

Autoradiographic studies with ^3H dihydrotestosterone in the brain of sex reversed mice, heterozygous for androgen insensitive testicular feminization (Tfm)

A comparison with normal female mice and sex reversed male mice

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Received June 8, 1985 / Accepted September 24, 1985

Summary. Specific binding sites for ^3H dihydrotestosterone are demonstrated by autoradiography in brain nuclei of sex reversed mice heterozygous for testicular feminization (Tfm) which are phenotypically intersexes with testes and accessory sex glands that consist of a mosaic of androgen insensitive Tfm cells which lack specific dihydrotestosterone binding and androgen sensitive normal cells. The nuclear group evaluated include: nucleus (n.) septi lateralis, n. interstitialis striae terminalis, n. medialis amygdalae, the hypothalamic n. arcuatus, n. ventromedialis lateralis, n. pre-mammillaris ventralis, n. preopticus medialis, and nuclei of the cranial nerves VII, X, and XII. In the sex reversed males and the female, used as controls, the frequency of neurons with specific DHT binding show a distinct male-female difference in the caudal part of the arcuate nucleus. In the sex reversed Tfm heterozygotes, in all brain nuclei studied, the frequency of labeled neurons is reduced. The extent of reduction of androgen binding in the different brain nuclei varies among as well as within individual sex reversed Tfm heterozygotes, suggesting variations of the ratio of normal to Tfm neurons in sex reversed Tfm heterozygotes. The differentially reduced androgen binding of different brain systems corresponds to a differentially reduced androgen dependent behaviour reported in the literature.

Introduction

Sex reversed mice, heterozygous for testicular feminization (Tfm) (Lyon and Hawkes 1970), carry two X-chromosomes, one of which is coding for androgen insensitive Tfm (Drews 1975). Androgen insensitivity of Tfm is caused by an androgen receptor deficiency (Attardi and Ohno 1974; Gehring and Tomkins 1974). Due to random X-inactivation in somatic cells with two X-chromosomes (Lyon 1961) the Tfm-heterozygotes are composed of a mosaic of androgen insensitive cells with an active X^{Tfm} chromosome (Tfm-cells) and of normal, potentially androgen sensitive cells with active X^+ chromosome (Drews 1975).

Supported by US PHS grant NS09914 to W.E.S. and Deutsche Forschungsgemeinschaft Dr94/4 to U.D.. The work of Dr. Schleicher and his stay in Chapel Hill were sponsored by Studienstiftung des Deutschen Volkes and Boehringer-Ingelheim Fonds

Sex reversed Tfm heterozygotes have testes, due to the action of the sex reversal factor (Sxr) (Cattanach et al. 1971), which is a translocation of testes determining sequences from the Y-chromosome to the X-chromosome (McLaren 1983). Therefore, in androgen target organs the mosaic of Tfm and normal cells is exposed in fetal life as in adulthood to testicular secreted androgens.

Since only normal cells, but not Tfm-cells, can respond directly to androgens in fetal life, the androgen target organs of the genital tract of these animals exhibit an intersexual phenotype (Drews 1975). Depending on the ratio of X^+ to X^{Tfm} cells in a sex reversed Tfm heterozygote, the phenotype ranges from a male-like phenotype with hypospadias and minor asymmetries of male sex organs to a heavily feminized phenotype with a short blind ending vagina and rudimentary male accessory sex organs.

Since the mosaic of X^+ and X^{Tfm} cells is visible by histological criteria (Drews 1975) and by autoradiography (Schleicher et al. in prep.) in the genital tract of the sex reversed Tfm heterozygotes, we examined in the present study by autoradiography with ^3H dihydrotestosterone in sex reversed Tfm heterozygotes, whether the mosaic of X^+ and X^{Tfm} cells can be demonstrated among potential androgen target cells in brain nuclei and to what extent the ratio of X^+ and X^{Tfm} cells varies for the same nucleus among different animals and among different nuclei of the same animal.

Materials and methods

The experiments were carried out with sex reversed Tfm heterozygotes and with Tfm hemizygotes, sex reversed male mice and female mice as controls. Sex reversed (genotype: $X^+X^+ - \text{Sxr}$) males show a full male phenotype (Cattanach et al. 1971). They will be subsequently called males. Tfm hemizygotes (genotype: $X^{\text{Tfm}}Y$) are androgen insensitive and therefore have female external sex organs with a short blind ending vagina and neither male nor female internal sex organs due to complete androgen insensitivity and the presence of the Anti-Müllerian hormone in fetal life (Lyon and Hawkes 1970).

The mice, 50 to 130-day old and weighing 20–40 g, were taken from the stock kept in Tübingen (Germany) which was derived from the stock of Dr. Ohno (Duarte, CA). In this colony Tfm and Sxr is carried. The X-linked coat markers blotchy and tabby are used for identification of the X-chromosomes present in an animal. We used four sex reversed Tfm heterozygotes (genotype

$X^{Tfm}X^+ - Sxr$), two sex reversed males without Tfm and two Tfm hemizygotes. Furthermore, a normal female (genotype X^+X^+), and a female heterozygous for Tfm (genotype X^+X^{Tfm}) were included.

Sex reversed males and sex reversed Tfm heterozygotes were castrated 36 h before the experiment in order to reduce endogenous androgen levels (Bouton et al. 1981). Tfm hemizygotes and female animals were not gonadectomized, since their androgen levels are lower than those of sex reversed males and sex reversed Tfm heterozygotes (Ohno et al. 1974).

The mice were injected i.v. with 0.5 μ g/100 g body weight of (1, 2, 4, 5, 6, 7, 16, 17) 3H -dihydrotestosterone (3H DHT), spec. act. 179 Ci/mmol (New England Nuclear), in 1:10 ethanol-isotonic saline. One hour afterwards, mice were decapitated, brains dissected, placed on tissue holders, then frozen in liquified propane and stored in liquid nitrogen. Four μ m serial frontal sections were cut in a cryostat at $-30^\circ C$ at 160 μ m intervals and thaw-mounted onto emulsion coated slides (Kodak NTB 2) under safe light, then stored in light-proof desiccator boxes for photographic exposure at $-15^\circ C$ between two months to two years. Slides were photographically processed at $16^\circ C$ for 60 s in Kodak D19 developer, rinsed, and fixed for 5 min in Kodak fixer, rinsed for 5 min and stained with methylgreen pyronin (MGP) or methylene blue-basic fuchsin (MBBF). The autoradiographic technique has been described in detail at Stumpf and Sar (1975).

The labeling index was determined with a Zeiss microscope, using a $40\times$ objective and an eye piece reticule. The *labeling index* is defined as the number of labeled neurons expressed in percent of the total number of neurons in a defined nucleus or subnucleus. A cell was considered labeled, when the number of nuclear silver grains was two times, or more, above the number of silver grains in a respective adjacent area. Only brain nuclei or subnuclei were evaluated which could be delineated anatomically and which showed in the male animals a homogeneous labeling index. In the results section labeling indices of whole brain nuclei are given and statistically compared as relative frequencies (Sachs 1968). In order to demonstrate possible variations in different areas of the same brain nucleus in Tfm heterozygotes, in Figs. 1 and 3 the labeling index is depicted as mean and standard deviation of the labeling index of single cryostat sections.

The labeling index was determined, for each brain half separately, in the following nuclei: Nucleus septi lateralis (two parts: sl 2 and sl 3), nucleus interstitialis striae terminalis (st), nucleus preopticus medialis (pom), nucleus hypothalamicus ventromedialis lateralis (hvml), nucleus premammillaris ventralis (pmv), nucleus arcuatus (arc), nucleus medialis amygdalae pars dorsalis (am), nucleus nervi facialis (VII), nucleus ambiguus (ab), nucleus dorsalis nervi vagi (X), and nucleus nervi hypoglossi (XII).

Results

In the autoradiograms of the males, the Tfm heterozygotes, and the normal female, accumulation of silver grains (nuclear labeling) appears in certain cell nuclei after a photographic exposure time of two months. Silver grains are found also over cytoplasm of labeled cells, over unlabeled cells and extracellular spaces, but with low density. After a photographic exposure of 8 months in the males and the sex reversed Tfm heterozygotes and after 10 months in the females, the number of cells with nuclear labeling did not increase with a further extension of the exposure time, while the intensity of nuclear labeling increased further. In the androgen-insensitive Tfm hemizygotes, no nuclear labeling is found, whereas diffusely distributed cytoplasmic and interstitial silver grains are present. In the male animals in the nuclei and subnuclei examined, not all nerve cells, but only a portion of them are labeled. Among different males, the labeling indices for a given nucleus show close correspondence.

In contrast, among sex reversed Tfm heterozygotes the

labeling indices of respective nuclei show considerable variations. The labeling indices of respective nuclei in sex reversed Tfm heterozygotes are, in general, lower than those of males, and in some cases show major differences between the left and the right side. In order to document these variations labeling indices of left and right nuclei are given separately and the variation between the sections is shown (Figs. 1 and 3).

The autoradiographic results for the individual nuclei are as follows:

The *lateral septal nucleus* (sl) is subdivided by morphological criteria and nuclear labeling intensity into three groups:

sl 1: This group is present in the rostral part of the septum and located in the dorsolateral part close to the corpus callosum. Nuclear labeling is occasionally seen with low or moderate intensity. This group was not evaluated due to the low intensity of nuclear labeling.

sl 2: This group is well developed in the medial and caudal lateral septum and located ventral to sl 1. When sl 1 disappears in the caudal part of the lateral septum, sl 2 extends dorsally to the corpus callosum. The intensity of nuclear labeling is moderate and sometimes high. The labeling index is considerably higher in sl 2 than in sl 1.

sl 3: This group comprises the most ventral part of the lateral septum. Cells are smaller and more variable in size when compared to sl 2. Intensity of nuclear labeling is higher than in sl 2.

In sl 2 the labeling indices of the males and of the normal female are not different from each other, ranging from 45% to 49% (Fig. 1.1). In the sex reversed Tfm heterozygotes, the labeling indices are slightly lower than those of the males. They range between 36% and 44% (Fig. 1.1), which is close to the indices for the males, while the female Tfm heterozygote shows lower indices (fig. 1.1).

In sl 3 the labeling indices in the males and the normal female range between 45% and 52% (Fig. 1.2). In sex reversed Tfm heterozygotes the labeling indices range between 19% and 28% (Fig. 1.2), which is distinctly below the labeling indices in the control group without Tfm. The labeling indices of the female Tfm heterozygote are similar to those of the sex reversed Tfm heterozygotes.

Of the nucleus *interstitialis striae terminalis* (st) only one part has been evaluated. This part is referred to in the description of Bleier et al. (1979) as part "a" and is found on cross sections between two branches of the stria terminalis and the anterior commissure. In this area the labeling index is slightly higher in males (57%) than in the normal female (50%) (Fig. 1.3). The difference is significant with a *p*-value smaller than 0.05. In sex reversed and in the female Tfm heterozygotes the labeling indices are significantly lower, ranging from 24% to 37% (Fig. 1.3).

The *medial part of the preoptic area* (pom) is, according to Bleier et al. (1979), a loose heterogeneous collection of cells lying between the anterior commissure and the optic nerves and the rostral optic chiasm. Ventromedially the pom is delineable from the pars suprachiasmatica of the preoptic nucleus by the higher density of neurons in the latter. Dorsomedially in the "septal hypothalamic nucleus" (Bleier et al. 1979) the cells are smaller in size and more homogenous than those in the pom. Dorsolaterally the part "c" of the nucleus interstitialis striae terminalis (Bleier et al. 1979) shows a higher density of nerve cells and therefore can be delineated. Only sections were evaluated, in which

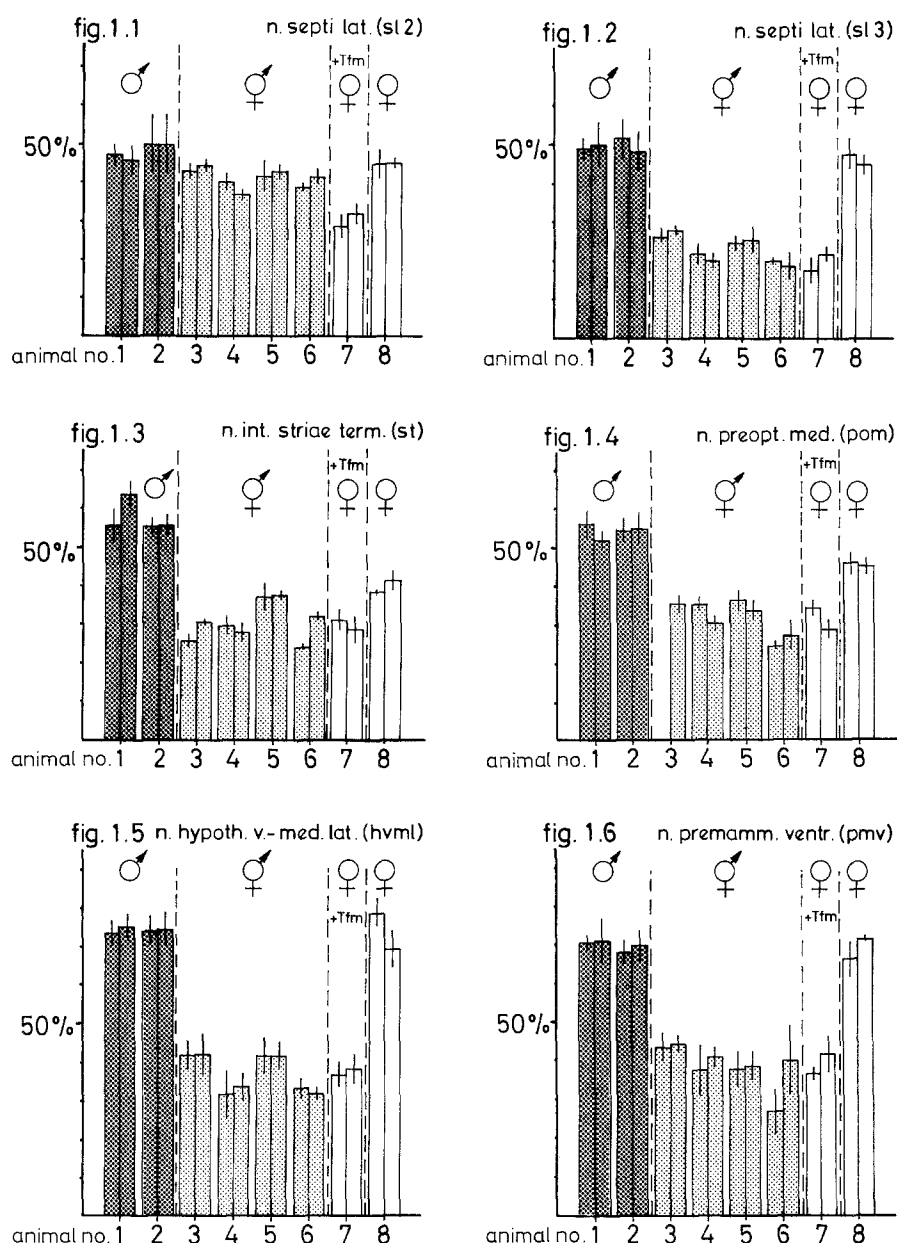


Fig. 1. Histograms of labeling indices (mean \pm standard deviation of 3 to 16 sections depending on size of the nucleus), after injection of ^3H dihydrotestosterone of the nucleus septi lateralis (two parts: *sl* 2, 1.1, *sl* 3, 1.2), nucleus interstitialis striae terminalis (*st*, 1.3), medial preoptic area (*pom*, 1.4), nucleus hypothalamicus ventromedialis lateralis (*hvm*, 1.5) and nucleus premammillaris ventralis (*pmv*, 1.6). Labeling indices are shown for the left side (left bar) and right side (right bar) of a corresponding nucleus. Animals number 1 and 2 are sex reversed males, number 3 to 6 sex reversed Tfm heterozygotes, number 7 is a female Tfm heterozygote and number 8 a normal female

the anterior commissure crosses the midline. Since the lateral border of *pom* is not easy to delineate, an imaginary line between the medial border of the nucleus interstitialis striae terminalis and the lateral end of the optic chiasm was used as the lateral boundary. In the examined region, the nuclear labeling indices in the males are significantly higher (54%) than in the normal female (45%) (Fig. 1.4, $p < 0.05$). In the sex reversed and the female Tfm-heterozygotes, labeling indices range from 24% to 36% (Fig. 1.4). In animal 3 only one side was evaluated.

The *ventromedial hypothalamic nucleus* (*hvm*) consists of three parts: the dorsomedial and the ventrolateral parts with a high density of nerve cells, and the central part with a low density of nerve cells (Bleier et al. 1979). Only the ventrolateral part (*hmv*) shows a distinct nuclear labeling, high enough for evaluation. The labeling indices in the males of the normal female are the highest (69%–75%; Fig. 1.5) among all of the nuclei examined. In sex reversed and the female Tfm-heterozygotes the labeling indices range from 32% to 42% (Fig. 1.5).

The *nucleus premammillaris ventralis* (*pmv*) shows high nuclear labeling in many cells. The labeling indices in the males and the normal female (70%; Fig. 1.6) are close to those of the *hvm*. In the sex reversed and the female Tfm heterozygotes the labeling indices range between 27% and 44% (Fig. 1.6).

Of the *nucleus arcuatus* (*arc*) only the caudal part has been used for evaluation, that is, the neurons at and caudal to the beginning of the infundibular stem along the mammillary recess. Nuclear labeling is found predominantly in the ventral part of the nucleus. In males, the labeling indices range from 16–23% (Fig. 3.1), in sex reversed Tfm heterozygotes they range between 7% and 11% (Fig. 3.1). In females, in both the normal and the Tfm heterozygote, labeled cells are only occasionally seen. The labeling index was 2% for the normal female and 1% for the female Tfm heterozygote (Fig. 3.1). The differences were significant on the 0.001 level.

The conspicuous differences between nucleus premammillaris ventralis and arcuatus are shown in the autoradio-

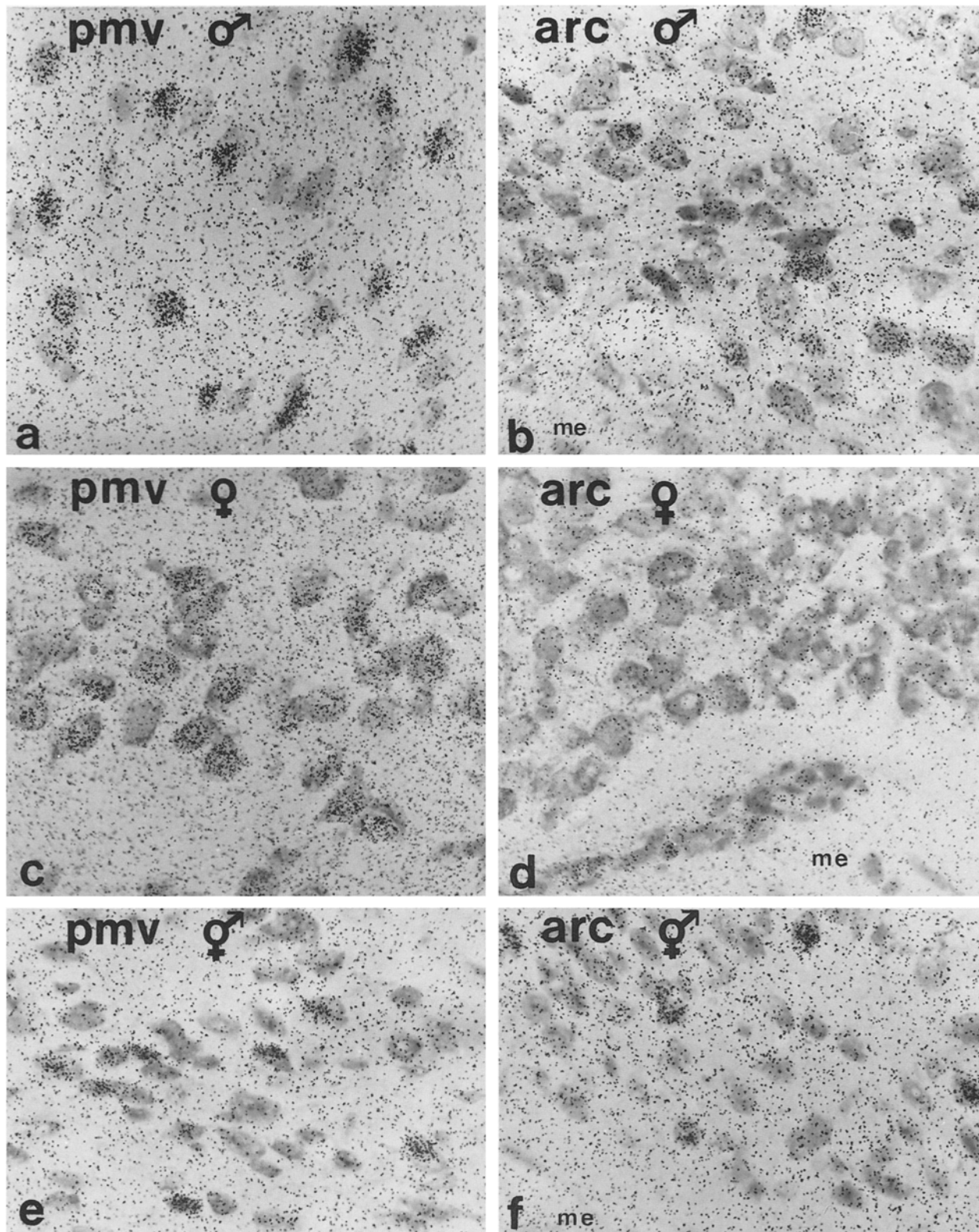


Fig. 2a-f. Autoradiograms after injection of ^3H dihydrotestosterone of the nucleus preammillaris ventralis (*pmv*; **a**, **c**, **e**) and the nucleus arcuatus (*arc*; **b**, **d**, **f**) in a sex reversed male (**a**, **b**), a normal female (**c**, **d**) and a sex reversed Tfm heterozygote (**e**, **f**) showing that the frequency of labeled cells is higher in the male (**a**) and the female (**c**) than in the sex reversed Tfm heterozygote (**e**) in the ventral preammillary nucleus. In the arcuate nucleus, however, the frequency is higher in the male (**b**) than in the sex reversed Tfm heterozygote (**f**), and higher in the sex reversed Tfm heterozygote (**f**) than in the female (**d**). *me*: median eminence. Magnification $\times 530$, exposure times: 1 year (**a**, **b**, **e**, **f**) or 2 years (**c**, **d**), stained with methylgreen pyronin

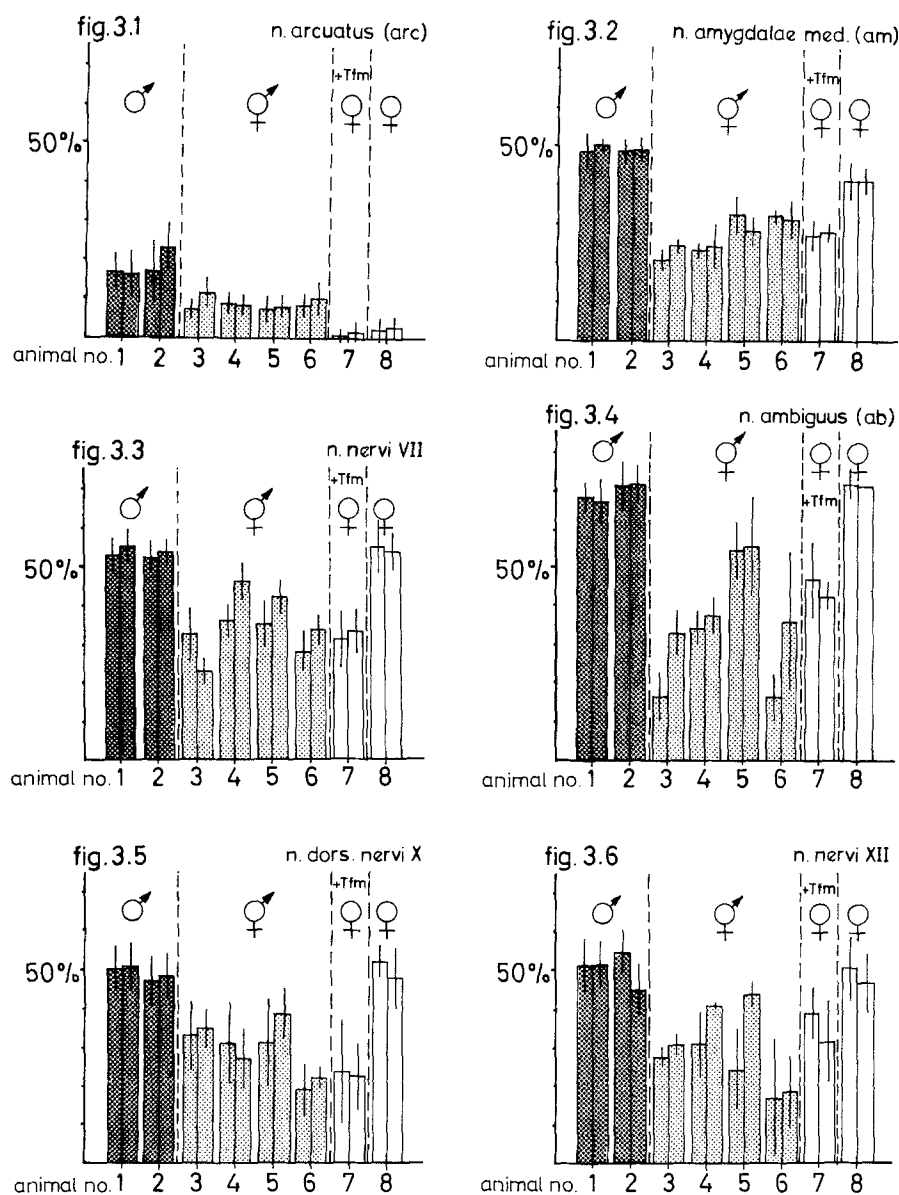


Fig. 3. Histograms of labeling indices (mean \pm standard deviations of 3 to 16 sections depending on the size of the nucleus) after injection of ^3H dihydrotestosterone of the nucleus arcuatus (*arc*, 3.1) nucleus amygdalae medialis pars dorsalis (*am*, 3.2), nucleus nervi facialis (*VII*, 3.3), nucleus ambiguus (*ab*, 3.4), nucleus dorsalis nervi vagi (*X*, 3.5) and nucleus nervi hypoglossi (*XII*, 3.6). Labeling indices are shown for the left (left bar) and the right (right bar) of a corresponding nucleus. Animals number 1 and 2 are sex reversed males, number 3 to 6 are sex reversed Tfm heterozygotes, number 7 is a female Tfm heterozygote, number 8 is a normal female

grams of Fig. 2. In frontal sections both nuclei can be found at the same level. In the nucleus premammillaris ventralis, the frequency of labeled neurons is about the same in the male and in the normal female but is lower in the sex reversed Tfm heterozygote. In the arcuate nucleus in the female only occasionally labeled neurons are present, whereas the male and the sex reversed Tfm heterozygote show a labeling pattern similar to the nucleus premammillaris ventralis.

The nucleus medialis of the amygdala (*am*) is subdivided into two parts regarding the intensity of nuclear labeling. The dorsal part shows a high or intermediate labeling in many cells, whereas in the ventral part only low or moderately labeled cells are found. Additionally, in the most caudal as well as the most rostral end of the dorsal medial nucleus, the number of labeled cells and the labeling intensity are lower than in its central portion. Only the central portion therefore has been evaluated. This portion is found at the level of the ventromedial hypothalamic nucleus and is bordered dorsally and laterally by the stria terminalis. The difference of labeling indices in the males (49%) and the normal female (41%) is significant ($p < 0.05$, Fig. 3.2).

In sex reversed and the female Tfm heterozygotes, the labeling indices range from 21% to 33%.

The nucleus nervi facialis (*VII*) is easily recognized by its large neurons and by its characteristic shape. The labeling indices in males and the female are similar (54%) (Fig. 3.3). In the sex reversed and the female Tfm heterozygotes the labeling indices range from 23% to 46%.

The nucleus ambiguus (*ab*) consists of densely arranged large neurons which extend rostro-caudally from the caudal end of the nucleus nervi facialis to the area of the inferior olive. The labeling indices in the males and the normal female range from 66%–71% (Fig. 3.4). In the sex reversed and in the female Tfm heterozygotes the labeling indices range from 16% to 55%. In animal 3 a significant side difference (left 16%, right 51%) with a $p < 0.05$ is observed. Figure 4 shows autoradiograms of the nucleus ambiguus with typical large motor neurons. In contrast to the female (Fig. 4a) the Tfm hemizygote (Fig. 4b) exhibits no nuclear labeling. Figure 4c and d demonstrate the exceptional difference between the right and the left nucleus of the sex reversed Tfm heterozygote No. 3.

The nucleus dorsalis nervi vagi (*X*) is distinguished from

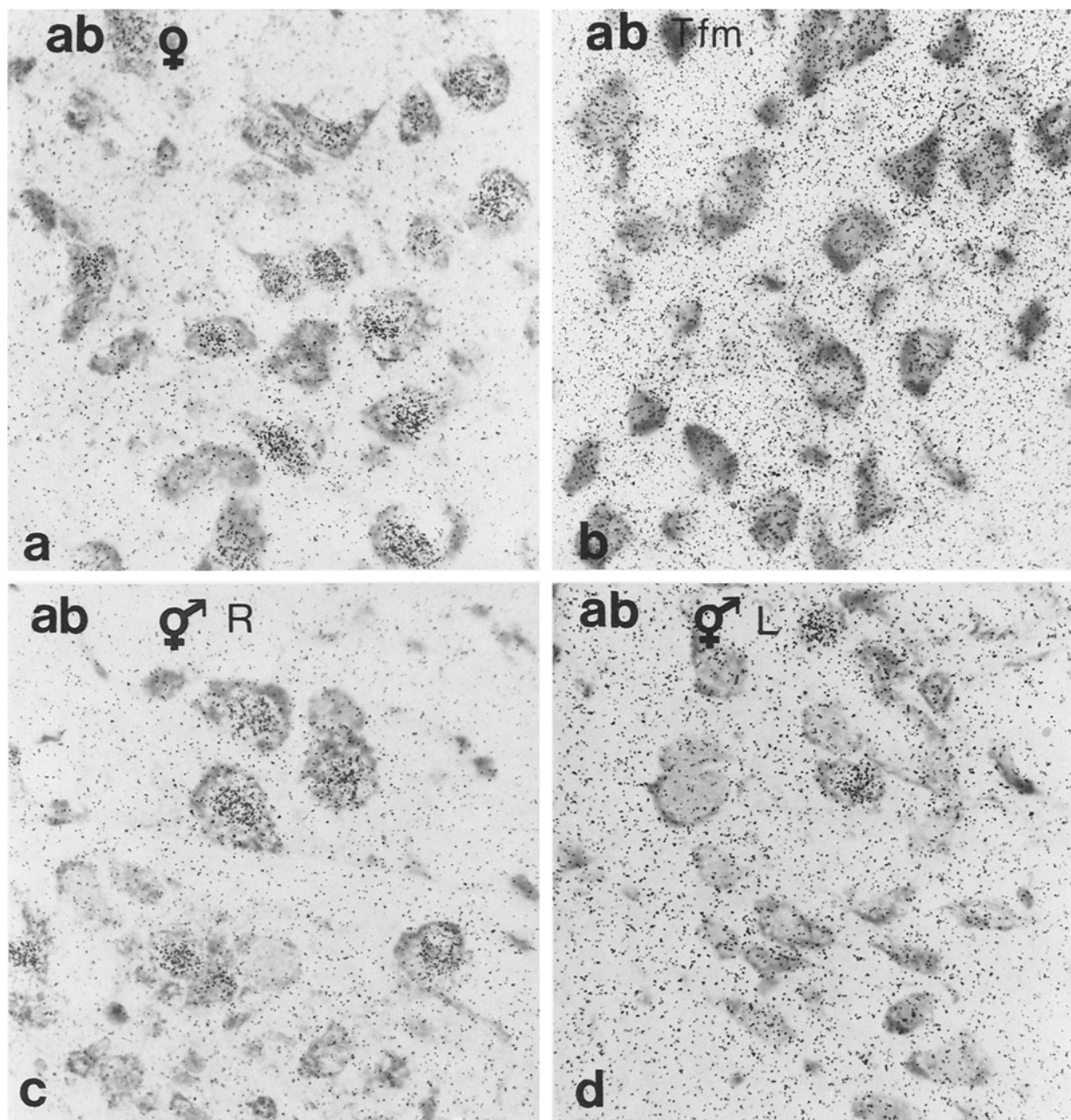


Fig. 4a-d. Autoradiograms after injection of ^3H dihydrotestosterone of the nucleus ambiguus (*ab*) of the normal female (**a**), a Tfm hemizygote (**b**) and of the right (**c**) and left (**d**) side of a sex reversed Tfm heterozygote, showing nuclear labeling in most of the cells in the female (**a**), no nuclear labeling in the Tfm hemizygote (**b**) and a higher number of labeled cells in the right side (**c**) than in the left side (**d**) of the sex reversed Tfm heterozygote. Magnification $\times 530$, exposure time 1 year, stained with methylgreen pyronin

the surrounding by the larger size and a horizontal orientation of its neurons and by the high intensity of nuclear labeling. The labeling indices in the males and the normal female is about 50%; and ranges from 14% to 39% in the sex reversed and the female Tfm heterozygote (Fig. 3.5).

The *nucleus hypoglossus* (XII) is subdivided into two parts regarding nuclear labeling intensity and labeling index. The rostral part shows a low number of labeled cells with a low nuclear labeling intensity. In the caudal part, nuclear labeling intensity is higher and labeled cells are more frequent. The border between the two parts is at the level of the caudal end of the nucleus raphae magnus. Only the caudal part of the hypoglossal nucleus had been evalu-

ated. The labeling index in the males and in the normal female range from 47% to 54%. In the sex reversed and the female Tfm heterozygotes it ranges from 17% to 44% (Fig. 3.6). A side difference is seen in animal 5 (left 24%, right 43%, $p < 0.05$; Fig. 3.6).

Discussion

The autoradiographic data demonstrate nuclear labeling after injection of ^3H dihydrotestosterone in males, Tfm heterozygotes, and females. In contrast, no nuclear labeling is observed in the Tfm hemizygotes, which are androgen insensitive. Lack of specific dihydrotestosterone binding

was also described by Sheridan (1978) who, in addition, demonstrated specific binding of estradiol in the brain of Tfm mice. The results of these studies indicate that nuclear labeling after injection of ^3H DHT is due to specific nuclear binding of labeled hormone and not due to unspecific hormone binding. Furthermore, it suggests that nuclear labeling with ^3H DHT is correlated with the biological function of androgens.

In males and the normal female of the present study, only a portion of the neurons in the brain nuclei examined exhibit specific ^3H DHT binding. The labeling indices vary among the brain nuclei examined. In the normal female, in most of the nuclei the labeling indices are as high as in the male. A major sex difference in the frequency of ^3H DHT binding neurons is found in the nucleus arcuatus. Slight but significant sex differences are also found in the nucleus interstitialis striae terminalis, in the medial preoptic area and in the nucleus medialis of the amygdala. Thus, only in certain brain nuclei androgen binding appears to be sex-specifically determined.

In the brain nuclei which show sex differences in the frequency of ^3H DHT binding neurons, binding of ^3H testosterone or its metabolites has been described in postnatal rats by Sheridan et al. (1975). In the preoptic area of the rat Gorski et al. (1975) described the sexual dimorphic nucleus with a higher number of neurons in the male.

In the sex reversed Tfm heterozygotes the labeling indices are generally lower than those in respective nuclei of the males. The primary difference between sex reversed Tfm heterozygotes (genotype $X^{\text{Tfm}} X^+ \text{Sxr}$) and the males is the presence of X^{Tfm} cells in the former. Tfm cells lack specific ^3H DHT binding and are insensitive toward androgen stimulation. This indicates that the difference observed in the present study between the sex reversed Tfm heterozygotes and the control males is due to the lack of specific ^3H DHT binding of Tfm cells, present among potential androgen target cells of the brain nuclei examined. The possible influence of perinatal androgens on the expression of the mosaic can be assessed by comparing the female Tfm heterozygotes which has no testes, with the sex reversed Tfm heterozygotes which have testes. No differences are found in all of the studied nuclei except in the arcuate nucleus. In the sex dimorphic arcuate nucleus, however, the labeling index of the female Tfm heterozygote is lower than in the sex reversed Tfm heterozygote and as low as in the normal female. There is no indication that the proportion of Tfm to normal cells was altered during the perinatal androgen dependent organizational phase.

In contrast to the other androgen target nuclei studied, in the septal nucleus part two (sl 2), against the expectation of randomness, the labeling indices of all four sex reversed Tfm heterozygotes are only slightly lower than those of the males. A clarification of this observation would require further studies.

Due to the mosaic, in the sex reversed Tfm heterozygotes the range of the labeling indices is considerably wider than in the males.

Considerable variations are observed not only among the sex reversed Tfm heterozygotes but also among different brain nuclei of a sex reversed Tfm heterozygote and between left and right brain nuclei of the same animal. This indicates that different ratios of X^+ to X^{Tfm} cells are found not only among the individual sex reversed Tfm heterozygotes but

also among brain nuclei of the same animal. A coarse mosaic leading to a major left-right difference is present in animals No. 3 and 5 (Fig. 3.4 and 3.6). Figure 4 demonstrates e.g. in the nucleus ambiguus of animal No. 3 that the random expression of the mosaic may lead to virtual androgen insensitivity of a single brain nucleus in an otherwise "male" animal. Thus the sensitivity toward androgen stimulation is variously reduced among the brain nuclei of a sex reversed Tfm heterozygote and androgen dependent brain processes are hampered to a different extent. This is in line with findings of Ohno et al. (1974) who reported that in a few sex reversed Tfm heterozygotes, either male copulation or aggression behavior was missing, while the alternate male behavior in the same animal appeared to be similar to that of males. No studies on the correlation between androgen dependent behavior and its morphological substrate in the brain exist. Direct correlation between behavior and androgen binding in sex reversed Tfm heterozygotes may be a tool in exploring the pathways of the different androgen dependent brain processes.

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