tissues from Tfm male animals. Implantation of testosterone-containing (T-containing) Silastic capsules elevated nuclear estrogen receptor (ERn) concentrations to a similar extent in the brains of all four genotypes studied (normal males, Tfm-affected males, normal females, and Tfm carrier females). Administration of T to normal males led to elevations of cytosolic progesterin receptors (PRc) and monoamine oxidase (MAO) activity in the brain, and of alcohol dehydrogenase (ADH) and glucose-6-phosphate dehydrogenase (G6PDH) in the kidney. In Tfm-affected males, only the PRc response to T was observed, at a somewhat reduced level compared to that in normal male and female controls: no effect of T was observed with respect to either MAO in the brain, or ADH and G6PDH activities in the kidney. Estradiol (E₂) administration increased the activity of pituitary G6PDH and lactic dehydrogenase (LDH) in all four genotypes. However, brain MAO activity was increased by E₂ *only* in normal and carrier females. The lack of an MAO response to E₂ in Tfm-affected males is consistent with the hypothesis that the central nervous system of this genotype is at least partially masculinized, despite the loss of androgen receptor-mediated responses—most probably as a result of local conversion of T to E₂ within the brain itself.

In mammals, sexual differentiation of the central nervous system is brought about primarily through the actions of testosterone (T) released from the gonads of the developing male. The diverse behavioral and neuroendocrine effects of this androgen are mediated through two receptor systems: androgen receptors, which preferentially bind T and its 5 α -reduced metabolite, 5 α -

dihydrotestosterone (DHT); and estrogen receptors, which respond to estradiol (E₂) synthesized from T by the aromatase enzyme complex (Bardin & Catterall, 1981; MacLusky & Naftolin, 1981; Naftolin et al., 1975). Aromatase and 5 α -reductase are both present in the developing brain (Denef, 1973; Naftolin et al., 1975). Data accumulated over the last 15 years suggest that androgens and estrogens may both contribute to the mechanism of sexual differentiation (MacLusky & Naftolin, 1981; McEwen, 1983; Olsen, 1979b). The contributions of androgen and estrogen receptor-mediated effects to the differentiation of individual sexually dimorphic characteristics are, however, difficult to define, and in most cases all that can be said is that effects *can* be elicited by either androgen or estrogen alone. As a result of metabolic interconver-

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sions and the possibility of cross-reactions between androgens and estrogens at their respective receptor sites (Ginsburg, MacLusky, Morris, & Thomas, 1977; Kao, Lloret, & Weisz, 1977; Rochefort & Garcia, 1976; Zava & McGuire, 1978), the ability to elicit a response to either estrogens or nonaromatizable androgens alone does not provide definitive proof of the primary involvement of the one or the other receptor mechanism.

Naturally occurring genetic defects in the androgen response mechanism provide a valuable approach to study of this problem. One such defect, the complete testicular feminization (Tfm) syndrome, is of particular interest in view of its biochemical specificity and wide species distribution. This X-linked genetic mutation occurs in several mammalian species, including man (see Lyon & Hawkes, 1970; Morris, 1953; Short, 1967; Stanley, Gumbreck, Allison, & Easley, 1973). Affected males fail to fully differentiate sexually, appear as phenotypic females, and are sterile. Complete Tfm appears to be caused by a profound insensitivity to androgen, associated in most instances with a drastic reduction in androgen receptor concentration in target tissues of the affected male (Attardi, Geller, & Ohno, 1976; Griffin & Wilson, 1980). Developmentally, the abnormal androgen receptor levels result in incomplete differentiation of the reproductive structures that are dependent on the actions of T or 5 α -DHT. Thus, Tfm-affected males lack a vas deferens, an epididymis, and male accessory glands. In adults, a lack of androgen receptors is consistent with the reported inability of T to induce androgen-dependent enzymes in the kidneys of Tfm-affected males (Dofuku, Terrenborn, & Ohno, 1971; Ohno & Lyon, 1970), and with their impaired androgen-dependent behaviors (Beach & Buehler, 1977; Krey, Lieberburg, MacLusky, Davis, & Robbins, 1982; Ohno, Geller, & Young-Lai, 1974; Olsen, 1979a, 1979b).

Since the genetic defect associated with the Tfm mutation appears to be specific to the androgen receptor system and is not accompanied by a loss of estrogen receptors (Attardi et al., 1976; Fox, 1975), Tfm males offer a valuable experimental model with which to test hypotheses concerning the specific roles of androgen and estrogen receptor-mediated effects in sexual differentiation. Several previous studies have used the specificity of the Tfm mutation to investigate the role of androgen receptor-mediated responses in the actions of T on the rat brain (Krey et al., 1982; Olsen, 1979a, 1979b). An unresolved issue, however, is the extent to which contributions from the "residual" androgen receptors found in the Tfm male may confound interpretation of the results from such studies in terms of purely estrogen-mediated responses. There is considerable variation—among species and even among different tissues within the same species—in the reported effects of the Tfm mutation on androgen receptor concentrations (Attardi & Ohno, 1978; Griffin & Wilson, 1980; Max, 1981). In the submaxillary gland, the reduction in receptor levels in the Tfm male is associated with a concomitant increase in a second, smaller androgen-binding protein (Verhoeven & Wilson, 1976). Depending on the species and target tissue, Tfm

males may retain some degree of androgen responsiveness (Naess et al., 1976), indicating potential functionality for the residual receptor molecules. These observations, however, must be interpreted cautiously, since the experimental conditions employed in the study of the residual androgen receptors in different tissues of the Tfm male have been somewhat variable, and the possibility remains that effects of high-dose androgen treatment observed in the Tfm male may not in fact be mediated through the androgen receptor system (Krey et al., 1982).

This article summarizes results obtained in our laboratories regarding the consequences of the Tfm mutation with respect to androgen binding, metabolism, and action in the brain and pituitary gland of the mouse. Androgen receptor concentrations in different brain regions as well as in nonneural androgen target tissues have been measured and correlated with the extent of nuclear androgen and estrogen binding after *in vivo* androgen administration. Finally, biochemical responses to androgen and estrogen have been studied in the brain and peripheral tissues to assess the consequences of the alterations in steroid binding. The results are considered in relation to gonadal steroid action in the adult and to the possible mechanisms underlying sexual differentiation of the brain.

MATERIALS AND METHODS

The tritium-labeled steroids and serotonin used in these studies were obtained from New England Nuclear (Boston, MA). Unlabeled R5020 was a gift from J.-P. Raynaud, Roussel-UCLAF, Romainville, France. Diethylstilbestrol (DES) and the other unlabeled steroids were purchased from Steraloids, Inc. (Pawling, NY). Precoated glass-based plates for thin layer chromatography (TLC) were obtained from Brinkmann (Westbury, NY). All other chemicals and solvents were of Analar grade and used as supplied.

Animals

Mice either were of the BALB-C strain (Jackson Labs, Bar Harbor, ME) or represented the offspring of matings between BALB-C or Tabby (Ta)/Y males and "carrier" females heterozygous for the Ta and Tfm genes. The carrier females were kindly provided by Jean Wilson of the University of Texas, Dallas, TX. Breeding pairs were housed either at the Rockefeller University or at Columbia University, in facilities kindly provided by Dominique Toran-Allerand. The offspring from the matings were segregated at the time of weaning (Postnatal Day 25) into Ta/Y males, Ta/X females, Tfm/Y males, and Tfm/X females. The first two of these genotypes were distinguished on the basis of their coat color. Distinction between the Tfm/Y and Tfm/X genotypes, which are identical in external appearance, was made by laparotomy under light methoxyflurane anesthesia and visual inspection of the gonads. All animals were housed, in groups of 4–5, in plastic cages (8 × 12 in.) under a 14:10-h light:dark cycle, and they were maintained on tap water and Purina Chow *ad lib*. Except where otherwise indicated, they were gonadectomized bilaterally under methoxyflurane anesthesia, 7–9 days before use.

Sucrose Density Gradient Analysis of Cytoplasmic [³H]DHT Binding

Gonadectomized (GDX) mice were killed by decapitation, and their brains, pituitaries, submaxillary glands, and kidneys were removed and immediately placed on ice. The brain was dissected into pooled hypothalamus, preoptic area, septum, and amygdala (HPAS),

midbrain plus brain stem, and a sample consisting of the remainder of the forebrain (RFB) after removal of the HPAS. The tissues were homogenized in a buffer containing 10 mM Tris (Tris hydroxymethyl aminomethane), 1.5 mM disodium-ethylenediamine tetra-acetate (disodium-EDTA), 1 mM dithiothreitol, and 10% (vol/vol) glycerol, adjusted to pH 7.4 with hydrochloric acid (TEGD buffer). Homogenization volumes were 2.5 ml/g for the brain, kidney, and submaxillary gland, 25 ml/g for the pituitary. The homogenates were centrifuged for 1 h at $106,500 \times g$ (average), using a Ti50 rotor in an L3-50 centrifuge (Beckman Instruments), at 1°C . The supernatant cytosol fractions were decanted and used immediately. The cytosols were incubated with 2 nM [^3H]DHT for 2 h at 4°C . Aliquots (250 μl) of the incubates were overlaid on 5-ml 5%-10% sucrose gradients in TEGD buffer, and centrifuged at $190,000 \times g$ (average) for 22 h at 1°C . The gradients were collected by tube puncture in 12-drop (0.2 ml) fractions. Each fraction was counted in 2.5 ml of toluene(4,228 ml)-Liquifluor (New England Nuclear; 160 ml)-Triton X-114 (Ruger Chemical Co., Irvington, NJ; 1,463 ml) scintillator at an efficiency of 32%. Sedimentation markers, run in parallel, were horse heart myoglobin (2.0S), bovine serum albumin (4.6S), and bovine liver catalase (11.3S).

Uptake and Metabolism of [^3H]DHT by Androgen Target Tissue in Vivo

Gonadectomized mice were injected via the jugular vein vein with 10 μCi (approximately 250 pmol) [^3H]DHT dissolved in 50 μl 30% ethanol-70% isotonic saline, with or without a 100-fold molar excess of unlabeled DHT, under light methoxyflurane anesthesia. One hour later they were reanesthetized with methoxyflurane and the chest cavity was opened. Blood (0.5 ml) was withdrawn from the right ventricle, and the animals were then killed by rapid perfusion through the left ventricle with 10 ml ice-cold 6% clinical dextran in 0.85% saline. Their brains, pituitaries, kidneys, and submaxillary glands were immediately removed and placed on ice. The brains were dissected as described above for the sucrose density gradient studies. The tissues from groups of 3-5 mice were pooled for subsequent analysis. Each tissue pool was homogenized in 5 ml of buffer containing 1 mM potassium phosphate, 3 mM MgCl_2 , 0.32 M sucrose, and 0.25% Triton X-100 adjusted to pH 6.5. Kidney and submaxillary gland homogenates were then filtered through two layers of nylon gauze (200- μm mesh). Aliquots (0.5 ml) of the homogenates were withdrawn into extraction tubes. Nuclei were isolated from the remaining nine-tenths of each homogenate, as previously described (Roy & McEwen, 1977). Purified nuclear pellets were transferred to extraction tubes with 3×0.5 ml 0.01 M citric acid. Blood was allowed to clot at 4°C for 2 h. Serum was separated by centrifugation at $1000 \times g$ for 20 min at 4°C . Fifty microliter serum aliquots were counted directly in 10 ml of toluene-Liquifluor-Triton X-114 at an efficiency of 40%. Whole homogenate samples, purified cell nuclei, and 200- μl aliquots of serum were extracted with 5×3 ml of toluene containing 200 $\mu\text{g}/15$ ml of the following unlabeled steroids: 5α -androstan- $3\alpha,17\beta$ -diol (3α -ADIOL), 5α -androstan- $3\beta,17\beta$ -diol (3β -ADIOL), DHT, androsterone (AONE), and 5α -androstan- $3,17$ -dione (ADIONE). Aliquots (5 ml) of these extracts were evaporated to dryness and counted in 15 ml of toluene-Liquifluor scintillator (4,228 ml toluene, 160 ml Liquifluor) at an efficiency of 50%. The metabolites present in the remainder of the toluene extracts were separated and quantified as described by Deneff, Magnus, and McEwen (1973). In one experiment, indicated below, nuclear radioactivity was analyzed by reverse isotope dilution and crystallization to constant specific activity from acetone and petroleum ether (BP 40°C - 60°C).

Autoradiographic Localization of [^3H]DHT Uptake

The procedures used for autoradiography were similar to those reported previously by Gerlach and McEwen (1972). Four GDX Ta/Y and 4 GDX Tfm/Y animals were injected via the jugular vein with 10 μCi [$1,2\text{-}^3\text{H}$]DHT (Specific Activity 46.5 Ci/mmol) dis-

solved in 50 μl of 30% ethanol-70% physiological saline, under light methoxyflurane anesthesia. One hour later the animals were killed by decapitation. Their brains and submaxillary glands were removed rapidly and frozen in liquid nitrogen. The frozen tissues were cut in a cryostat into 4- μm thick sections and mounted, unfixed, on slides coated with Kodak NTB-3 nuclear emulsion. The brains were cut in the coronal plane; the submaxillary glands were cut in the transverse plane. Autoradiograms were exposed for 375-586 days, then developed and stained with cresyl violet acetate. To control for artifacts due to negative chemography, some autoradiograms from each tissue were air-dried and exposed to light before storage. No negative chemography was seen.

Cell Nuclear Retention of [^3H]Estradiol (E_2)

Ovariectomized (OVX) mice were injected subcutaneously in the back with 10 μCi of [$6,7\text{-}^3\text{H}$] E_2 (Specific Activity 47.5 Ci/mmol) dissolved in 50 μl of 30% ethanol-70% physiological saline. One hour later they were killed by decapitation and their brains were removed. Cell nuclei were isolated from pooled hypothalamus, preoptic area, and amygdala (HPOA) tissue from 2-3 animals, as described above. The nuclear pellets were resuspended in 0.5 ml of 0.01 M citric acid, then extracted with 2×1 ml ethanol. The pooled ethanol extracts were counted with 10 ml Hydrofluor (National Diagnostics, Somerville, NY).

Exchange Assay of Cell Nuclear Estrogen Receptors

Mice were gonadectomized under light methoxyflurane anesthesia. One month later they were implanted subcutaneously with Silastic capsules (Dow Corning ID 0.058 in., OD 0.077 in., 1 cm long) containing either cholesterol or pure testosterone. One week after implantation, the animals were killed by decapitation, and their brains and pituitaries were removed. Cell nuclear estrogen receptor (ERn) concentrations were measured essentially as described by Roy and McEwen (1977) for nuclear receptors from rat brain and pituitary. Purified cell nuclei were prepared from pooled HPOA from 3-5 mice, as described above. The cell nuclear pellets were thoroughly resuspended in 0.25 ml of ice-cold TDB buffer (10 mM Tris base, 1 mM dithiothreitol, 0.5 mM bacitracin, adjusted to pH 7 with hydrochloric acid), and then mixed with 0.25 ml of TDBK 0.8 buffer (as for TDB, with the addition of 0.8 M KCl, adjusted to pH 7.6 with hydrochloric acid). After 30 min on ice with frequent vortexing, the nuclear residue was removed by centrifugation at $16,000 \times g$ for 10 min. The supernatants were decanted and incubated for 4.5 h at 25°C with 5 nM [^3H] E_2 . Control incubates, used in assessing the contribution from low-affinity binding, also contained a 50-fold molar excess of unlabeled DES. Bound radioactivity in 100- μl aliquots of the incubates was measured with gel filtration on columns of Sephadex LH-20 equilibrated with an equal parts mixture of TDB and TDBK 0.8 buffers.

Cytosolic Progesterone Receptor Assays

Mice were treated as described for the ERn study, above. The hypothalamus and preoptic area were dissected using a scheme analogous to that described by Luine, Khylichevskaya, and McEwen (1974) for the rat. Tissues were homogenized in TEGD buffer (10 mM Tris, 1.5 mM disodium-EDTA, 10% vol/vol glycerol, and 1 mM dithiothreitol adjusted to pH 7.4 with hydrochloric acid), with a motor-driven Teflon-glass homogenizer. Tissues from 2-3 animals were pooled for each milliliter of homogenization buffer. The homogenates were centrifuged at $105,000 \times g$ (average) for 1 h in a Sorvall OTD-75 ultracentrifuge at 2°C . The supernatant "cytosols" were decanted and used immediately. Cytosols were incubated for 4 h at 4°C with a fixed concentration (0.4 nM) of [^3H]R5020. Control incubates, used in assessing the contribution from low-affinity binding, contained 20 nM unlabeled R5020 in addition to the labeled steroid. Bound [^3H]R5020 in 0.2-ml aliquots of the incubates was measured by gel filtration at 4°C on small columns of Sephadex LH-20 equilibrated with TEGD buffer, as

previously described (MacLusky & McEwen, 1980). Macromolecular bound radioactivity was extracted into 5 ml of Betafluor (National Diagnostics) and counted at an efficiency of 55%.

Enzyme Assays

Adult mice gonadectomized 9 days previously received subcutaneous implants of Silastic capsules containing cholesterol, testosterone, or E_2 (Sigma, St. Louis, MO). Five days after implantation, the mice were sacrificed by decapitation, and the brains, pituitaries, and kidneys were removed. The hypothalamus, medial preoptic area, and corticomedial amygdala (HPOA) were pooled and immediately homogenized (1:7, w/v) in 50mM Tris-HCl, pH 8. The parietal cortex was dissected and homogenized separately (1:7). The pituitaries and kidneys were frozen on dry ice and stored in a Revco freezer (-40°C). Within 1 week they were homogenized in the same buffer (pituitary, 25 μl ; $\frac{1}{2}$ kidney, 1.8 ml), and the enzyme activities were measured.

Type A monoamine oxidase (MAO) in HPOA and cortex was assayed radiochemically, using [^3H]serotonin as substrate (Luine, Khylchevskaya, & McEwen, 1975b). Glucose-6-phosphate dehydrogenase (G6PDH) and lactic dehydrogenase (LDH) were assayed fluorimetrically (Luine, Khylchevskaya, & McEwen, 1975a). Alcohol dehydrogenase (ADH) was assayed according to Ohno, Stenius, and Christian (1970), by monitoring the oxidation of NADH in the presence of acetaldehyde at pH 7. Two modifications were made in the method to allow for fluorometric instead of spectrophotometric analysis. Concentrations of acetaldehyde and NADH were reduced to 1 mM and 10 mM, respectively. Because of the high sensitivity of fluorometric as opposed to spectrophotometric assay, sodium amytal was added to the reagent to inhibit nonspecific NADH oxidase. In addition, blanks that contained buffer, NADH, and amytal (no acetaldehyde) were monitored for each homogenate, and this rate was subtracted from the complete reagent values. Enzyme activities are expressed as nmol substrate consumed/mg protein/h.

Other Procedures

Radioactivity was counted in either a Packard Tri-Carb 3375 or a Beckman LS8000 liquid scintillation spectrometer. Efficiencies were calculated by internal standardization, using a tritiated toluene standard (New England Nuclear). Cytosol protein was measured by the method of Bradford (1956), homogenate protein concentrations by the method of Lowry, Rosebrough, Farr, and Randall (1951). DNA was assayed by the method of Burton (1956). The results were analyzed with Bartlett's test for homogeneity of variance, analysis of variance, Duncan's and Newman-Keuls multiple range tests, and Student's *t* test.

RESULTS

Cytosolic Binding of [^3H]DHT in Normal and Tfm Mice

Cytosol [^3H]DHT binding was detected in all tissues examined, from all four mouse genotypes. In confirmation of findings from previous studies, however, there were quantitative and qualitative differences between the genotypes in the sedimentation profiles of the bound [^3H]DHT complexes. Representative profiles for cytosols from the submaxillary gland, the kidney, and the HPAS region of the brain are shown in Figure 1. In submaxillary gland cytosols from Ta/Y males, two peaks of macromolecular bound radioactivity were observed, one at 8-9S and the other at 2-3S. In the Tfm/Y mice, there was a reduction in the size of the 8-9S peak and a con-

comitant increase in the binding observed in the 2-3S region. The results from the Tfm/X carrier females fell between those from the Tfm/Y and Ta/Y males. In HPAS cytosols there was no evidence of binding in the 2-3S regions of the gradients, but in the 8-9S region, a major peak of radioactivity was observed, which was reduced in the Tfm/Y and Tfm/X animals. In the other tissues examined (the pituitary, mid-brain + brain stem, and residual forebrain), the results conformed to the pattern seen in HPAS cytosols, with the majority of bound [^3H]DHT sedimenting at around 8-9S. The results in the Ta/X females were indistinguishable from those in the Ta/Y males (data not shown). Addition of a 100-fold molar excess of unlabeled DHT to the cytosol incubates completely suppressed [^3H]DHT binding in the 8-9S region of the gradients (Figure 1). Quantitation of the 8-9S binding in each genotype was achieved by integration of the area between the curves obtained in the presence and absence of unlabeled DHT. The results are summarized in Table 1. In all tissues, cytosol concentrations of 8-9S binding were indistinguishable in the Ta/X females and Ta/Y males. The Tfm/X "carrier" females yielded about half the binding observed in the Ta/X females, while in the Tfm/Y males cytosol binding in all tissues was reduced by approximately 90%.

Tissue Uptake of [^3H]DHT and Its Metabolites

In all tissue and serum samples, the radioactivity recovered after [^3H]DHT administration represented a mixture of toluene-extractable and toluene-inextractable metabolites. The bulk of the toluene-extractable radioactivity comigrated on TLC with authentic 5 α -DHT, 3 α -DIOL, and 5 α -ADIONE, with little or no radioactivity in the 3 β -DIOL and AONE regions of the chromatograms.

Among the four groups of mice studied, there were no significant differences in the serum concentrations of radioactivity at sacrifice. The majority of the serum [^3H]DHT metabolites remained in the aqueous phase after toluene extraction. The toluene extracts contained predominantly polar metabolites, which did not migrate from the origin in the TLC systems used, together with small quantities of 3 α -DIOL, ADIONE, and unchanged [^3H]DHT (Table 2). In the tissue homogenate samples, a considerably higher proportion of the radioactivity was toluene-extractable than was the case in the serum from the same animals. In the Ta/Y males, the highest levels of [^3H]DHT uptake per milligram of protein were observed in the pituitary and submaxillary gland, followed by the kidney, HPAS, and remaining brain structures. In all six tissue samples studied, concentrations of [^3H]DHT were much lower in the Tfm males than in the Ta/Y males, Ta/X females, or Tfm/X carrier females. Slight reductions in the tissue levels of 3 α -DIOL were also apparent in the kidneys and submaxillary glands of the Tfm/Y animals. In addition, in the submaxillary gland there was an almost 5-fold increase in the levels of polar (toluene-inextractable) metabolites recovered in the whole

Figure 1. Sucrose density gradient sedimentation profiles for bound $[^3\text{H}]$ DHT complexes in cytosols from tissues of normal male (Δ), Tfm/X female ($\circ$), and Tfm/Y male ($\bullet$) mice. Cytosols were incubated with 2 nM $[^3\text{H}]$ DHT for 2 h at 4°C . In an additional series of incubates, cytosols from tissues of Ta/Y males were also incubated with 2 nM $[^3\text{H}]$ DHT in the presence of 200 nM unlabeled DHT ($\diamond$). Aliquots (250 μl) of the incubates were then sedimented through linear 5-ml 5%-20% sucrose gradients for 22 h at $190,000 \times g$. The gradients were collected by the puncture in 12-drop (about 0.2 ml) fractions and counted for radioactivity. In the case of the brain and kidney results, the data for Fractions 4 to 17 are also presented, inset, on an expanded scale. Sedimentation is from right to left. The sedimentation markers equine myoglobin (2.0S), bovine serum albumin (4.6S), and bovine liver catalase (11.3S) were run in parallel and sedimented in Fractions 22, 17, and 5, respectively. Limbic brain = pooled hypothalamus, preoptic area, septum, and amygdala.

homogenate fractions, compared to the levels in the Ta/Y males.

Cell Nuclear $[^3\text{H}]$ DHT Binding

More than 90% of the radioactivity present in cell nuclei isolated from the submaxillary gland, kidney, pituitary, and brain of the GDX Ta/Y males killed 1 h after i.v. $[^3\text{H}]$ DHT comigrated on TLC with authentic unlabeled DHT. The identity of the nuclear bound radioactivity was confirmed by crystallization to constant specific activity from acetone and petroleum ether. In all cases, the specific activity of the crystallized steroid did not change by more than 5% over three successive crystallizations (data not shown). The levels of cell nuclear bound radioactivity recovered from tissues of the Ta/X, Ta/Y, Tfm/X, and Tfm/Y mice are presented in Figure 2. The effects of the Tfm mutation on nuclear $[^3\text{H}]$ DHT retention were similar in all tissues and mirrored the results obtained for cytosol 8S $[^3\text{H}]$ DHT binding. Cell nuclear concentrations of radioactivity were indistinguishable in the Ta/Y males

and Ta/X females. In the Tfm/X carrier females, nuclear radioactivity was about half those seen in Ta/X animals, while in the Tfm/Y males relatively low levels of nuclear uptake were observed.

To test the saturability of cell nuclear $[^3\text{H}]$ DHT uptake in normal and Tfm pseudohermaphrodite male animals, mice were given i.v. $[^3\text{H}]$ DHT injections with or without a 100-fold molar excess of unlabeled DHT. One hour later they were killed, and cell nuclei were prepared from their brains, kidneys, and submaxillary glands. In this experiment, the brains were not dissected into subregions, in order to maximize the quantities of radioactivity present in the nuclear pellets and hence to achieve optimal conditions for detecting competition from the unlabeled DHT injections. The results are shown in Table 3. In the male mice, the unlabeled DHT injection reduced the levels of cell nuclear $[^3\text{H}]$ DHT retention by more than 90%. In contrast, in the Tfm/Y animals, the already low cell nuclear concentrations of radioactivity were only slightly reduced by the unlabeled DHT treatment. This reduction

Table 1
Cytosol 8S [³H]DHT Binding in Normal and Tfm Mice

	[³ H]DHT Bound (femtomoles/mg protein)			
	Ta/Y	Ta/X	Tfm/Y	Tfm/X
Pituitary	15.9±3.4	12.1±0.3	0.7±0.3	7.8±2.5
Limbic Brain	15.3±3.4	14.6±0.3	0.8±0.2	5.8±1.2
Remaining Forebrain	10.8±0.9	9.1±0.2	1.4±0.1	3.5±0.6
Mid-Brain + Brain Stem	8.0±0.4	9.1±0.8	1.1±0.5	4.0±0.7
Submaxillary Gland	30.8±6.7	27.7±7.6	2.6±0.4	17.6±5.4
Kidney	8.5±1.3	9.3±1.0	1.4±0.4	4.5±1.9

Note—Estimates of cytosol 8S binding were obtained by calculating the total quantity of radioactivity sedimenting between Fractions 5 and 15 of density gradient profiles such as those presented in Figure 1, subtracting the radioactivity sedimenting from the control incubates containing excess unlabeled DHT. Binding is expressed in terms of femtomoles per mg protein applied to the gradients. The data represent means ($\pm$ SEM) of results from 3 experiments.

was not statistically significant in any of the three tissues examined ($p > .05$, Student's t test).

Autoradiographic Localization of [³H]DHT

Autoradiograms prepared from the brains and submaxillary glands of wild-type and Tfm male mice are shown in Figure 3. In accord with previous studies (Sheridan,

1978), nuclear retention of [³H]DHT was observed in the brains of the wild-type animals, but not in the Tfm males. In the submaxillary glands, nuclear labeling was again observed in the normal males. In the Tfm animals, overall grain densities over the submaxillary gland sections were at least as high as those in the normal males; but there was no evidence of nuclear concentration of radioactivity.

Table 2
[³H]DHT Uptake and Metabolism in Normal and Tfm-Bearing Mice

	Toluene Inextractable	Polar	Toluene Extractable		
			3 α -ADIOL	DHT	ADIONE
Pituitary					
Ta/Y	6.2 $\pm$ 0.1	6.2 $\pm$ 2.4	1.6 $\pm$ 0.9	26.5 $\pm$ 3.5	1.7 $\pm$ 1.0
Ta/X	8.2 $\pm$ 0.8	7.3 $\pm$ 3.1	1.9 $\pm$ 1.0	19.2 $\pm$ 8.2	2.4 $\pm$ 0.6
Tfm/Y	3.1 $\pm$ 1.6	8.0 $\pm$ 3.0	2.3 $\pm$ 0.5	7.2 $\pm$ 1.9†	2.6 $\pm$ 1.2
Tfm/X	4.0 $\pm$ 2.4	7.7 $\pm$ 2.6	2.4 $\pm$ 0.6	14.5 $\pm$ 0.5*	1.8 $\pm$ 0.7
HPAS					
Ta/Y	0.6 $\pm$ 0.2	1.7 $\pm$ 0.1	0.3 $\pm$ 0.1	4.5 $\pm$ 0.4	0.2 $\pm$ 0.1
Ta/X	0.8 $\pm$ 0.6	1.2 $\pm$ 0.2	0.5 $\pm$ 0.3	2.6 $\pm$ 0.4	0.2 $\pm$ 0.1
Tfm/Y	0.9 $\pm$ 0.5	1.0 $\pm$ 0.2	0.3 $\pm$ 0.1	0.5 $\pm$ 0.1†	0.3 $\pm$ 0.1
Tfm/X	0.4 $\pm$ 0.1	1.2 $\pm$ 0.7	0.6 $\pm$ 0.1	2.5 $\pm$ 0.4*	0.2 $\pm$ 0.03
Kidney					
Ta/Y	5.8 $\pm$ 1.5	4.2 $\pm$ 0.4	1.1 $\pm$ 0.1	4.3 $\pm$ 0.8	0.10 $\pm$ 0.06
Ta/X	4.9 $\pm$ 1.4	2.5 $\pm$ 0.7	1.3 $\pm$ 0.1	2.5 $\pm$ 0.2*	0.06 $\pm$ 0.01
Tfm/Y	3.9 $\pm$ 0.9	3.4 $\pm$ 0.6	0.4 $\pm$ 0.1†	0.4 $\pm$ 0.1†	0.09 $\pm$ 0.01
Tfm/X	6.9 $\pm$ 1.1	3.5 $\pm$ 1.0	1.5 $\pm$ 0.5	2.7 $\pm$ 0.7*	0.10 $\pm$ 0.02
Submaxillary Gland					
Ta/Y	1.8 $\pm$ 0.5	3.2 $\pm$ 0.9	2.0 $\pm$ 0.8	17.8 $\pm$ 1.3	0.28 $\pm$ 0.11
Ta/X	1.1 $\pm$ 0.3	4.6 $\pm$ 0.9	2.0 $\pm$ 0.9	17.8 $\pm$ 2.9	0.40 $\pm$ 0.22
Tfm/Y	8.3 $\pm$ 1.7†	3.7 $\pm$ 1.3	0.9 $\pm$ 0.6†	0.6 $\pm$ 0.2†	0.55 $\pm$ 0.48
Tfm/X	2.6 $\pm$ 1.1	3.0 $\pm$ 0.9	3.0 $\pm$ 0.9	20.8 $\pm$ 5.4	0.40 $\pm$ 0.07
Serum					
Ta/Y	3564 $\pm$ 367	587.2 $\pm$ 61.0	48.5 $\pm$ 7.0	53.0 $\pm$ 23.3	9.0 $\pm$ 3.9
Ta/X	3726 $\pm$ 319	337.4 $\pm$ 131.5	72.1 $\pm$ 31.2	50.5 $\pm$ 17.8	9.7 $\pm$ 5.0
Tfm/Y	2997 $\pm$ 99	521.8 $\pm$ 67.1	37.0 $\pm$ 10.0	28.9 $\pm$ 1.0	8.4 $\pm$ 2.4
Tfm/X	3709 $\pm$ 647	354.0 $\pm$ 92.4	64.4 $\pm$ 19.0	44.6 $\pm$ 18.5	8.8 $\pm$ 3.4

Note—Metabolites recovered from the tissues and serum of gonadectomized normal and Tfm-bearing mice, 1 h after the i.v. administration of 10 μ Ci [³H]DHT. Results represent the means $\pm$ SEM of 3 observations in each case, and are expressed in terms of either femtomoles/mg protein (tissue samples) or femtomoles/ml serum. Each observation was obtained from material pooled from 3–5 animals. Results for the mid-brain and remaining forebrain samples were similar to those for the pooled hypothalamus, preoptic area, septum, and amygdala (HPAS): to conserve space, the results for the former two tissue samples are not shown. *Significantly different from results in Ta/Y males. †Significantly different from results in all other genotypes (Newman-Keuls Test, $p < .05$ level).

Table 3
Saturability of Cell Nuclear [³H] Retention in Normal and Tfm Mice

	Cell Nuclear [³ H]DHT (femtomoles/mg DNA)		
	Brain	Kidney	Submaxillary Gland
Ta/Y	21.7±3.1	38.9±8.0	356.1±17.5
Ta/Y + Unlabeled DHT	1.3±0.3	1.2±0.1	3.8±1.2
Tfm/Y	1.4±0.4	1.4±0.4	1.8±0.5
Tfm/Y + Unlabeled DHT	0.9±0.1	0.8±0.1	1.3±0.1

Note—Gonadectomized mice were injected via the jugular vein with either [³H]DHT alone (10 μ Ci; 250 pmoles) or [³H]DHT + a 100-fold molar excess of unlabeled DHT, and killed 1 h later. The radioactivity present in purified cell nuclei from the entire brain, the kidney, and the submaxillary gland was extracted and counted. Results represent the means $\pm$ SEM of 3 observations in each case.

Labeling appeared to be evenly dispersed across the entire gland, including the extracellular spaces and lumina of the ducts.

Nuclear Estrogen Receptors

In OVX BALB-C mice, injections of [³H]E₂ followed by extraction of isolated cell nuclei gave estimates of nuclear binding in pooled HPAS similar to those obtained by exchange assay after an identical dose of unlabeled E₂ (data not shown). These observations confirmed the validity of applying the exchange assay of Roy and McEwen (1977) to mouse brain tissue. Levels of cell ERn were measured by exchange assay in the HPOA of intact, GDX, and GDX T-implanted mice. The T implants were placed subcutaneously in the back 1 week prior to sacrifice. In normal males, gonadectomy resulted in a significant decline (approximately 3-fold) in ERn concentrations (Figure 4A). In contrast, intact and GDX Tfm

males gave similarly low values, within the range observed in GDX wild-type Ta/Y males. In all four genotypes (Ta/Y, Tfm/Y male; Ta/X, Tfm/X female), ERn concentrations were elevated approximately 4-fold after T implantation (Figure 4A). Radioimmunoassay measurements revealed that blood levels of T increased from < 0.2 ng/ml in the GDX animals to 1.5–3 ng/ml after T implantation.

Cytosol Progesterin Receptors

Previous studies have demonstrated that estrogens induce progesterin receptor synthesis in the hypothalamus and preoptic area of several mammalian species, including the mouse (MacLusky & McEwen, 1980; Roselli & Snipes, 1983). Progesterin receptor concentrations thus provide an index of estrogen action within the hypothalamus. To complement the measurements of ERn binding in wild-type and Tfm animals, the effects of T on cytosol progesterin

Figure 2. Cell nuclear radioactivity 1 h following administration of 10 μ Ci [³H]DHT, i.v. via the jugular vein. Results represent the means ($\pm$ SEM) of three observations. Limbic brain = pooled hypothalamus, preoptic area, septum, and amygdala. RFB = forebrain remaining after removal of the limbic structures.

Figure 3. Autoradiographic localization of radioactivity retained in the brains and submaxillary glands of gonadectomized wild-type and Tfm male mice 1 h after i.v. administration of [^3H]DHT. Top left: wild-type; Nucleus preammillaris ventralis, 375 days exposure. Top right: Tfm/Y; nucleus arcuatus, 486 days exposure. This nucleus is heavily labeled in normal male mice after [^3H]DHT administration (Sheridan, 1978). Lower left: wild-type; submaxillary gland, 586 days exposure. Lower right: Tfm/Y; submaxillary gland, 586 days exposure. Original magnification for all four panels 570 $\times$.

Figure 4. Nuclear estrogen receptor (ERn; Panel A) and cytosolic progesterin receptor induction (PRc; Panel B) in normal and Tfm-bearing males and females after gonadectomy (GDX) and testosterone (T) treatment. Animals were studied either intact, or after GDX with and without T treatment from a subcutaneous Silastic capsule. Mice gonadectomized 1 month previously were implanted subcutaneously with Silastic capsules containing either cholesterol or pure T. One week after implantation, the animals were killed. ERn were measured by exchange assay, in the pooled hypothalamus, preoptic area, and amygdala (HPOA) from GDX animals. Cytosolic progesterin receptors were measured in the pooled hypothalamus and preoptic area. Results represent the means $\pm$ SEM of 4–6 observations in each case. Each observation was based on tissue pooled from 2–3 animals.

receptor (PRc) levels were studied in a separate series of experiments. The results are shown in Figure 4B. In the wild-type Ta/Y animals, progesterin receptor levels were significantly reduced by gonadectomy. In contrast, in the Tfm/Y mice, levels of [3 H]R5020 binding in both intact and GDX animals were indistinguishable from those found in GDX Ta/Y males. T implantation markedly elevated progesterin receptor concentrations, to levels above those seen in the intact male. The level of induction observed in the Tfm/Y male, however, was approximately 50% less than that observed in either the wild-type Ta/Y male or the Tfm/X carrier female (Figure 4B). In all four genotypes, low levels of R5020 binding were observed in the pituitary gland after gonadectomy, with no significant effect of T implantation on these levels (data not shown).

The somewhat reduced PRc response of the Tfm/Y mice suggested that the estrogen sensitivity of this genotype might have been impaired, relative to that of normal male and female controls. To test this possibility, additional

groups of GDX Ta/X, Ta/Y, and Tfm/Y mice were injected subcutaneously with a low dose (1 μ g) of E_2 and assayed 24 h later for PRc levels in the hypothalamus and preoptic area. The results for the three groups of animals were indistinguishable. Means $\pm$ SEMs for [3 H]R5020 binding, expressed in fmol/mg protein, were as follows: for Ta/X (GDX), 16.2 ± 1.2 ; for Ta/X (GDX + E_2), 22.67 ± 2.2 ; for Ta/Y (GDX), 15.1 ± 1.0 ; for Ta/Y (GDX + E_2), 21.0 ± 2.7 ; for Tfm/Y (GDX), 14.9 ± 0.8 ; for Tfm/Y (GDX + E_2) 22.1 ± 2.2 ; there were four observations in each case.

T-dependent Enzymatic Changes in Ta/Y and Tfm/Y Males

A number of previous studies have shown that T administration to castrate male rats or mice results in an increase in the activity of specific enzymes in gonadal hormone target organs. Some of these changes include increased G6PDH and LDH in the pituitary (Luine et al., 1975a), ADH in the kidney (Ohno et al., 1970), and MAO in the hypothalamus-preoptic area of the brain (Luine et al., 1975b). In this section of experiments, we castrated mouse siblings of the Ta/Y and Tfm/Y genotypes. Nine days later they were implanted with Silastic capsules containing 1.5 cm of packed cholesterol or T for 5 days. We then examined the activity of the above mentioned enzymes. Previous studies by Ohno and Lyon (1970) established that ADH in the kidney of Tfm-affected male mice does not respond to T treatment. We were interested to determine whether other target tissues would show the same pattern of response: an increase in enzyme activity after T in normal castrate mice, but no change in Tfm males.

In the kidney, T dramatically increased ADH activity, approximately 6-fold, in Ta/Y males. However, ADH in T-treated castrate Tfm males was unchanged (see Figure 5). G6PDH activity in the kidney also increased after T administration in castrate Ta/Y mice, but not in castrate Tfm/Y mice. The change in normal mice, an approximately 30% increase, was much smaller than the ADH change.

The activity of Type A MAO was investigated in the HPOA in the same group of mice. In the HPOA, MAO activity in Ta/Y males was increased approximately 20% by T (see Figure 5). T administration was without effect on MAO activity in the HPOA from the Tfm/Y males. In the cerebral cortex, T administration did not alter MAO activity in either the normal or the Tfm males (data not shown). The lack of an effect of T on HPOA MAO in the Tfm/Y mice suggested that this genotype might resemble the normal female with respect to this endpoint. In rats, only the male responds to T with a change in hypothalamic MAO activity (Luine et al., 1975b), the female being unaffected by such treatment. To determine whether a similar sexual dimorphism might exist in the mouse, we implanted OVX female BALB-C mice with 1.5-cm Silastics, which either were empty ($n = 11$) or contained T ($n = 8$) and measured HPOA MAO. No T

Figure 5. Effects of testosterone (T) administration on enzyme activities in kidney and combined hypothalamus-preoptic area-amygdala (HPOA) of male mice. Normal male (Ta/Y) and Tfm-affected male (Tfm/Y) mice were gonadectomized (GDX). Nine days later they received Silastic capsules containing cholesterol (open bars) or T (solid bars). Five days later they were killed and enzyme activities measured. Panel A: Activity of kidney ADH and G6PDH. Panel B: Activity of HPOA MAO. Enzyme activity is expressed as μmol substrate consumed/mg protein/h and is represented by the average $\pm$ SEM for determinations in 5 mice/group. Data were analyzed by Student's *t* test (GDX vs. T for Ta/Y and GDX vs. T for Tfm/Y, where **p* < .05, ***p* < .01, and ****p* < .001).

effect was observed (MAO activity in nmol/mg protein/h: for OVX $M \pm \text{SEM} = 70.8 \pm 3.7$; for T-implanted, 66.1 ± 5.6).

The same enzymes were also measured in the brains and kidneys of mice receiving a Silastic implant containing 0.5 cm of testosterone. Significant but smaller changes were noted in enzyme activities of T-treated Ta/Y, but not Tfm/Y, mice. Pituitary G6PDH and LDH activities were investigated in males receiving both T regimens. In contrast to previous results in male rats (Luine et al., 1975a), T administration was without effect on either enzyme in Ta/Y or Tfm/Y mice (data not shown).

Estradiol Dependent Enzymatic Changes in Ta/Tfm Females and Tfm/Y Males

The lack of effect of the Tfm mutation on ER α binding suggested that, while androgen responses were impaired in the Tfm mice, the enzymatic responses to estrogen should be similar in wild-type and Tfm animals. To test this hypothesis, E₂ dependent enzymatic responses in the pituitary, kidney, and brain were compared. Tfm/Ta females and Tfm/Y males were gonadectomized for 9 days, received Silastic implants containing 1 cm of cholesterol or E₂ for 5 days, and then the kidneys, brains, and pituitaries were removed for enzymatic analysis.

In the kidney, the activity of G6PDH was increased in both the Ta/Tfm and the Tfm/Y animals by approximately 30% and 20%, respectively. ADH activity was less

responsive to E₂ treatment, and no effect was noted in either genotype. In the pituitary, E₂ treatment increased the activity of G6PDH and LDH by 2–3-fold in both genotypes (see Figure 6). In contrast, HPOA MAO activity was increased by E₂ (30%) *only* in the carrier females, and not in the Tfm/Y males (see Figure 6).

To determine whether the lack of an HPOA MAO response in the Tfm male was due to expression of the Tfm gene or to some aspect of sexual differentiation in these animals, normal BALB-C male and female mice were also examined for their responses to E₂ administration, using an identical experimental protocol. The results were similar to those in the Tfm/X and Tfm/Y animals. Although no differences were observed between males and females with respect to pituitary G6PDH responses to E₂, only the genetic females exhibited an increase in HPOA MAO activity in response to E₂ exposure. The activity of MAO in the males was unchanged (data not shown).

DISCUSSION

This study has explored the consequences of the Tfm mutation in four androgen target tissues: the brain, pituitary, submaxillary gland, and kidney. The results amplify and extend previous data on the effects of this mutation in the mouse, and they provide further support for the validity of using Tfm males in studies aimed at dissecting apart the contributions of androgen- and estrogen-

Figure 6. Effects of estradiol (E_2) administration on enzyme activities in the pituitary and the pooled hypothalamus, preoptic area, and amygdala (HPOA) of Tfm carrier female and Tfm-affected male mice. Carrier female (Tfm/Ta) and affected male (Tfm/Y) mice were gonadectomized (GDX). Nine days later they received Silastic capsules containing cholesterol (open bars) or estradiol (E_2 ; solid bars). Five days later they were killed and enzyme activities measured. Panel A: Activity of pituitary G6PDH and LDH. Panel B: HPOA MAO. Enzyme activity is expressed as μmol substrate consumed/mg protein/h and is the average $\pm$ SEM for determinations in 9 mice/group, except for GDX Tfm/Ta, which was 6 mice. See legend to Figure 5 for statistical analysis.

mediated responses to the mechanisms underlying sexual differentiation of the brain. The androgen receptor data confirm previous studies and, furthermore, establish that the consequences of the Tfm defect on receptor concentrations in different tissues are remarkably similar, when these tissues are analyzed simultaneously. In Tfm/Y males, 8S androgen receptor levels are reduced by approximately 90% (range 83.5%–95.6%) in different regions of the brain, as well as the pituitary, submaxillary gland, and kidney (see Table 1). In Tfm/X carrier females, androgen receptor concentrations fall approximately midway between those for wild-type and Tfm/Y males, which presumably reflects the process of random X-chromosome inactivation (Lyon, 1961; Takeda, Suzuki, Lasnitzki, & Mizuno, 1987). In confirmation of the findings of Verhoeven and Wilson (1976), the loss of androgen receptors in the submaxillary gland is accompanied by an increase in binding to a protein sedimenting at 2–3S. The autoradiographic results suggest, however, that unlike the receptor, this binding is not associated with the cell nucleus, and that much of the radioactivity retained in the submaxillary gland of the Tfm male is extracellular. The 4-fold increase in the concentration of polar DHT metabolites observed in the submaxillary gland of the Tfm/Y mouse suggests that much of the extracellular radioactivity observed on the autoradiograms from the submaxillary gland of the Tfm male may in fact represent conjugated metabolites of the injected steroid.

Although some residual androgen binding was consistently observed in all tissues from the Tfm/Y males, this binding did not appear to be associated with saturable nuclear $[^3\text{H}]\text{DHT}$ binding, suggesting that the “residual” receptor may be defective in terms of its interaction with the chromatin. If the residual androgen binding was in fact capable of interacting with the nucleus, we should have expected a reduction in saturable nuclear binding in proportion to the reduction in cytosol binding capacity. This was not the case. This is most clearly illustrated by the data for the submaxillary gland, where the cytosol results suggested that we should have observed approximately 30 fmol/mg DNA of saturable nuclear binding, if the 8–9S cytosol $[^3\text{H}]\text{DHT}$ binding species represented a functional androgen receptor. In fact, only a slight (0.5 fmol/mg DNA) reduction in nuclear binding was observed in this tissue after injection of excess unlabeled DHT (see Table 3). These results indicate that, at least in the mouse, the Tfm mutation results not only in a reduction in androgen receptor concentrations but also in the synthesis of “receptors” that retain the capacity to interact with androgens, but do not bind normally within the cell nucleus.

This conclusion is consistent with the results of a number of other studies on the synthesis and properties of androgen receptors in tissues from Tfm/Y males. The Tfm androgen receptor exhibits properties closely similar to those of the wild-type receptor, in terms of its steroid af-

finitly and specificity as well as its molecular size and charge characteristics (Attardi, Geller, & Ohno, 1976; Attardi & Ohno, 1978). Differences between the Tfm and wild-type proteins have, however, been reported with respect to their DNA binding affinities and isoelectric points (Fox, Blank, & Politch, 1983; Max, 1981; Weiland & Fox, 1979). A very recent report by Tindall, Young, and Prescott (1988) indicates that the Tfm receptor has a reduced number of immunodeterminants, when compared with androgen receptors from normal males, suggesting an abnormality in the receptor molecule. The question of how this occurs remains unresolved, although the available evidence points to a failure in androgen receptor mRNA production, rather than in the synthesis and subsequent processing of the receptor protein itself. Expression of full-length androgen receptor mRNA is markedly deficient in the Tfm male mouse, compared to the level for normal littermate controls (Tan, Joseph, & Wilson, 1988). This loss of mRNA could result from either a mutation in the mechanisms that control androgen receptor gene expression, or a structural defect within the gene itself, leading to reduced levels of synthesis of mRNA species that encode abnormal receptor proteins. The latter hypothesis is supported by studies in man, which show that the androgen receptor gene maps to the X-chromosome (Lubahn et al., 1988; Meyer, Migeon, & Migeon, 1975), and that, at least in some instances, complete androgen insensitivity is associated with the deletion of a portion of this gene (Brown, Lubahn, Migeon, & French, 1988). There appears, however, to be considerable heterogeneity in the various forms of partial and complete receptor-based androgen insensitivity that have been described in the human population, so it may be premature to assume that the mouse defect also involves a deletion mutation (Griffin & Wilson, 1980; Grino, Griffin, & Wilson, 1988; Stamatiadis, Portois-Bulteau, & Mowszowicz, 1988).

In contrast to the results for androgen binding, nuclear estrogen binding following T administration to the Tfm male appears to be normal, which suggests that aromatization and nuclear estrogen binding are both present in the brains of these animals. These findings are consistent with previous results in Stanley-Gumbreck Tfm/Y rats, which also demonstrated normal ERn levels in the hypothalamus and preoptic area after T replacement therapy (Krey et al., 1982). At first sight, they appear discordant with direct measurements of aromatase in both the mouse (Rosenfeld, Daley, Ohno, & Young-Lai, 1977) and the rat (Roselli, Salisbury, & Resko, 1987), which have demonstrated lower aromatase activities in the brains of Tfm/Y males than in normal male controls. The data of Roselli, Salisbury, and Resko (1987), however, suggest that this difference primarily reflects androgen action in the normal animals, and that it is region-specific, being less pronounced in the amygdala than in the hypothalamus and preoptic area. In the present studies, ERn levels were measured in the pooled HPOA, in which possible hypothalamus differences may have been diluted

by the presence of the amygdala. Our PRc data (see Figure 4B) in fact suggest that the estrogenic stimulus in the hypothalamus and preoptic area resulting from T implantation may have been somewhat reduced in the Tfm male as opposed to the three other genotypes studied (Ta/Y, Tfm/X, and Ta/Y), since lower PRc induction was observed in the Tfm/Y animals. Tfm male mice do not exhibit a measurable deficit in the ability of their PRc systems to respond to low doses of E₂ administered through the general circulation, as opposed to wild-type controls, and thus it seems likely that the difference in PRc induction following T implantation probably reflects local E₂ concentrations.

The enzyme assay data confirm the reported deficit of ADH induction in kidney from Tfm males and indicate that G6PDH in kidney also responds to T in normal but not in Tfm mice. In addition, the lack of T-dependent enzymatic responses in Tfm males has been extended to include the CNS where Type A MAO activity is increased by T in the HPOA of normal males but not in the same tissues from Tfm-affected males. While the pituitary contains high levels of androgen receptors, the two enzymes examined in the pituitary—G6PDH and LDH—did not respond to androgen in either normal or Tfm-affected males. These enzymes do respond to T in the pituitaries of normal male rats (Luine et al., 1975a).

This aspect of the study provides additional evidence that the Tfm mutation involves a specific defect on the X-chromosome. The allele-regulating G6PDH activity is also located on the X-chromosome (McKusick & Ruddle, 1977). We found no deficiencies in kidney, pituitary (see Figures 5 and 6), or brain (Luine, unpublished observations) G6PDH activities in castrate Tfm/Y or Tfm/X mice, as compared with the other genotypes, indicating that the mutation responsible for the Tfm defect does not have a generalized effect on the functions of the X-chromosome.

The response of the tissue enzymes to E₂ was also examined. Since the Tfm mutation does not affect levels of kidney or brain E₂ receptors (Attardi et al., 1976; Fox, 1975), normal enzymatic responses to E₂ should be expected in the Tfm mutant. In the pituitary and kidney, this prediction was supported: the Tfm males, like heterozygous females, normal females, and normal males, responded to E₂ with increased G6PDH activity. In the brain, however, HPOA MAO was not increased by E₂ in Tfm males. Heterozygous and normal females did show increased HPOA MAO after E₂ administration. Thus, the Tfm male responded like the normal male, which also showed no increase in HPOA MAO activity after E₂ administration. However, in contrast to the Tfm, the normal male *did* respond to T implantation. The insensitivity of the Tfm/Y male to either T or E₂ administration distinguishes this genotype from either of the normal sexual phenotypes. In both rats (Luine et al., 1975b) and mice (the present study), MAO activity in the brain appears to be regulated by the gonadal hormone associated with the homotypic sex—T in males and E₂ in females. The

Tfm/Y male falls somewhere between these two conditions, MAO activity responding to neither of these two steroids.

These findings provide a striking parallel to observations on the behavioral and neuroendocrine consequences of the Tfm mutation. Paradoxically, despite the almost complete failure of normal masculinization of the internal and external genitalia in the Tfm male, the brain nevertheless appears to undergo at least some aspects of normal androgen-induced sexual differentiation, as a result of the greater dependence of T actions within the brain on local estrogen biosynthesis. In rodents, testicular feminization is associated with low levels of expression of both masculine and feminine sexual behaviors (Beach & Buehler, 1977; Ohno, Geller, & Young-Lai, 1974; Olson, 1979a, 1979b). Because T production from the testes of the Tfm male is normal during early development (Goldstein & Wilson, 1972), and the mechanisms required for T action through local estrogen biosynthesis are present in the brains of testicular feminized animals, it is reasonable to suppose that such animals should undergo those aspects of sexual differentiation that depend on local aromatization. Several studies have demonstrated that, in the Stanley-Gumbreck Tfm rat, the developing testes are responsible for defeminization of the mechanisms that control sexual behavior and cyclic gonadotrophin release. Olsen (1979a) reported that, after castration on the day of birth, Tfm male rats gain the capacity to express high levels of feminine sexual behavior in response to estrogen and progesterone priming in adulthood. Likewise, Krey et al. (1982) reported that castration of Tfm male rats on Postnatal Day 2, but not at Day 10, prevented the losses of feminine sexual behavior and positive feedback control of gonadotrophin release normally observed in these animals. Although similar studies on the effects of neonatal gonadectomy have not been performed in the mouse, the available evidence suggests that Tfm/Y male mice probably also undergo central defeminization: in adulthood, they exhibit little or no feminine sexual behavior in response to estrogen and progesterone priming (Ohno, Geller, & Young-Lai, 1974) and show a male-like acyclic pattern of ovarian regulation after heterotopic ovarian transplantation under the kidney capsule (Ohno, 1977; MacLusky, unpublished observations, 1988).

The aromatization hypothesis provides a reasonable explanation for the impaired feminine behavior of the Tfm male rat, but it is difficult to reconcile with a number of other aspects of sexual differentiation in these animals. Some sexually dimorphic behaviors appear to remain feminine in the Tfm male, including saccharin preference (Shapiro & Goldman, 1973) and social play behavior (Meaney, Stewart, Poulin, & McEwen, 1983). Particularly puzzling is the failure of these animals to express normal levels of masculine sexual behavior. Previous studies from several laboratories (Paup, Mennin, & Gorski, 1975; Sodersten, 1973) have demonstrated that the full pattern of masculine sexual behavior can be re-

stored in castrate male rats by administration of estradiol benzoate (although there have also been reports of incomplete responses to this steroid—e.g., Olsen, 1979a). In contrast, the nonaromatizable androgen, DHT, is relatively ineffective (McDonald et al., 1970; Parrott, 1975; Whalen & Luttge, 1971). Administration of the aromatase inhibitor ATD impairs T-induced differentiation of masculine sexual behavior (Booth, 1978). These observations clearly suggest that aromatization may play a role in both the differentiation and the induction of masculine sexual behavior in normal male rats. Thus, it is somewhat surprising that the Tfm male exhibits a reduced capacity to express masculine behavior compared to that of its normal male siblings, even when the steroid used to induce this behavior is E_2 (Olsen, 1979b).

A possible explanation for these apparently discordant results is that normal masculinization of sexual behavior may require actions of T mediated through *both* the androgen and the estrogen receptor systems. There is a good deal of evidence to support this viewpoint. Aspects of sexually differentiated CNS function that remain phenotypically female in the Tfm male, such as play behavior, may be primarily dependent on androgen action per se (Meaney et al., 1983). DHT synergizes with estrogen with respect to both the induction of masculine sexual behavior in adulthood (Baum & Vreeburg, 1973; Lodder & Baum, 1977) and the differentiation of reproductive behavior patterns during early life (Booth, 1977). Testosterone action through the estrogen receptor system may be indirectly impaired in the Tfm male, as a function of the androgen dependence of the aromatase enzyme. As indicated above, hypothalamic aromatase activity is reduced in the Tfm male, probably because the androgen receptor-mediated induction of aromatase activity observed in normal male rats is attenuated or absent in the Tfm (Roselli & Resko, 1984; Roselli et al., 1987). Since androgen induction of aromatase may play a role in some T responses (MacLusky, Philip, Hurlburt, & Naftolin, 1985; Weisz, Brown, & Ward, 1982), lack of this induction could attenuate the estrogen receptor-mediated actions of T.

This represents the major theoretical weakness of the Tfm male as an experimental model for discrimination of the relative contributions of androgen and estrogen receptor-mediated responses to the actions of circulating T. The effects of T mediated through androgen and estrogen receptors are obviously not independent: androgens regulate estrogen biosynthesis and action, and they have been shown to exert direct effects on the estrogen receptor system at high dose levels (Roselli & Resko, 1984; Roselli et al., 1987; Zava & McGuire, 1978). Recent studies suggest that estrogens may also influence the central actions of androgen, with estrogen treatment increasing androgen receptor concentrations in several regions of the rat brain (Handa, Stadelman, & Resko, 1987). Thus, the reduction in the number of androgen receptors observed in the Tfm male cannot be considered solely in terms of its impact on androgen receptor-mediated responses.

Nevertheless, there is clearly a considerable separation of the aromatase- and androgen receptor-dependent actions of T in the Tfm male. Although aromatase activity may be reduced in the brains of androgen receptor-deficient animals, the remaining activity is sufficient to generate biologically active local concentrations of estrogen. Krey et al. (1982) observed induction of ER α and PR α , as well as changes in circulating LH levels and feminine sexual behavior, in castrated Tfm male rats treated with T-containing Silastic capsules. While in the present study the PR α response to T of the Tfm male mice was somewhat diminished, it still represents an almost 3-fold increase compared to that of the gonadectomized male controls. This is in stark contrast to the complete absence of significant responses to T in the three nonneural androgen target tissues studied. Thus, despite the loss of androgen's inductive effects on aromatase activity, the Tfm male remains a useful model for discriminating between responses to T that can be mediated by estrogen alone, and those that also require androgen receptor-mediated effects. If a particular response to T is present in the Tfm male, then it is reasonable to suppose that it may be mediated through local estrogen biosynthesis. It is only with respect to the alternative situation, in which T responses are impaired in Tfm-affected individuals, that ambiguities of interpretation may occur. Unfortunately, masculine sexual behavior presents just such an ambiguity. The limited masculine sexual behavior of the Tfm/Y animal suggests that the androgen receptor system is involved in some way in the differentiation and/or activation of this behavior pattern. This involvement need not, however, be based on a direct androgen-receptor mediated action of T. It is also possible that reduced aromatase activity in the brain of the Tfm might lead to generation of insufficient estrogen to bring about normal masculine differentiation. This issue could be resolved by examining the effects of neonatal hormone administration in the Tfm male, since, if the failure to undergo normal masculine differentiation in this genotype is due solely to insufficient estrogen biosynthesis, neonatal treatment with either T or E $_2$ should eliminate the observed behavioral deficit in response to E $_2$ administration in adulthood.

Further study of the characteristics of Tfm-affected individuals may be particularly informative in terms of the possible mechanisms underlying sex differences in higher CNS functions. Studies of the distribution of aromatase in the brain suggest that androgens may influence the development of a number of brain structures besides those most directly associated with the sexual differentiation of reproductive function. In both rodents (MacLusky, Clark, Naftolin, & Goldman-Rakic, 1988) and primates (Clark, MacLusky, & Goldman-Rakic, 1988; MacLusky, Naftolin, & Goldman-Rakic, 1986; Roselli & Resko, 1984), aromatase activity appears to be present in the developing cortex and hippocampus, as well as in the HPOA. The physiological significance of these observations remains to be elucidated, but an intriguing

possibility is that local estrogen biosynthesis may play a role in the genesis of sex differences in cognitive function. In man, males and females differ significantly with respect to a number of aspects of cognitive development, including visuospatial and verbal abilities, cerebral lateralization, and the incidence of developmental learning disorders (Maccoby, 1966; McGlone, 1980). Androgen levels during early development have been suggested as a possible causal factor for these differences (Geschwind & Behan, 1982). In rodents, sex differences with respect to maze-learning performance (Williams, Barnett, & Meck, *in press*) and right-left differences in the thickness of the cerebral cortex (Stewart & Kolb, 1988) have both been shown to be dependent on testosterone secretion in the male during early life. The presence of aromatase activity in the cortex and hippocampus clearly suggests that the actions of testosterone on higher CNS functions may be mediated through mechanisms essentially similar to those for the sexual differentiation of neuroendocrine and reproductive behavior patterns, involving effects of locally synthesized estrogen (MacLusky & Naftolin, 1981). A corollary of this hypothesis is that cognitive function in the Tfm-affected individuals should develop along masculine lines. In man, the available evidence is inconsistent with this idea: the reports of Money, Ehrhardt, and Masica (1968) and Masica, Money, Ehrhardt, and Lewis (1969) suggest that the Tfm condition is associated with female gender identity and sexual orientation, as well as a typically feminine pattern of visuospatial and verbal ability. However, additional studies, including work in experimental animals, will be required to fully elucidate the consequences of the Tfm mutation on higher CNS functions.

More detailed examination of the consequences of the Tfm mutation may also prove informative in terms of elucidating the cellular response mechanisms underlying the differentiation and activation of sexually dimorphic CNS functions. As previously mentioned, the Tfm male is characterized by incomplete masculinization of both central and peripheral androgen-dependent, sexually dimorphic characteristics. This pseudohermaphroditic condition provides the opportunity not only to distinguish between androgen- and estrogen-mediated responses, but also potentially to study the mechanisms responsible for these responses in the absence of some of the complexities presented by the normal male phenotype. In many species, it is difficult to separate the central effects of gonadal steroids from the indirect consequences of peripheral masculinization. In man, the sex of rearing, determined by physical secondary sexual characteristics, has a major impact on psychosexual development (Ehrhardt & Meyer-Bahlburg, 1981). In experimental animals, the presence of masculine external genitalia and testosterone-induced pheromonal secretions have undeniable effects on the expression of sexually dimorphic behaviors (Baum & Vreeburg, 1973; Larsson, Sodersten, & Beyer, 1973; Olsen, 1979b). In Tfm males, the brain is subject to effects of locally synthesized estrogen; but

the peripheral androgen-dependent organs remain essentially female, allowing separation of the direct central effects of aromatized androgen from the consequences of peripheral masculinization. Comparisons of the cellular (biochemical and morphological) effects of gonadal steroids in normal and Tfm-affected males could lead to a greater understanding of the role of these effects in the mediation of behavioral and neuroendocrine responses to steroid hormone exposure, as well as their steroid specificity. Schleicher, Stumpf, Drews, and Sar (1986) have suggested that sex-reversed mice heterozygous for the Tfm gene could be used to better define the role of androgen receptors in specific regions of the central nervous system, since such mice exhibit differing degrees of impairment of androgen binding in different regions of the brain. The potential usefulness of the Tfm gene in elucidating the functions of specific biochemical androgen responses is illustrated by the MAO data presented above. The finding of an impaired MAO response to T as well as E₂ in Tfm male mice suggests that steroidal regulation of MAO activity may be involved in the behavioral responses of both males and females to gonadal steroid administration. Thus, loss of E₂ sensitivity in the Tfm in terms of feminine sexual behavior is accompanied by loss of the HPOA MAO response to the same steroid. Conversely, the deficit in masculine sexual behavior in the Tfm, as opposed to the intact male, is associated with an impaired MAO response to T. Taken together, these findings are consistent with the view that sexual differentiation may involve a shift, in the steroid sensitivity of MAO-containing systems within the brain, from estrogen to androgen dependency (Luine et al., 1975b). In the case of the Tfm male, the response to T remains like that of the female, a result of either incomplete masculinization of the brain or the loss of activational androgen receptor-mediated effects.

In summary, the work presented in this paper provides further confirmation of the specificity and completeness of the androgen-insensitivity syndrome produced by the Tfm mutation in the male mouse. While detectable levels of androgen receptor are present in Tfm/Y males, nuclear androgen binding and tissue enzymatic responses to T administration are essentially abolished. Despite the drastic reduction in androgen receptor concentrations, Tfm males remain capable of responding to estrogen synthesized in the brain from circulating androgen. As a result, the brain is partially differentiated towards the normal masculine phenotype. These properties make the Tfm a unique model for investigation of the mechanisms that underlie gonadal steroid action on the central nervous system.

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