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The special case of hormonal imprinting, the neonatal influence of sex

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Key words. Alpha-fetoproteins; androgens; brain; estrogens; estrogen antagonists; estrogen receptors; genitalia; gonads; hypothalamus; preoptic area; receptor imprinting; reproductive tract; sexual differentiation; sexually dimorphic nucleus.

The chain of events leading to reproductive success is based on the participation of a variety of organs and tissues with different structures and functions. The *brain* controls behavioral orientation and sexual identification as well as gonadotropic hormone (GTH) release from the pituitary gland. A sex specific pattern of gonadotropic hormone release will stimulate maturation and release of male or female germ cells respectively. A properly developed *internal duct system* will then transport the germ cells to the outside. Species with internal fertilization need appropriately developed *external genitalia* for the transfer of germ cells from the male to the female individual.

Historical perspectives

The question about which factors may determine the fate of a developing fetus, causing it to become either male or female, has occupied many previous cultures and scientists. Ancient Greek ideas about sexual differentiation centered mainly around two hypotheses. The 'hypothesis of laterality', established by Anaxagoras of Clazomenae (about 440 BC), claimed that semen from the right testis would produce male offspring, semen from the left testis would produce females. This hypothesis further claimed that male fetuses are carried in the right horn of the uterus, females in the left[1]. The 'thermal hypothesis' of Empedokles of Akras (about 460 BC) claimed that temperature was an important factor in sex determination[85]. Conception in a hot uterus would produce a male, in a cold uterus a female. Aristotle of Stagirus (384 to 322 BC) favored the thermal hypothesis. He observed in sheep and goats that they would produce male offspring when warm winds were blowing from the south during copulation, but female offspring when cold winds were blowing from the north[4]. Plato postulated that the first human generation consisted only of men. Those men of the first generation, who had been cowardly or had spent most of their lives in wrong-doing, were reborn in the second generation as women[86].

Environmental influences on sexual differentiation

The 'thermal hypothesis' of Empedokles may actually not be that far off after all. It has been shown that frog larvae develop a male phenotype when raised at an elevated water temperature; at a low temperature they develop into females[83]. In some species of lizards breeding of the eggs at temperatures below 26 °C will prime the embryos for female development, whereas at temperatures above 26 °C the embryos will develop into males[95]. In two species of turtles, *Emys orbicularis* and *Testudo graeca* the temperture effect on sexual differentiation is reversed. Male development is induced during breeding at temperatures below 28 °C and female development is induced during breeding at above 32 °C[82].

Another environmental influence which may effect sexual differentiation is the concentration of potassium and calcium ions in the water. Three- to four-fold elevation of calcium ions in the water will stimulate the larvae of *Discoglossus pictus* to develop into females. Five- to

sex-fold elevation of calcium ions will stimulate the same larvae to develop into males[98].

Genetic influences on sexual differentiation

After the ancient times of the early Greek philosophers it took almost 2500 years before the role of the Y-chromosome in masculinization of the gonads was discovered. In mammals, a gene on the male Y-chromosome stimulates the production of a cell surface antigen, the histocompatibility-Y-antigen (H-Y-antigen). Under the influence of H-Y-antigen the gonads will differentiate into testes. Lack of H-Y-antigen, as in the female, will result in differentiation of ovaries. Although differentiation of the mammalian gonads is under chromosomal control, differentiation of other sexual structures (reproductive tract, external genitalia and even the brain) is now known to be controlled by an imprinting action of hormones during fetal or neonatal life.

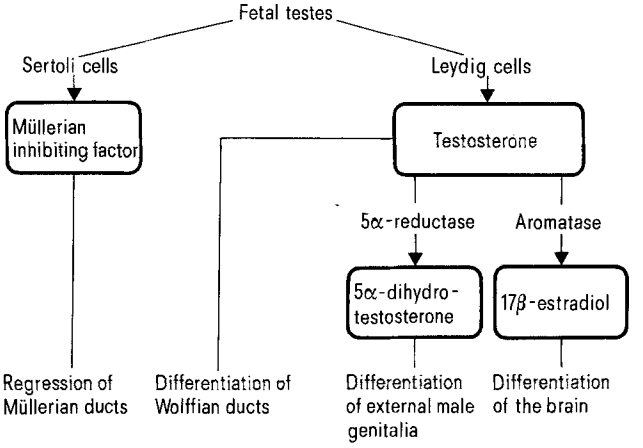

Figure 1. Influence of testicular hormones on sexual differentiation of sex ducts, genitalia and brain.

Hormonal influences on sexual differentiation

1. Differentiation of the gonads. Differentiation of the mammalian gonads is under chromosomal control and cannot be influenced by the action of hormones. Although in lower vertebrates gonadal differentiation may also be primarily under genetic control, it was shown in several species of fish and amphibians that early hormonal influences may sex-reverse the differentiation of the gonads without sex-reversing the genetic information. When female larvae of the medaka fish *Oryzias latipes* are treated with androgens, they will develop into fully reproductive males. These males will produce only female offspring after breeding with normal females. When male larvae of *Oryzias latipes* are treated with estrogens, they will develop into fully reproductive females. During breeding with normal males these females (XY genotype) will produce 25% female offspring (XX genotype) and 75% male (XY and YY genotype) offspring[114-116]. Gonadal sex reversal was also observed in several amphibian species when the larvae were raised in water which contained estrogenic or androgenic hormones[14,15].

2. Differentiation of reproductive tract and external genitalia. In mammals, differentiation of the gonads cannot be influenced by hormones, but differentiation of the reproductive tract and the external genitalia depends exclusively on the imprinting (priming) action of gonadal hormones. The *sertoli* cells in the testes produce a locally acting substance, the Müllerian inhibiting factor. This factor, the structure of which is still unknown, causes regression of the female embryonic reproductive tract, the Müllerian ducts (fig. 1). Without the priming action of this factor, which does not exist in females, the Müllerian ducts will develop into fallopian tubes, uterus and upper vagina.

The *leydig cells* in the testes produce testosterone, a male sex hormone (androgen). Under the priming influence of testosterone the male embryonic reproductive tract, the Wolffian ducts, will develop into vasa deferentia, seminal vesicles and epididymis (fig. 1). Differentiation of the external male sex organs (penis, scrotum and sinus urogenitalis) occurs under the priming influence of another androgen, 5α-dihydrotestosterone (DHT). In the target cells this androgen is converted from testosterone by the enzyme 5α-reductase. DHT is subsequently bound by intracellular cytosolic androgen receptors and this receptor-hormone complex is then translocated into the cell nucleus. Interaction of the receptor-hormone complex with the genetic material of the nucleus stimulates sexual differentiation of the respective organ irreversibly. The exact molecular mechanism of this irreversible imprinting action is still unknown. In cases where DHT is absent during the sensitive developmental phase – this is normally the case in females, but also in males with deficiency in 5α-reductase[79] – the external genitalia will develop in the female direction (vulva and lower vagina). The female type of genital development also occurs when the genital tissue is non-responsive to androgens, owing to an androgen receptor defect, as it is in the syndrome of testicular feminization[5,71].

3. Differentiation of the brain. Sexually dimorphic brain functions. The most obvious functional differences between male and female animals are those involved in reproductive physiology and reproductive behavior. The best-studied animal model in this respect is the rat. In the female rat, rising plasma titers of estrogens trigger a cyclic neural stimulus which activates the release of gonadotropin-releasing hormone(s) (GnRH) from the hypothalamus (positive feedback). GnRH, in turn, stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary gland. The gonadotropins FSH and LH stimulate follicular maturation in the ovaries and trigger ovulation. In the male rat, rising plasma titers either of estrogens or of androgens are unable to stimulate the release of GnRH. The neural substrate which controls GnRH release has apparently developed differently in males and females. The neural substrate which controls sexual behavior has also developed along different lines in males and females. Under the influence of estrogens and progesterone, adult female rats will respond to the mounting attempts of a sexually active male by an arching of the back, the so-

called lordosis reflex. Adult male rats will hardly show any lordosis behavior, even if given the same hormone treatment. Under the influence of testosterone, adult male rats will show vigorous mounting, intromission and ejaculatory behavior towards a receptive female, whereas female rats will show little or no such response when treated with testosterone.

Organization of sexually dimorphic brain functions. Sexual differentiation of the brain has been studied most thoroughly in the rat. In 1936 Pfeiffer[81] presented evidence that there is a critical period during early postnatal development of the rat, during which differentiation of the pattern of anterior pituitary hormone secretion can be influenced permanently by testicular hormone action. He removed the testes of newborn male rats and replaced them with ovaries when the animals were adult. These male animals showed the female capacity to form corpora lutea in the grafted ovarian tissue. Newborn females, implanted with testes from littermate males, did not show estrous cycles or form corpora lutea in their ovaries when adult. Pfeiffer[81] had originally concluded that the pituitary gland is sexually dimorphic in function, but it was shown later that hormone release from the pituitary gland and, thus, ovulation is under the control of the central nervous system[42].

Present knowledge of hormonal influences on the development of sexually dimorphic brain functions is based on a great number of studies, most of which have been carried out in the last 25 years. The individual contributions to the field of sexual brain differentiation have been discussed in several excellent reviews[13, 37, 50, 51, 84]. In summary, there is a sensitive developmental period during which sexual differentiation of neural substrates proceeds irreversibly under the influence of gonadal hormones. In the rat this period starts a few days before birth and ends approximately 10 days after birth. Female rats, treated during this sensitive period with testosterone or estradiol, will permanently lose the capacity to release GnRH in response to estrogenic stimulation, and will lose the capacity to show lordosis behavior ('defeminization'). Instead, they will develop the capacity to show the complete masculine sexual behavior pattern following administration of testosterone in adulthood ('masculinization'). If castrated perinatally, male rats become unable to display male sexual behavior patterns after treatment with testosterone in adulthood ('demasculinization'). Instead, they will develop the capacity to show lordosis behavior, and to respond in adulthood with a positive GnRH feedback to estrogen treatment ('feminization').

These studies indicate that androgens and/or estrogens, whether released by the testes or applied exogenously during the perinatal period, will permanently defeminize and masculinize neural substrates controlling sexually dimorphic brain functions (fig. 2).

Sexually dimorphic brain structures. Despite the well-known sex differences in brain functions, brain structure was for a long time believed to be essentially the same in males and females. The first anatomical sex differences observed in the mammalian brain were rather subtle. In rats, Pfaff[80] as well as Dörner and Staudt[38, 39] observed differences between the sexes in the size of nerve cell nuclei. Sex-linked differences in the pattern of neuronal connections were observed in rodents by Raisman and

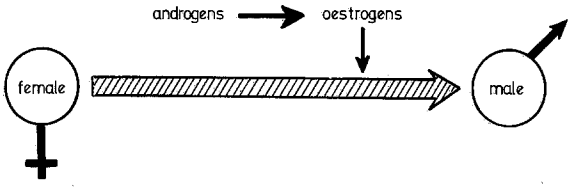

Figure 2. Diagram summarizing contemporary thinking on the mechanism of sexual brain differentiation. The genetic program for brain development is thought to be inherently female. It will remain female unless male differentiation tendencies are epigenetically triggered by androgens or estrogens during a sensitive period. The organizational effects of androgens are thought to be mediated by intracellular conversion of these hormones in certain brain areas to estrogens (aromatization hypothesis). Diagram from Döhler and Hancke[29] after modification.

Field[87], Dyer et al.[40], Dyer[41], Greenough et al.[52], Nishizuka and Arai[76] and by De Vries et al.[21, 22]. In all these cases the sex differences proved to be dependent upon the degree of androgen exposure during the perinatal period. The first discovery of a gross sexual dimorphism of the brain was made by Nottebohm and Arnold[77] on two species of song birds. During a reinvestigation of the male and female rat brain, Gorski et al.[49] observed a striking sexual dimorphism in the gross morphology of the medial preoptic area. The volume of an intensely staining area, now called the sexually dimorphic nucleus of the preoptic area (SDN-POA), is several times larger in adult male rats than in females[47–49]. The development of this nucleus starts during late fetal life[54, 56, 57] and depends on the hormonal environment during the critical period of sexual differentiation[24–28, 32, 34, 49, 55].

The sexually dimorphic nucleus of the preoptic area (SDN-POA): development and differentiation. Development of the SDN-POA was shown to start during late fetal life and to extend throughout the first ten days of postnatal life[54, 56, 57]. This developmental period is identical with the period when sexual differentiation of brain function proceeds under the influence of gonadal hormones.

A series of studies was performed during recent years in order to test the influence of hormones perinatally on development and differentiation of the SDN-POA. Neonatal castration of male rats reduced the volume of the SDN-POA permanently[49, 55]. Reimplantation of a testis or treatment with a single injection of testosterone propionate (TP) one day after neonatal castration restored SDN-POA volume in male rats to normal[55]. Treatment of female rats with a single injection of TP either prenatally (F.C. Davis, unpublished observations) or postnatally[49, 55] increased SDN-POA volume significantly; however, the volume of the SDN-POA in these animals was still significantly smaller than that in normal male rats. Only the extended pre- and postnatal treatment of female rats with TP resulted in SDN-POA differentiation equivalent to that of normal males[24, 26]. The treatment of male rats pre- and postnatally with TP did not increase the size of their SDN-POA above normal[24, 26].

A closer look at female sexual differentiation of the brain. Sexual organization of the brain is thought to be inherently female unless male differentiation is superimposed by androgens or estrogens during a critical period of development. The organizational effects of androgens are thought to be mediated by intracellular conversion of

these hormones in certain brain areas to estrogens. In other words, female differentiation is thought to proceed in the absence of specific hormonal influences, whereas male differentiation requires estrogenic stimulation (fig. 2).

The assumption that female sexual differentiation proceeds normally in the absence of gonadal hormones is based upon the early observation by Jost[60] that gonadectomy of female rabbit fetuses does not interfere with female differentiation. Estrogen concentrations in mammalian fetuses are known to be very high, often higher than during later reproductive live[9, 89, 90, 99, 110, 111], and fetal ovariectomy was assumed to clear the fetal blood circulation of estrogens. Recently it was shown, however, that the fetal ovaries are in fact not the major source of the estrogens found in the fetal circulation[45, 63]. In the best-studied species, the human, the primary source of estrogens during pregnancy are the fetal and, to a lesser degree, the maternal adrenals. The adrenals secrete aromatizable androgens (mainly dehydroepiandrosterone sulfate) which are aromatized to estrogens in the placenta[63]. There is, therefore, no reason to believe that fetal gonadectomy renders the fetus free from estrogens.

In the rat, the determination of sexually dimorphic brain differentiation occurs mostly after birth. The assumption that female sexual differentiation of the rat brain would proceed in the absence of gonadal hormones is based on the early observation by Pfeiffer[81], that ovariectomy of newborn female rats did not interfere with female differentiation of the brain.

The role of alpha-fetoproteins. Meanwhile we know very well that postnatal ovariectomy of rats does not clear the blood circulation of estrogenic hormones[113]. This is due to the presence of high levels of estrogen-binding alpha-fetoproteins (AFP)[78, 88] which protect circulating estrogens from metabolism. In fact it was shown that the ovaries of newborn rats do not secrete any estrogens before day 7 of life[64], and it seems most likely that the high levels of estrogens observed in newborn male and female rats[36] are actually remainders of maternal/placental origin from prenatal life (for discussion see ref. 31). The biological role of AFP during the fetal and neonatal period is rather speculative. It was originally assumed that AFP may prevent circulating estrogens from interacting with the developing brain, thus protecting it from masculinization[68]. This assumption becomes highly dubious, however, in view of the intra-neuronal localization of AFP[10, 104]. It should also be considered that without AFP there would not be any estrogens in the blood circulation of postnatal female rats, since the ovaries do not release estrogens during the first week of life[64].

Recent investigations favor the proposition that the biological purpose of AFP may actually be to protect estrogens from enzymatic degradation and to inhibit estrogen uptake by the liver, thus precluding metabolism and excretion (see refs. 31 and 105 for reviews). AFP may even act as carrier for the transport of estrogens into brain cells, as suggested by Döhler[23] and by Toran-Allerand[105]. The overall result would be the conservation of vital estrogens, which are crucial not only for female differentiation of the brain, but also for certain aspects of brain development.

The necessity for perinatal imprinting by estrogenic hormones. Studies of the hormonal influence on female differentiation of the brain have been hampered by the fact that gonadal/placental hormones cannot effectively be removed from the blood circulation of fetal mammals and of postnatal rats and mice. In a series of studies, therefore, Döhler and co-workers[27, 28, 31, 33, 34, 53, 109] adopted the approach of inactivating the endogenous estrogens by treating newborn female rats with the estrogen antagonists tamoxifen or LY 117018 respectively. Both estrogen antagonists inhibit the biological effects of estrogens by competing with estrogens for intracellular estrogen receptor binding sites[58, 59, 75, 101].

Postnatal treatment with tamoxifen[31, 34, 53] or with LY 117018 (Ganzemüller, Veit and Döhler, unpublished) inhibited permanently the differentiation of a positive feedback mechanism for the estrogen-stimulated release of luteinizing hormone (LH), and it inhibited differentiation of the capacity to show female sexual receptivity. These results confirm and extend studies which had been performed with other estrogen antagonists[16, 44, 69]. Since female sexual differentiation of the brain is thought to occur in the complete absence of hormones, the dramatic effects of estrogen antagonists on the developing female brain seem surprising.

On the other hand, the initial steps of the intracellular action of estrogens and estrogen antagonists are similar to some extent. Tamoxifen and LY 117018 are known to bind to intracellular estrogen receptors in an apparently similar fashion as is done by estradiol and other estrogens[58, 59, 75, 101]. On the basis of such similarities one is tempted to consider the possibility that the defeminizing effects of tamoxifen on the developing female brain may actually be due to estrogenic effects rather than to estrogen antagonism. The available results indicate, however, that the permanent biological effects, induced by postnatal treatment of female rats with tamoxifen or LY 117018, are different from the permanent biological effects which have previously been shown to occur after postnatal treatment of female rats with estrogens[13, 26-28, 32, 34, 84]. Although tamoxifen and LY 117018 inhibited differentiation of female sexual behavior patterns, they did not stimulate the organization of male sexual behavior patterns[53], an event which is known to be stimulated by postnatal action of estrogens[13, 84].

The conclusion that the two estrogen antagonists did not act like estrogens, but instead prevented the activity of estrogens postnatally, is supported by the finding that development and differentiation of the SDN-POA is stimulated by perinatal treatment with an estrogen[26, 28, 32], but inhibited by similar treatment with tamoxifen[27, 28, 34]. Furthermore, the defeminizing effect of tamoxifen on organization of female sexual brain functions was actually attenuated by concomitant treatment with estradiol[31, 53].

The conclusion that female sexual differentiation of the brain may not proceed without hormones, but may need estrogenic stimulation, is supported by the results from several other studies. Toran-Allerand[103, 105] demonstrated that hypothalamic neurons of newborn mice do not develop neurite processes in vitro when the culture medium is devoid of estrogens. Vom Saal et al.[110, 111] observed that female mice which were located in utero between two other females had higher levels of estradiol in their am-

nionic fluid, and showed better adult sexual performance, than did their female litter mates which had been located in utero between two male fetuses.

In summary, the available data[10, 16, 27, 28, 31, 33, 34, 44, 53, 69, 103–105, 109–111] suggest that female sexual differentiation of the brain, or even brain development per se, may require perinatal estrogenic stimulation for its full expression. Therefore, the capacity for the normal display of female sexual behavior and for the cyclic release of gonadotropins is not, as has been assumed, inherent to central nervous tissue, but depends on active hormonal induction during a sensitive period of development. Perinatal antagonism of estrogenic activity thus produces animals which are neither male nor female, behaviorally and physiologically speaking. In adulthood they respond neither to estradiol nor to testosterone. Requirements for estrogenic influences on male and female brain differentiation, functional and structural, may be quantitative rather than qualitative[23, 29, 31].

A closer look at male sexual differentiation of the brain. Male sexual differentiation is controlled by testicular hormon. Previous observations, indicating that non aromatizable androgens are not able to stimulate masculinization or defeminization of brain functions, whereas estrogens or aromatizable androgens are quite effective in this regard, generated the hypothesis that male differentiation of the brain is exclusively estrogen dependent. Androgens are thought to be active in brain differentiation only after being enzymatically aromatized to estrogens (fig. 2). The following paragraphs will indicate, however, that the processes of masculinization or defeminization of different brain structures and functions are under the control of more complex hormonal mechanisms.

Structural development and differentiation depends on estrogens. Although pre- and postnatal treatment of rats with TP was shown to substitute fully for testicular activities in stimulating SDN-POA development[24, 26], the prime candidates for the control of SDN-POA differentiation do not seem to be androgens as such, but rather estrogens. This conclusion is supported by several observations: 1) Female rats which had been treated pre- and postnatally with the synthetic estrogen diethylstilbestrol developed a significantly enlarged SDN-POA, which was similar in volume to that of control males[26, 28, 32]. This observation indicates that estrogens can stimulate SDN-POA development directly. The treatment of male rats pre- and postnatally with diethylstilbestrol did not increase the size of their SDN-POA above normal[26, 32]. 2) Male rats, treated pre- and postnatally with the androgen antagonist cyproterone acetate, developed female genitalia, but the volume of their SDN-POA was not reduced[27, 28]. 3) Male rats, treated pre- and postnatally with the estrogen antagonist tamoxifen, developed male genitalia, but the volume of their SDN-POA was significantly reduced and was similar to that of control female rats[27, 28]. The normal development of male genitalia in these animals and the observation that pre- and postnatal treatment of male rats with tamoxifen did not influence serum levels of testosterone[27], indicate that tamoxifen did not act via inhibition of testosterone release from the testes. Instead, the growth inhibiting influence of the estrogen antagonist on the SDN-POA, a brain area with known sensitivity to estrogens[26, 32, 48, 100], seems most likely to be due to local interference with the activity of estrogens, which may have derived via enzymatic conversion from circulating androgens.

In the adult organism tamoxifen is known to bind to intracellular estrogen receptors and to prevent estrogen uptake as it inhibits cytosol receptor replenishment[58, 59, 75]. Tamoxifen may act similarly in the developing organism. After aromatization of testicular androgens into estrogens tamoxifen may have interfered with estrogen uptake into cell nuclei of the SDN-POA by occupying intracellular estrogen receptors. The inhibitory effect of pre- and postnatal tamoxifen on growth and differentiation of the SDN-POA in male rats indicates that not only functional, but also structural differentiation of the male rat brain may be dependent on aromatization of testicular androgens into estrogens and the subsequent interaction of these estrogens with the nuclear material.

The observation that the androgen antagonist cyproterone acetate did not interfere with growth and differentiation of the SDN-POA indicates that androgens are not the primary stimulators of SDN-POA differentiation. Androgens seem to be the substrate, which has to be converted into estrogens before being able to activate SDN-POA differentiation.

Functional differentiation: estrogens alone are without effect. The fact that cyproterone acetate did not inhibit development of the SDN-POA indicates further that cyproterone acetate did not interfere with androgen entry into preoptic-hypothalamic nerve cells or with aromatization of androgens into estrogens. Aromatization of androgens into estrogens is generally considered to be a prerequisite for preoptic-hypothalamic masculinization and defeminization of brain functions. Pre- and postnatal treatment of rats with cyproterone acetate was repeatedly shown to feminize permanently sexual behavior patterns and the mode of gonadotropin release in males[73, 74], and to inhibit the defeminizing action of exogenous testosterone in females[3].

Since differentiation of the SDN-POA was more complete after treatment of male rats perinatally with cyproterone acetate[27] than after treatment of female rats postnatally with a single, anovulation-inducing dose of testosterone propionate[49, 55], estrogenic interaction with preoptic-hypothalamic tissue must have been more intense (and/or more physiological?) during treatment with cyproterone acetate than during treatment with testosterone propionate. Nevertheless, the intensive hypothalamic interaction with estrogens during cyproterone acetate treatment did not disrupt differentiation of the cyclic mode of gonadotropin release or differentiation of female sexual behavior patterns[46, 73, 74], whereas the less intensive (or less physiological?) estrogenic interaction during postnatal treatment with TP interfered with both events.

Thus the question arises, how exactly does cyproterone acetate interfere with sexual differentiation of brain function?

Functional differentiation; the necessity for androgens. The observation that only aromatizable androgens stimulate differentiation of male sexual behavior patterns in female rats, and that non-aromatizable androgens seem to be without this capacity (for reviews see refs. 13, 51, 84), and the observation that masculinization of sexual

behavior patterns can be inhibited by postnatal treatment of rats with estrogen antagonists[11, 69, 97, 109], seem to indicate that estrogens are the major effective hormones which stimulate the differentiation of male sexual behavior patterns. However, this conclusion does not fit with the observation that perinatal treatment of male rats with an aromatization inhibitor did not inhibit differentiation of the capacity of male sexual behavior[112], whereas perinatal treatment with an androgen antagonist inhibited differentiaton of the capacity for male sexual behavior, despite its inability to prevent aromatization of testicular androgens into estrogens[27, 28]. The latter observations suggest that estrogens per se may be less important and androgens per se may be more important for the organization of male sexual brain functions, than has been assumed.

Testosterone is known to enter androgen target cells in the brain. Within the target cells testosterone is subjected to aromatization or to 5α-reduction, the principal metabolites being estradiol and 5α-dihydrotestosterone (DHT). Both hormones are bound with high affinity to specific cytoplasmic receptor proteins and are then translocated into the cell nucleus where they stimulate a characteristic biological response. Cyproterone acetate does not prevent androgen entry into hypothalamic cells, nor does it influence androgen metabolism[67, 102]. Its main antagonistic activity seems to be based on interference with intracellular androgen binding to specific androgen receptors in the cytosol and prevention of the translocation of the receptor-androgen complex into the cell nucleus[67, 102]. Thus, the activity of cyproterone acetate is directed against androgen-mediated events, but is not directed against estrogen-mediated events.

With reference to sexual differentiation of the brain, this discussion points to one necessary conclusion: masculinization and defeminization of sexual brain functions in the rat seem to be mediated not only by estrogens alone, but also seem to require the participation of androgens per se. Androgenic and estrogenic components seem to be required for complete masculinization and defeminization of sexual brain functions. Interference of hormone antagonists with one or the other component results in incomplete organization of the male brain (fig. 3).

Metabolism of non-aromatizable androgens is very rapid in the rat, but slow in the rhesus monkey[51]. The fact that non-aromatizable androgens are quite effective in stimulating differentiation of male sexual behavior patterns in monkeys[51, 84], but not in rats, may be a result of the different speed of metabolism, rather than an indication of a different mechanism of brain differentiation. The inefficiency of DHT, in contrast to testosterone propionate, in stimulating the male type of genital differentiation in female rats, when given at high daily doses during the last week of fetal life[27, 28], is a strong indication that the non-aromatizable androgen never reaches the target organ. In conclusion, during differentiation of male sexual brain function estrogens may be supportative to the primary action of androgens, rather than directive.

Hormonal imprinting and hormone receptors. The intracellular and molecular mechanisms of hormonal imprinting during the process of sexual differentiation are poorly understood. We know that steroid hormones stimulate

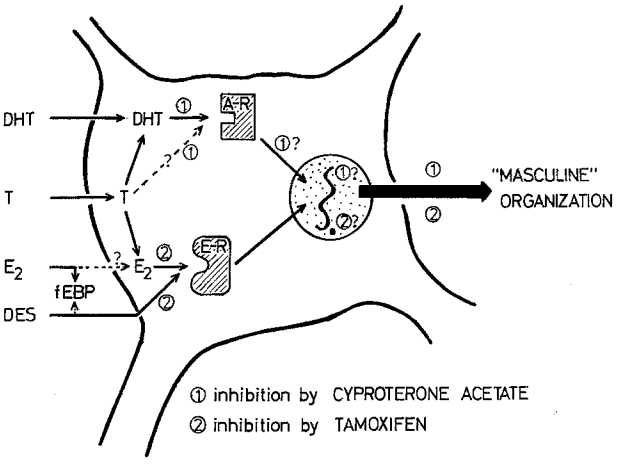

Figure 3. Schematic representation of the androgenic and estrogenic hormone environment with plasma and neuronal compartments in the perinatal rat in relation to masculine organization of the brain. The diagram indicates that interference with androgen action by the androgen antagonist cyproterone acetate (1) or interference with estrogen action by the estrogen antagonist tamoxifen (2) will interfere with masculine organization of the brain. The possible steps of interference are listed A-R = cytoplasmic androgen receptor; DES, diethylstilbestrol; DHT, 5α-dihydrotestosterone; E_2, estradiol; E-R, cytoplasmic estrogen receptor; fEBP, fetal estrogen-binding protein; T, testosterone.

neurite outgrowth in the central nervous system[103, 105], a process which may result in the development of sex specific neural circuitries. Whether this influence of steroids may be mediated via effects on the plasma membrane, or via effects on the genetic code, receptor-mediated or not receptor-mediated, is still a matter of speculation. It is a fact that development of the central nervous system and programing of neural circuitries takes place in parallel with dramatic changes in steroid hormone receptor levels in various target tissues (see ref. 66 for review), and with changes in serum concentrations of estrogen binding proteins[78, 88]. All of these events occur against a background of greatly elevated plasma estrogen concentrations in both sexes[36], elevated plasma androgen levels in males[36], and dramatic increases in serum levels of triiodothyronine and thyroxine in both sexes[35].

It is reasonable to assume that steroid receptors are involved in the process of steroid-induced sexual differentiation, since the syndrome of testicular feminization is due to a defect in androgen receptor development[5, 70]. Individuals with testicular feminization are genetically males, but since their tissues are unresponsive to androgens they do not develop male genitalia or male internal sex ducts. Now the question arises, which mechanism stimulates induction and development of reproductive hormone receptor systems? The answer is, we do not really know. Since, however, the syndrome of testicular feminization is originally a genetic defect, it can be concluded that the genetic code participates in some way in the formation of androgen receptors. The absence of known genetic defects of estrogen receptors suggests that such defects would be incompatible with life and underlines the possible fundamental importance of estrogen receptors for the development and differentiation of the central nervous system.

Since the genetic code is identical in each cell of one organism, and since steroid receptor development does not occur in each cell of the body, but is restricted to cells in specific steroid target tissues, it can be concluded, that the development of steroid receptors is not due exclusively to genomic influences. Instead, activation of genomic information must depend on particular intracellular environments. It was mentioned previously that in lower vertebrates the hormonal environment can totally override the sex-determining genetic mechanism, even in germ cells, without altering the genetic code[116]. These observations indicate that, in regard to sex-determining mechanisms, the genetic code does not provide the final anatomical or physiological substrate. Instead, the genetic code only provides 'precursor information' which, during early development of an individual organism, will be shaped (imprinted) into the final substrate by hormones. It has been shown that estrogen receptors in cells of the adult uterus are present in different configurations[65]. The original receptor, synthesized in the endoplasmatic reticulum, has been described as being of precursor nature[65]. This receptor is modified at least twice before it is released into the cytosol[65]. In the absence of estradiol, the original cytosol receptor is compounded in some unknown fashion into a storage form, which can be recalled at any time. In the presence of estradiol the cytosol receptor dimerizes into the active form and translocates into the nucleus[65]. These data indicate that, even in the adult organism, it is not the active receptor which is synthesized as a result of genetic information, but rather a precursor form of the receptor. The final shaping of the receptor is performed by the cellular (hormonal) environment.

Ontogeny of estrogen receptors. In prenatal rats estrogen receptors are still undetectable, but they increase in number substantially in hypothalamus, amygdala and cortex during the postnatal period. Development and maturation of estrogen receptor systems involves two well-defined phases. The initial phase, extending up to postnatal day 5, is characterized by a rapid increase in receptor concentrations throughout the brain, from undetectable levels 2 to 4 days before birth. The second phase, which occurs between postnatal days 5 and 25, transforms the neonatal receptor distribution into the adult pattern through a series of dynamic changes in estrogen receptor levels in different brain regions. In the cerebral cortex, receptor concentrations first increase to a peak at day 10 and then decline rapidly between days 10 and 15. Hypothalamic and pituitary receptor levels also increase up to day 10, but decline only slightly between days 10 and 25. In the preoptic area estrogen receptor levels increase steadily throughout the first 25 days of life; in the amygdala and midbrain they do not change significantly from post natal day 3 onward (see ref. 66 for review).

Alpha-fetoprotein, a precursor for estrogen receptors? The fact that estrogen receptors are as yet undetectable prenatally does not exclude the possibility that they may actually be present in some precursor-form. Good candidates for such precursors would be the different molecular forms of alpha-fetoproteins, which have been detected in serum[78, 88, 108] and in uterus[7, 106, 107] and brain cells[6, 100,, 104, 105]. It was demonstrated by Vallette et al.[108] that high and low carbohydrate forms of alpha-fetoprotein exist in the serum of developing rats. The different forms of alpha-fetoprotein have estrogen-binding affinities, which range from 10^{-7} to 10^{-9} M/l. The estrogen-binding affinity of intracellular estrogen receptors is one order of magnitude higher (10^{-10} M/l) than the highest affinty of alpha-fetopnitein[2]. Uriel[106] demonstrated that in the uterus of immature rats in intracellular cytosol receptor was transformed by 0.4 M KCl into an estrogen-binding protein, whose antigenicity, sedimentation coefficient and binding specificity resembled closely that of alpha-fetoprotein. These data were confirmed by some groups[8, 96], but not by others[6]. Sometimes methodological problems may mask the true results, and if something cannot be seen with the methodology used, it does not necessarily mean that it does not exist.

A controversy of similar nature was raised by Toran-Allerand[104], who observed intracellular location of immunologically active alpha-fetoprotein in neuronal groups throughout the developing rodent brain of both sexes, with the exception of certain areas, which are known to contain high affinity estrogen receptor sites and to be specific targets for estrogens. The significance of this absence of immunoreactive alpha-fetoprotein within the presumed target regions for estrogens during the critical period for sexual differentiation is not known. It is conceivable that estrogen-sensitive regions may be 'protected' by the specific inability of their neurons to take up alpha-fetoproteins. On the other hand it is just as conceivable, that within specific estrogen target cells alpha-fetoproteins may lose their immunoreactivity, because they may be transformed into some other molecular entity. That such transformation of estrogen-binding proteins is not only possible, but may, in the presence of estradiol, actually alter the binding affinity and generate the active form of an estrogen-receptor, has been demonstrated previously[65]. The fact that alpha-fetoproteins in the serum of immature rats exist in several different molecular forms with different affinities and binding capacities for estradiol[108] proves the ability of alpha-fetoproteins to undergo structural and functional transformations.

The decrease in serum concentrations of alpha-fetoproteins during postnatal development is accompanied by a parallel increase in the concentration of specific intracellular estrogen receptors in the same brain regions, in which immunologically active alpha-fetoproteins could not be detected. This leaves space for the speculation that alpha-fetoproteins, after undergoing multiple conformative changes in the blood circulation, enter estrogen target cells in the brain, where they are transformed into estrogen receptors of even higher affinity. The serum fraction of alpha-fetoprotein with the lowest glycosylation was shown to carry the highest-affinity estrogen binding sites[108]. Taken together with the well-known facilitated entry of deglycosylated glycoproteins into cells, this observation advocates the facilitated entry of those alpha-fetoprotein molecules with the highest-affinity estrogen binding sites into estrogen target cells. The second hypothetical step, intracellular transformation of alpha-fetoproteins into specific cytoplasmic estrogen receptors, has been shown repeatedly to be reversed at high salt concentration[8, 96, 106].

In summary, estrogens, via their ability to alter the affin-

ity of estrogen-binding sites, may act as catalysts for passing binding proteins through membranes, be it cell membranes or the membranes of the cell nucleus.

Defeminization is linked to impaired function of the estrogen receptor system. It was previously mentioned that female rats, which had been treated pre- and/or postnatally with estrogen antagonists, like tamoxifen or LY 117018, remain permanently irresponsive to later estrogen treatment with regard to the expression of reproductive functions. It was also shown that uptake of ^{3}H-estradiol into the nuclei of preoptic and ventromedial hypothalamic cells was permanently inhibited in these animals[33]. Since uptake and retention of estradiol is predominantly receptor-mediated, these results suggest that development of the estrogen receptor system was permanently impaired. In the adult organism, estrogenic interaction with the genetic material in the cell nucleus stimulates DNA synthesis and stimulates estrogen receptor resynthesis in the cytosol[58, 59, 75]. Since, during the perinatal phase of development, many steroidal effects are of permanent (imprinting) nature, the well-known effect of estrogens on resynthesis of their own receptors in adulthood may have an equivalent of a permanent imprinting nature during development. Since tamoxifen inhibits the stimulatory effects of estrogens on DNA synthesis and estrogen receptor resynthesis in adulthood[58, 75], this estrogen antagonist may act similarly in the developing organism. The result is a permanent impairment in the formation of estrogen receptor systems. It remains uncertain, however, whether the impairment developed because estrogenic interaction with the genetic material was prevented postnatally, or whether tamoxifen may have induced its own receptor system at the expense of the estrogen receptor system. The existence of anti-estrogen binding sites in various estrogen target tissues, which bind tamoxifen, but not estradiol, was recently revealed by Sudo et al.[101].

Clark and Peck[17] previously concluded that the estrogen receptor system develops without the influence of postnatal estrogens. Their conclusion was based on the assumption that gonadectomy would clear the postnatal rat of estrogens. It was, however, discussed previously that postnatal gonadectomy does not clear the developing rat of estrogens (see also ref. 31).

Ontogeny of androgen receptors. Concurrently with the developing estrogen receptor system there is also the development of an androgen receptor system in the hypothalamus[61, 62]. The similarity of estrogen and androgen receptors in the affinity for their respective hormones[20, 72], and the fact that androgen receptors possess affinity for estradiol[43, 72], and estrogen receptors possess affinity for androgens[43, 91, 92], may indicate that both receptor systems may derive from a common undifferentiated precursor. Already Sheridan et al.[93, 94] and Fox[43] have considered the possibility that androgens and estrogens may compete with each other for their receptor during the period of sexual differentiation. Döhler and Hancke[30] demonstrated that hypothalamic masculinization of female rats due to postnatal treatment with testosterone was prevented by concomitant postnatal treatment with estradiol, and vom Saal et al.[111] reported that prenatal estrogens interfered with the effect of androgens in the differentiation of aggressive behavior in male mice. The observation by Csaba (for reviews see ref. 18 and 19) that hormones may induce their own receptors in unicellular organisms may also be valid for receptor induction in higher organisms. There are, however, no studies yet which would prove or disprove the possibility that initial contact of a steroid with a binding protein would imprint this protein in such a way that binding affinity to the particular steroid in question is increased and a specific receptor is formed.

Conclusion

Sexual differentiation of the mammalian brain seems to be more complex than has generally been taken for granted. The available data indicate that the capacity for the display of female sexual behavior and for the cyclic release of gonadotropic hormones is not, as has been assumed, inherent to central nervous tissue. Instead the embryonic brain seems to be as yet undetermined for either a masculine or feminine course of development.

Female differentiation of the brain. Under the influence of moderate levels of estrogens, the embryonic sex centers in the brain differentiate into neural substrates and circuitries which will be able, in adulthood, to respond to female sex hormones (i.e. estrogens and gestagens) with a display of characteristic female sexual functions. During the period of sexual differentiation of the brain moderate levels of estrogens in the fetal blood circulation are provided by the placenta. In rodents with a short gestation period, such as the rat, mouse, and hamster, prenatal estrogens of maternal or placental origin are carried over into the postnatal period, probably by alpha-fetoproteins, which protect the estrogens from metabolic degradation. Although the majority of these estrogens circulate in a biologically inactive form, they are always 'on-call' and are immediately available at the target site either by rapid dissociation from the estrogen-alpha-fetoprotein complex, or by catalyzing the entry of alpha-fetoprotein into target cells.

Male differentiation of the brain. Under the influence of sufficiently high levels of estrogens, on the other hand, the embryonic sex centers in the brain will develop and differentiate into neural substrates and circuitries which are able, in adulthood, to respond physiologically in a male fashion to estrogens and to aromatizable androgens. High levels of estrogens are delivered perinatally for local action on the brain by means of aromatizable androgens, released from the developing testes. Since androgens are not bound by alpha-fetoproteins, they can reach the brain fairly rapidly, and be aromatized intracellularly into estrogens within certain brain areas. Via this mechanism, estrogens can act specifically and at high concentrations on selective brain regions without influencing other estrogen-sensitive target tissues. Structural differentiation of the male brain seems to depend exclusively on the priming action of estrogens.

For masculinization and defeminization of brain functions, however, the available data suggest that androgens per se may be more important, and estrogens per se may be less important, than has been assumed. Additional information about molecular mechanisms beyond ste-

roid-induced transcription, and about the possible influence of neurotransmitters and neuropeptides, is needed for further elucidation of the mechanism of hormone induced sexual differentiation of the brain.

Sexual differentiation: controled by receptor imprinting? It is reasonable to assume that steroid receptors are involved in the process of steroid-induced sexual differentiation. In prenatal rats specific estrogen receptors are still undetectable, but they increase in number substantially in hypothalamus, amygdala and cortex during the postnatal period. Since, during the perinatal phase of development, many steroidal effects are of permanent (imprinting) nature, the well-known effect of estrogens on resynthesis of their own receptors in adulthood may have an equivalent of a permanent imprinting nature during development. Such an imprinting mechanism could explain the ontogeny of different affinity forms of estrogen-binding proteins in serum and in estrogen target cells and non-target cells against a background of greatly elevated circulating estrogen levels. The serum fraction of alpha-fetoprotein with lowest glycosylation was shown to carry the highest-affinity estrogen binding sites. Since deglycosylated glycoproteins show facilitated entry into cells, it is very suggestive that the alpha-fetoprotein molecules with the highest affinity estrogen binding sites will preferentially enter estrogen target cells. In other words, the binding affinity for estradiol increases the closer the binding protein gets to the target cell and to the nucleus respectively. Thus, the perinatal rat possesses an estrogen binding system, which prevents degradation of estrogens in peripheral organs, but conveys estrogens through its affinity gradient directly into target cells. Another possibility is that estrogens, via their ability to alter the affinity of estrogen-binding sites, may act as catalysts for passing binding proteins through membranes and, thus, may convey alpha-fetoproteins into estrogen traget cells. It should be mentioned, however, that there are no studies yet which would prove or disprove the possibility that initial contact of a steroid with a binding protein would imprint this protein in such a way that binding affinity to the particular steroid in question is increased and a specific receptor is formed.

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