ANDROGEN RECEPTOR DYNAMICS IN BRAIN AND PITUITARY OF FETAL GUINEA PIGS

by

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CHAPTER I

INTRODUCTION AND STATEMENT OF THE PROBLEM

Sexual differentiation in mammals is a complex developmental process resulting in an organism with a male or female phenotype. Sexually differentiated anatomic structures include: the gonads, the external genitalia, some brain nuclei, the reproductive tract and accessory organs of reproduction, as well as nonreproductive organs and tissues. The primary brain functions organized during sexual differentiation are the pattern of gonadotropin release (tonic or cyclic) and stereotypical sexual behaviors. Male sexual differentiation occurs when undifferentiated tissues are exposed to secretions of the fetal testis. Individuals not exposed to threshold amounts of androgen (e.g. genetic females, males unable to produce androgens, or males unable to respond to androgens) develop as females. Androgen exposure must occur during a tissue-specific "critical period" or period of maximal susceptibility to androgen.

Steroid hormones bind specific receptors in target tissues; receptor/hormone complexes interact with cellular DNA to regulate protein synthesis. Androgen receptors are necessary for normal male development. Men with complete androgen resistance possess feminine external genitalia, are usually reared and behave as females, and have abnormal gonadotropin secretion. The ontogeny of androgen receptors may be important in determining the critical period by: 1) conferring initial sensitivity and steroid specificity to target tissues or 2) signalling the beginning and end of the critical period when receptor concentration exceeds/drops below a minimum level.

In rats and mice, in situ metabolism of testosterone to estradiol (aromatization) appears to be essential for brain masculinization. Testosterone then serves as a pro-hormone. However, in other rodents, such as guinea pigs and ferrets, and in non-human primates, androgens alone appear sufficient for masculinization. In these animals testosterone either acts directly or serves as a pro-hormone for dihydrotestosterone formation. In these latter cases, an understanding of the ontogeny and dynamics of the androgen receptor will lead to a better understanding of cellular mechanisms involved in brain sexual differentiation. There are currently no reports on androgen receptor distribution, specificity, or capacity for activation in the fetal guinea pig brain.

The objective of this study is to provide new information pertaining to the above-mentioned parameters for androgen receptors in brains of fetal guinea pigs. The specific aims are: 1) To determine the ontogeny of androgen receptors in specific regions of the fetal guinea pig brain, and 2) To assess the specificity and "maturity" of androgen receptors in the fetal brain, i.e. do receptors bind only androgens and become "activated" (increase the number of nuclear receptors) in the fetus.

To address these questions we measured in vitro binding of radioactive androgen in guinea pig brains throughout gestation and at four months of age. We describe the ontogeny and distribution of this binding in males and females. Males showed increased amounts of nuclear androgen receptor binding in brain areas associated with reproductive functions. Treatment of pregnant guinea pigs with androgens activated androgen receptors in the brains of female fetuses; activation was demonstrated to be specific for androgens. The effects of maternal androgen treatment on fetal serum steroid levels were also observed.

CHAPTER II

REVIEW OF SEXUAL DIFFERENTIATION IN MAMMALS

HISTORICAL BACKGROUND

It is written in the Bible that "God created man...male and female he created them"[1]. For centuries, man has tried to understand the biological mechanisms which constitute this differentiation process, whereby with each new birth either a male or female is produced. Early attempts to explain sexual differentiation were mostly philosophical. Plato believed "The males were descended from the Sun, the females from the Earth, and the hermaphrodites from the Moon, which partakes of either sex,..."[2], or alternatively, that "Of the men who came into the world, those who were cowards or led unrighteous lives may with reason be supposed to have changed into the nature of women in the second generation."[3]. The philosopher/naturalist Aristotle (384-322 B.C.), through his observations, came to believe that the sex of lambs and goats was determined by which waters their parents had consumed and which way the wind was blowing during copulation[4,5]. Aristotle also noted that castration results in feminization of males, and concluded that certain organs are important for sex determination.

Early in the twentieth century scientists began to concentrate on addressing the origins of sexual differentiation experimentally. In 1903, Bouin and Ancel[6] examined developing gonads in the fetal pig and found that testes, but not ovaries, develop interstitial cells; they postulated that the secretions of these cells during uterine life determined certain male characteristics of the adult.

In 1916 Frank Lillie in the United States and Keller and Tandler in Germany, working independently, proposed the "hormone theory" of sexual differentiation from their observations on the freemartin[7-9]. The freemartin is the female of a pair of opposite-sex twins which develop with placental vascular anastomoses[10]. The freemartin's mammary glands and external genitalia are female; however, the male accessory organs of reproduction also develop, and the atrophic gonads resemble testes[11]. Approximately 90% of freemartins are sterile. The probable origin of the term "freemartin" is from the Scottish flearr, meaning a dry cow, and mart, which means meat: These dry, infertile cows were slaughtered for beef. Lillie asked "...how the association of a male and female in utero may so transform the female."[8]. The hypothesis that androgens from the male twin entered the circulation of and masculinized the female twin was supported by the presence of vascular anastomoses between their placentas; in male/female twins without these anastomoses the females are unaffected. Although it is no longer believed that androgens derived from the male twin are responsible for the freemartin phenomenon[10,12,13], the "hormone theory" which had its origins in these observations stimulated much research into the possible role of gonadal steroids as mediators of normal sexual differentiation.

In the 1930's, Vera Dantchakoff studied the reproductive anatomy of hermaphrodites, organisms with both male and female reproductive systems[14]. The isolation of testosterone had made it possible to study the effects of testicular secretions without actually transplanting the testis[15-17]. Dantchakoff injected pregnant guinea pigs with testosterone and observed the effects on fetal development; these early studies demonstrated that treatment of pregnant animals with exogenous testosterone androgenizes the external genitalia of female fetuses, and results in some "hypermasculinization" of the male genitalia[18,19]. Dantchakoff concluded that the male hormone stimulated genetically predetermined growth in the male and that the same hormone produces the same effects in females by acting on "polyvalent" or plastic tissues[19]. Dantchakoff also found that females exposed to exogenous androgen treatment _in_ _utero_ displayed male sexual behavior in adulthood[20]. At the same time, Pfeiffer (1936)[21] hypothesized that pituitaries of male rats showed different patterns of gonadotropin secretion compared to females. He postulated that the pituitary is not sexually determined genetically but is differentiated by secretions of the gonad that occur at birth[21]. The hypothesis that the pituitary gland itself is sexually differentiated by androgens was later proven incorrect when Harris[22] transplanted male pituitaries into the sella turcica of females and found that the male pituitary supports cyclic release of gonadotropin under these circumstances. This was confirmed by the work of Martinez and Bittner[23] and Yazaki[24]. Regardless, Pfeiffer's work was important because it indicated that male differentiation of both sexual behavior and gonadotropin release is due to the presence of male hormones during early development. Everett and workers (1949) also expressed the view that sexual differentiation of the nervous system occurs because of testosterone's action during fetal or neonatal life[25].

In the 1940's Alfred Jost, using rabbits, demonstrated that the internal organs of reproduction develop as female unless a testis is present, regardless of the genetic sex. This female development occurs, in the absence of a testis, even if no ovary is present[26,27]. This work led to the theory that the mammalian reproductive anlagen is inherently "programmed" to develop as female unless testicular secretions are present during development to "override" the basic programming. This concept of a bipotential undifferentiated stage, where the anlagen of developing tissues can be either masculinized or feminized, is now widely accepted. To summarize this central dogma of sexual differentiation: <u>regardless of the genetic sex of the fetus</u>, the inherent mammalian pattern of development is female, and the presence during development of local testicular secretions (testosterone and Müllerian inhibiting hormone) causes male differentiation of the reproductive tract.

Jost also described normal sexual development as consisting of three sequential processes[13]:

1. The establishment of chromosomal sex at fertilization.
2. The development of gonadal sex.
3. The translation of gonadal sex into phenotypic sex.

In mammals, males are the heterogametic sex (XY) and females are homogametic (XX); this is opposite to what is found in birds, where females are heterogametic (ZO) and males are homogametic (ZZ)[28]. In mammals the Y chromosome carries genes that direct differentiation of the indifferent gonad as a testis; the specific mechanisms by which this chromosome regulates testicular development are not clear. At one time it was believed that the H-Y antigen gene was the primary determinant of testicular differentiation[29]. Recently it has been shown that the H-Y antigen gene is instead located near the gene required for male sexual differentiation, which has been called the testis-determining factor (TDF)[30]. It is not known how many genes are required for full expression of the male phenotype[31].

The final process of sexual differentiation, determination of phenotypic sex, is directly dependent on the type of gonad formed. In 1959, Phoenix and workers presented the "organizational hypothesis" which stated that not only is this determination by the gonad true for the anatomy of the reproductive tract, as demonstrated by Jost, but for the neural processes mediating sexual behavior as well[32]. Also, they stated that these brain tissues go through a restricted period when they are bipotential, and during which circulating androgens can act on the tissue anlagen to organize them permanently as male.

The nature of the restricted period when tissues are susceptible to androgen action during development is still not fully understood. Barraclough and Leatham (1954) found that while a single injection of testosterone propionate caused permanent sterility in 5 day old female mice, the same treatment in 20 day old mice had no effect[33]. Barraclough[34] and Goy, Phoenix, and Young[35] later demonstrated a spectrum of responsiveness to single androgen injections in female rats during the first 20 days of life. In the female guinea pig, treatment with androgens before birth causes decreased female sexual behavior (lordosis) and increased masculine sexual behavior (mounting) as adults[36]; androgen exposure after birth has no permanent effects on sexual behavior. Specifically, Goy, Bridson, and Young (1961) showed that the "critical period" for testosterone action on the guinea pig brain fell between days 30 and 65 of fetal life[37]. Subsequently, Goy, Phoenix, and Young showed that defeminization by androgens was most effective from day 25 (post-fertilization) in the rat and from day 30 in the guinea pig[35]; this period in the rat includes the first ten days postpartum. In 1964 Goy and workers proposed the term "critical period" be replaced by "period of maximal susceptibility" (or sensitivity) to the organizing actions of androgen[38]. The "short-gestation" mammals whose young are born immature (with closed eyes, little hair) are susceptible to androgenization in the neonatal period; these include the rat[39,40], mouse[41], hamster[42], and ferret[43]. "Long-gestation" mammals such as sheep[44], guinea pigs[36,38,45,46], and primates[47], on the other hand, require androgen *in utero* for male development.

The work cited above indicated that the fetal testis plays a major role in sexual differentiation and that exogenous androgen can replace some but not all of the effects mediated by the fetal testis. It was not known, however, whether the fetal or neonatal testis actually secretes testosterone during the "critical period" for sexual differentiation. The first report of endogenous androgen secretion in the neonatal rat appeared in 1968 by Resko, Feder, and Goy[48] and was followed in 1970 by their report of a sex difference in the amounts of testosterone in circulation in male and female rhesus monkey fetuses[49], a species in which sexual differentiation occurs before birth. Differences in fetal male and female levels of testosterone have also been reported in the mouse[50], guinea pig[51-53] and human[54].

SEXUAL DIFFERENTIATION OF THE BRAIN

Anatomical

The presence of a sexually dimorphic nucleus in the preoptic area (SDN-POA) of rats was reported by Gorski[55]. This nucleus is larger and more dense in males than females and this difference is dependent on androgens in the perinatal period. Since then, analogous regions have been reported in the gerbil[56], ferret[57], guinea pig[58,59], and human[60,61]. The function of this area remains unknown, but may be associated with male behavior. In rats, an area corresponding to the medial preoptic nucleus which seems to regulate cyclic release of gonadotropins is larger in females than in males, and this decreased volume in males is also due to perinatal androgens[60]. Sex differences in the volume of other brain areas are also seen in the ventromedial nucleus, medial amygdaloid nucleus, and the bed nucleus of the stria terminalis as well as the corpus callosum[62]. There is also a sexually dimorphic area in the hypothalamus of gerbils[63].

Synaptic organization in specific brain nuclei is also sexually differentiated by neonatal androgens[60]. For example, the number of spinal and somatic synapses is greater in the arcuate nucleus of female compared to male rats; other brain nuclei show different ratios of spinal synapses between males and females, with males having more spinal synapses in the SCN and females having more in the dorsomedial POA. Arai et. al. state "These findings suggest that synaptic organization may vary according to the genomic responses of the individual nuclei to organizational action of sex steroids": Also "A high correlation between the sexually dimorphic synaptic pattern and the presence of neurons containing sex steroid receptors indicates that the occurrence of synaptic sexual differentiation is rather specific to the sex steroid-sensitive neuronal system"[60].

Physiological

The two most-studied functional parameters of brain sexual differentiation, and the ones we will focus on, are the regulation of gonadotropin secretion and reproductive behavior. There are, however, other brain functions which show sexual dimorphisms and whose development appears to be steroid-dependent including aggressive behavior[41,64], social and play behaviors[65], and body weight and food intake[66]. Pituitary secretion of growth hormone[67,68] and prolactin[69] are also sexually differentiated.

From the examples of functional sexual differentiation given above, Gorski[70] drew three generalizations. First, as discussed in the previous section, critical periods vary between species, being either prenatal, perinatal (both pre- and postnatal), or postnatal, depending on the maturity of the central nervous system at the time of birth. Secondly, in any single species critical periods for different functions may vary temporally and may be sensitive to different steroid hormones. Lastly, some functions may be sexually differentiated in one species and not in another; for example, the mechanism that mediates positive feedback of steroids on gonadotropin secretion is not differentiated in rhesus monkeys[71,72] but is in various rodent species[73-75].

Another distinction that is useful for discussing the effects of hormones in sexual development is the use of the term "masculinization" or the enhancement of male traits (tonic gonadotropin secretion, aggression, male sexual behavior) versus "defeminization" or the diminishing of female traits (lordosis, gonadotropin surges in response to estrogen). Masculinization and defeminization are individual processes whose critical periods may be sensitive to different steroids, as mentioned in the previous paragraph.

ROLE OF ANDROGEN METABOLISM IN SEXUAL DIFFERENTIATION

Testosterone metabolism has two primary bioactive steroid products. The enzyme 5α-reductase catalyzes the conversion of testosterone to the androgen 5αdihydrotestosterone (5α-DHT), while the aromatase enzyme complex converts testosterone to an estrogenic metabolite, estradiol. While androgens are responsible for male differentiation of the reproductive tract, testosterone and DHT each play a unique role in the development of different parts of the reproductive tract. Specifically, testosterone directs the differentiation and development of the Wolffian duct structures (epididymis, ductus deferens, and seminal vesicles) while DHT guides differentiation of the external genitalia[76]. In human males with 5α-reductase deficiency, the external genitalia are ambiguous at birth due to low levels of DHT production, although testosterone levels are normal and differentiation of testosterone-dependent structures is normal.

Male differentiation of the brain proceeds by mechanisms analogous to those found in the reproductive tract, i.e., by androgens acting to virilize previously bipotential tissues. Experimentation with rats and mice, however, has made it clear that the testosterone metabolite responsible for male sexual differentiation of the brain in these species is estradiol rather than an androgen[60,61,70,77-80]. It was found that when 5α-reductase inhibitors are administered to rats and mice during the critical period male brain differentiation proceeds normally, whereas administration of aromatase inhibitors during this period blocks male sexual differentiation. Estradiol alone, but not DHT, given to neonatal female rats can induce male brain differentiation (i.e. tonic gonadotropin secretion, an inability to respond to an estrogen/progesterone challenge with an LH surge, decreased female sexual behavior, and an increased frequency of male sexual behaviors, such as mounting, in adulthood)[60,81-83]. In the rat, estradiol is responsible for masculinization of the SDN-POA[84]. Also, in male rats, inhibition of aromatase activity during fetal life leads to increased female sexual behavior as an adult[85,86]. An interesting question raised by these data is whether estradiol is the masculinizing agent in all rodents or whether a difference between short and long-gestation mammals exists.

The ferret is a short gestation rodent in which the critical period for differentiation of male sexual behavior occurs postnatally (approximately day 5-15 postpartum)[43]. Both aromatization and 5α-reduction of testosterone occur in the perinatal ferret brain[87]. Greater activities are found in male compared to female brains; however, aromatase activity in the fetal ferret brain is unaffected by the plasma testosterone concentration[87]. Also, when either aromatization or 5α-reduction are blocked in the neonatal ferret, male sexual behavior develops normally[88]. These results suggest that testosterone itself is the active agent for masculinization of behavior in the ferret brain.

In the guinea pig, male sexual behavior develops normally when 5α-reduction is inhibited[89,90]. Female fetuses in this study were more responsive to the positive feedback effects of estradiol benzoate, indicating a possible role for endogenous 5α-DHT in defeminization of normal females. There have been some recent reports that the synthetic estrogen, diethylstilbestrol (DES) dipropionate can cause masculinization (of sexual behavior and the sexually dimorphic nuclear region of the preoptic area) and defeminization (delayed vaginal opening, decreased lordosis) in fetal female guinea pigs[91,92]; however, females treated with an antiestrogen (tamoxifen) continue to show some male sexual behavior without the defeminization observed after DES treatment[92]. This data suggests that while estrogen can masculinize guinea pigs, the role of estrogen in normal sexual differentiation of the guinea pig is less clear. These results conflict with earlier data indicating that testosterone but not estrogen or DHT abolish lordosis behavior in female guinea pigs[93].

Androgens administered _in utero_ masculinize brain areas controlling gonadotropin release in guinea pigs. Female fetuses exposed to testosterone propionate _in utero_ have androgenized external genitalia and do not release LH in response to an estradiol benzoate challenge[90]; sensitivity of the gonadotrophs to gonadotropin releasing hormone (GnRH), however, is unaffected.

In rhesus monkeys, the ability to release gonadotropin after an estrogen challenge is not sexually differentiated[71,94]. The behavioral masculinization found in this species is androgen-dependent since testosterone or DHT are equally effective[95]. Prenatal exposure to androgens masculinize both mounting behavior and rough-and-tumble play behavior in monkeys but there is no evidence that this treatment decreases female receptive behavior (i.e. no defeminization of behavior)[96]. Female proceptive behaviors (solicitation), however, are diminished in androgen-treated females[97,98]. Also, in these so-called "pseudohermaphrodites", the external genitalia are masculinized, and menarche is delayed but there is no anovulatory syndrome as occurs in laboratory rodents[96]. In human females androgenized as a consequence of the adrenogenital syndrome, menarche is delayed but ovulation does occur[99].

Information from the five species mentioned above enable us to make generalizations concerning hormonal control of sexual differentiation, differences between species, and the class of steroid which is responsible for this process. Primates and the smaller rodents (rats, mice, and hamsters) seem to differ most, while the ferret and guinea pig share some characteristics of both groups. Similar to other rodents, guinea pig behavior is not masculinized by exposure to 5α-DHT, but the capacity to release LH in response to an estradiol challenge is sexually differentiated by DHT. On the other hand, the guinea pig resembles the other long-gestation animal in this group, the monkey, in that estradiol alone cannot masculinize behavior in females. Therefore, in the monkey and guinea pig, androgen plays the major role in masculinization of the areas of the brain that control sexual behavior. This species diversity for sexual differentiation of the brain contrasts to the situation in the reproductive tract, where all mammals utilize the same mechanisms of differentiation[100].

ANDROGEN RECEPTORS

Androgen target tissues, such as the prostate, of rats[101,102], rabbits[103], guinea pigs[104], and man[105] retain exogenously administered androgen, hence the concept that androgen-dependent tissues contain specific binding molecules called androgen receptors. In 1969, Fang and co-workers demonstrated that the ability to retain androgens in the prostate was due to a protein in the cell nuclei which retained 5α-DHT for at least six hours after testosterone injection[106]. Anti-androgens such as cyproterone will inhibit nuclear androgen retention[107-109]. The hypothalamus and pituitary are also androgen target tissues in the guinea pig[104].

The three known functions of steroid receptors are hormone binding, DNA binding, and regulation of gene transcription. Androgen receptors, like other steroid hormone receptors, are thought to be synthesized on cytoplasmic ribosomes from one strand of mRNA[110]. Early workers believed that steroid hormone receptors resided in the cytoplasm of cells and that upon hormone binding, the receptor-hormone complexes were translocated to the nucleus[111]. Development of monoclonal antibodies to steroid receptors and the use of immunocytochemistry revealed that steroid hormone receptors are found only in the nucleus, with or without hormone treatment, and that unoccupied receptors are associated loosely with nuclear DNA[112,113]. Human androgen receptors possess amino acids with a high percentage of homology to nuclear translocation signals, supporting the idea that receptors are transported to the nucleus during or shortly after their synthesis[114,115]. When isolated in the absence of steroids, androgen receptors are associated in large oligomeric complexes along with a 90 kDa heat shock protein (HSP 90); these complexes are unable to bind DNA[116]. Addition of hormone causes dissociation of HSP 90. In glucocorticoid receptors, loss of HSP 90 from the oligomeric receptor complexes correlates with loss of hormone binding activity; this information led to the hypothesis that HSP 90 is involved in maintaining steroid receptors in an inactive state (unable to bind DNA), and that HSP 90 is required for the receptors to have the correct conformation for hormone binding[117].

The androgen receptor gene has been cloned and shown to be a member of a superfamily of receptors including those for steroids, thyroid hormone (T_3), vitamin D, retinoic acid, and other similar molecules[113-115,118-121]. The androgen receptor gene is located on the X chromosome[115,122], and it contains three regions that are highly homologous between members of this superfamily, known as regions C1-C3. The C1 region is most highly conserved and is the DNA binding region, which forms two zinc fingers[110]. This region may act to repress constitutive gene expression of the receptor; one function of androgens is to suppress the steady-state level of androgen receptor mRNA expression[113]. The less homologous C2 and C3 regions are believed to participate in hormone binding, while the hypervariable amino-terminal region is probably involved in the ligand specificity of each receptor[110]. Two forms of androgen receptor have been reported, with the minor form comprising 10-15% of total androgen receptor content[123].

"The ultimate function of receptors is to elicit specific effects at the transcriptional level"[110]; this appears to be a complex process. Once the androgen receptor binds T or DHT, the receptor is said to be "activated" (vs. the earlier "translocated") and will bind tightly to specific DNA sequences on the genes called hormone responsive or regulatory elements (HRE) and affect transcription[124-126]. Estrogen and glucocorticoid receptors bind DNA as a dimer[127,128]. At least two distinct steps are involved in the translocation of steroid receptors[129]. The nuclear matrix is the major organizer of DNA in the nucleus[130]. It has been hypothesized that the different nuclear matrix proteins found in various tissues may organize the DNA differently in these cells, allowing tissue-specific interactions between androgen receptors and the genome to express genes for cell-specific products[130,131].

A single base mutation in the androgen receptor gene is capable of altering the receptor sufficiently to cause the androgen insensitivity syndrome[122,132-134].

Ontogeny of Androgen Receptors

The ontogeny of AR has been described in androgen-sensitive tissues such as the mouse mammary gland[135,136] and the rabbit genital tubercle[137], as well as in the brain of the mouse, rat[138,139] and rhesus monkey[140]. Although specific ARn were not found in the primate brain[140,141], the ARn in the rodent brain was found to be qualitatively like the adult receptor in its ability to bind DNA[142]. The androgen receptor in developing mouse mammary gland exerts its effects shortly after its appearance and is then inactivated[136].

No sex differences in the amount of ARc have been reported. Except in the primate cortex[140], the AR first appears shortly before the time the tissue differentiates (or the "critical period" for that tissue)[137,143], and the amount of AR binding increases throughout gestation[138,140,142]. In developing non-nervous tissue, insensitivity to androgens ensues during development without a decrease in AR number or affinity[136,137].

The appearance of AR just before differentiation in these tissues supports the idea that the appearance of AR could mediate tissue sensitivity to androgens. However, in mouse mammary gland, the epithelium (which stimulates the increase in AR in the mesenchyme) must be associated with mesenchyme by a specific time during gestation in order for the gland to respond to hormones later via the AR[136]. These results suggest that while the presence of AR could signal the onset of tissue sensitivity to androgen, at least one other process limits the length of time that the tissue remains sensitive.

Regulation of Androgen Receptors

Androgen target tissues contain measurable cytosolic androgen receptors, and androgen treatment leads to increased nuclear androgen retention[144]. Castration leads to an increase in cytosolic and a decrease in nuclear androgen receptor content in the prostate[145]. Complete replenishment of cytosolic receptor levels is seen within 3-4 hours after androgen administration[146]. Testosterone treatment in castrated mice leads to an immediate dose-related decrease in kidney cytosolic androgen receptors and a dose-independent increase in nuclear androgen receptor content; duration of nuclear androgen retention is dose-dependent after one hour[147]. It appears that both the number of androgen receptors accumulated and their nuclear retention time regulate the extent of the biological response to androgens[147]. However, a relatively small nuclear androgen receptor content appears to be sufficient to elicit androgen effects[144,148].

Because the relationship between the androgen-induced decrease in cytosolic and increase in nuclear receptor content is not stoichiometric, a two-component mechanism was suggested: testosterone 1) translocates cytosolic receptors, and 2) stimulates synthesis of new cytosolic receptors[145]. Step 2 of this mechanism, however, appears to conflict with reports of autoregulation of the androgen receptor gene, whereby androgen stimulation decreases synthesis of androgen receptor mRNA[113]. An alternative explanation may be that nuclear androgen retention increases processing of nuclear receptors, inactivating excess nuclear complexes which are not involved in chromatin interactions and causing dissociation of hormone from the receptor[146]. It has also been suggested that proteins and RNA produced by interactions of androgen receptors and nuclear acceptor sites may be involved in regulating the cell's response to androgens[149]. Interestingly, while androgens are the primary regulators of nuclear androgen receptor levels in the dorsal and ventral lobes of the rat prostate, in the lateral lobe there is evidence of testosterone-independent regulation of nuclear androgen retention[150].

Estrogen treatment increases cytosolic androgen receptors in the rat anterior pituitary, medial preoptic area, and medial amygdala[151-153]. This increase in receptor number has been postulated to increase tissue sensitivity to androgen; receptor affinity is unchanged[151]. Estrogen treatment also increases progestin receptor content in anterior pituitary and hypothalamus-preoptic area[151].

Progesterone treatment of adult male guinea pigs results in decreased male sexual behavior and a concomitant decrease in nuclear androgen receptor content in the preoptic area, medial basal hypothalamus, and anterior pituitary without changes in cytosolic androgen receptor content[154,155]. These changes in nuclear androgen receptor retention are believed to be mediated through the progesterone receptor and not by direct competition of progesterone for the androgen receptor[155]. Neither estrogen nor progesterone treatment increased androgen receptor content in the rat prostate[145,151].

There is recent evidence which indicates that catecholamines may be involved in the regulation of androgen receptor and estrogen receptor transformation in the rat brain, as well as in male sexual behavior[156-158]. Therefore, while androgen receptors bind androgens specifically, translocation of the androgen-receptor complex in the brain appears to be influenced by other steroids and neurotransmitters.

Androgen Insensitivity

The requirement for androgen receptors in human male sexual differentiation becomes evident when we examine the phenomenon of androgen insensitivity. Failure of a steroid hormone to induce its effects can be due to interruption of any part of the receptor-mediated process: Inability of the receptor to bind hormone, instability of the receptor/hormone complex or its inability to bind DNA, or post-receptor defects wherein DNA binding does not affect transcription. Indeed, all of these possibilities have been reported in man, inducing partial to complete androgen insensitivity[133,134,159-162]. The complete form of androgen insensitivity produces a stereotypical syndrome in man which Morris named "testicular feminization"[163]. These patients are male pseudohermaphrodites who develop physically and psychologically as females despite having androgen-producing testes; they often marry and adopt children (being unable to bear children since they lack female reproductive structures such as the oviduct and uterus).

Some degree of androgen insensitivity has also been reported in the cow, dog, mouse (Tfm/Y), and rat (Tfm)[164]. The syndrome has been studied extensively in rodents. The first insight into the mechanism of the defect came when it was demonstrated that Tfm rats are unable to accumulate androgen in nuclei of target tissues[165,166], a phenomenon which also occurs in the mouse brain[167]. The Tfm mouse represents a complete form of androgen insensitivity, which is expressed in utero (androgens given to the mother masculinize normal females but not Tfm males); the hypothalamic-pituitary axis is unresponsive to negative feedback by androgen in Tfm males but estrogen is still capable of suppressing LH[164]. The Tfm rat, on the other hand, is partially responsive to androgens due to a small amount of residual androgen receptor activity[168]. High doses of exogenous androgens are capable of decreasing gonadotropin concentrations in castrated Tfm rats, presumably through the action of these residual receptors. A similar phenomenon has been observed in some variants of human androgen insensitivity syndrome[133].

It had been suggested at one time that the Tfm syndrome was due to the inability of androgen target organs to convert testosterone to DHT, i.e. a defect in the 5α-reductase enzyme that mediates this conversion[169]. Indeed, a deficiency in 5α-reduction has been reported in these patients[170]. However, as discussed above, subjects with 5αreductase insufficiency syndrome exhibit distinctly different symptoms than Tfm subjects, as well as a different mode of genetic transmission (autosomal recessive vs. X-linked)[171,172]. Also, the symptoms of 5α-reductase deficiency are not due to a lack of DHT receptors[173]. Defective androgen receptors have been described in human patients with Tfm[133,134,161,174].

It is apparent, then, that androgens are the agent of masculinization in the human male, and we assume that androgen receptors play an essential part in regulating the critical periods for sexual differentiation of brain areas that are sensitive to masculinization. The purpose of this work is to obtain a better understanding of the role of androgen receptors in brain masculinization in an androgen-dependent species, the guinea pig. This information should be useful for understanding the cellular mechanisms involved in sexual differentiation of the brain.

CHAPTER III

ONTOGENY OF ANDROGEN RECEPTORS IN THE FETAL GUINEA PIG BRAIN

ABSTRACT

Sexual differentiation of the guinea pig brain is androgen dependent. In order to understand the cellular mechanism of androgen action, we studied the ontogeny of cytosolic (ARc) and nuclear (ARn) androgen receptors in the brains and anterior pituitaries of fetal, neonatal, and adult guinea pigs. Using cytosol from the hypothalamus-preoptic area-amygdala-septum of 60-65 day fetuses and nuclear preparations from 6 day old neonates treated with testosterone propionate, validation studies revealed an AR with an apparent K_d of 1.9±1.1 (mean ± SEM,n=3) x 10^{-10} M (ARc) and 3.4±3.2 (n=3) x 10^{-10} M (ARn). The cytosolic receptors were highly specific for androgens. After assay validation, AR content was determined from specific brain regions of fetuses obtained on days 30, 40, 50, and 59 of gestation and on days 6 and 120 postpartum. Cytosolic AR differed significantly ($p<.05$) between brain regions and times of gestation but no sex differences were apparent. In contrast, ARn showed little difference between tissues or with gestational age but there were significant differences between males and females, especially in late gestation and early postnatal life, with males having greater ARn binding ($p<.05$). These data demonstrate the presence of cytosolic

and nuclear AR in the fetal brain and pituitary gland during the "critical" period of sexual differentiation (days 30-37 of gestation), thus establishing the identity of cellular structures that are involved in androgen action.

INTRODUCTION

Sexual differentiation of the mammalian brain is an androgen-mediated process that depends upon testosterone (T) secretion by the fetal testis[40,44,93,94,175,176]. In rats and mice, aromatization of T to estrogen within the central nervous system (CNS) is a necessary prerequisite for androgenization[177-180]. The guinea pig, however, like the primate, does not require aromatization since a non-aromatizable androgen, 5α-dihydrotestosterone (DHT), given prenatally will androgenize female brains so that male behavior is elicited in adult females in response to appropriate hormonal stimuli[93,95].

The response of the fetal guinea pig brain to androgens occurs during a circumscribed prenatal "critical period" with maximal susceptibility between days 30 and 55 of gestation[38,77]; female fetuses exposed to androgens during this time will exhibit masculine behavior as adults in response to exogenous T[36,93]. Sex differences in fetal plasma T concentrations can be found as early as day 27 of gestation[52]. However, the cellular mechanisms that mediate androgen sensitive sexual differentiation are as yet unknown. Vito and Fox[139] suggested that the development of hypothalamic androgen receptors initiates the critical period for androgen action in the brain.

The ontogeny of brain cytosolic androgen receptors (ARc) has been described in the rhesus monkey[140] as well as in several rodents[32,138,139,142]. In these studies ARc were shown to be present in the hypothalamic-preoptic area (HPOA) during the critical period for sexual differentiation.

To determine if androgen receptors are present in the guinea pig CNS during the "critical period" for sexual differentiation, we validated a DHT binding assay in fetal guinea pig brain and anterior pituitary and measured DHT binding throughout fetal and early postnatal life. Data that describe the ontogeny of both ARc and nuclear androgen receptors (ARn) in different regions of the brain are presented.

METHODS

Animals

Female Duncan-Hartley guinea pigs (Simonsen, Gilroy, CA) were housed overnight with males on the first day of vaginal opening which was designated day 0 of gestation. Pregnancy was confirmed by radioimmunoassay (RIA) of plasma progesterone prior to day 30[181] or by palpation after day 30; non-pregnant animals were bred a second time. A progesterone concentration of 10 ng/ml or greater was used as the criterion for a positive pregnancy test.

Tissue preparation

Pregnant guinea pigs were killed by decapitation on day 30,40,50, or 59 of gestation (±1 day; average gestation is 68-70 days in this strain), trunk blood collected, and the fetuses removed and weighed (Table 2). Fetuses were immediately decapitated, trunk blood was collected, and the brain and pituitary removed and placed on ice. When available, tissues from two fetuses of the same sex were combined. Additionally, tissues from neonatal (postnatal day 6±1 day) and adult guinea pigs (postnatal day 120±7 days) were obtained under similar conditions.

Five brain areas: medial-basal hypothalamus (MBH), preoptic area (POA), amygdala (AMYG), septum (SEPT), and frontal cortex (FRCTX) and anterior pituitaries (PIT) were dissected for analysis of AR. Brain areas (except for FRCTX) were dissected according to previously published methods[182]. The most anterior 1-2 mm of cortex were designated frontal cortex. At 30 days gestation, a single block of hypothalamus-preoptic area (HPOA) was obtained by removing the ventral surface of the brain with a cut immediately anterior to the optic chiasm and one immediately anterior to the mammillary bodies at a width of approximately 1 mm and a depth of 1-2 mm. No pituitary or septal tissue was taken from 30 day fetuses.

Cytosolic androgen receptors

Dissected tissues were homogenized in 200 µl of ice cold TEGMD buffer (10 mM Tris, 10 mM EDTA, 10% glycerol [v/v], 25 mM molybdate, and 1 mM dithiothreitol [DTT]; pH 7.4) in cold Dounce tissue grinders (Wheaton, Millville, NJ) using 15-20 strokes of the pestle. The homogenates were transferred to 8 x 34 mm polyallomer centrifuge tubes (Beckman, Palo Alto, CA). This and all subsequent procedures were performed on ice. The homogenizers and pestles were rinsed with 100 µl of TEGMD and the combined homogenate and wash were centrifuged at 1000 x g in a Beckman J6-B centrifuge for 10 min at 4°C. The supernatant was centrifuged at 106,000 x g for 10 min at 4°C in a Beckman TL-100 tabletop ultracentrifuge and the resultant purified cytosol was used for measurement of ARc and soluble protein[183].

Nuclear androgen receptors

The crude nuclear pellet from the low speed centrifugation was suspended in 100 µl of Buffer A (1 mM KH_2PO_4, 0.32 M sucrose, 3 mM $MgCl_2$, 10% glycerol [v/v], 1 mM DTT; pH 6.8) and centrifuged at 1000 x g for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 25 µl of Buffer A containing Cellex 410 (120 mg/500 µl; Bio-Rad Labs., Richmond, CA). The Cellex is washed before use in distilled ethanol, then distilled water (2x), and Buffer

A. Two hundred μl of Buffer B (1 mM KH_2PO_4, 2.4 M sucrose, 1 mM $MgCl_2$, 10% glycerol [v/v], 1 mM DTT; pH 6.8) were added and the solution mixed and centrifuged at 55,000 x g for 10 min in a Beckman TL-100 ultracentrifuge yielding a purified nuclear pellet. The pellicle was removed with a pointed wooden applicator stick, the sucrose buffer removed with a Pasteur pipette and the tube walls dried with a cotton swab.

Nuclear androgen receptor was salt-extracted from the purified nuclear pellet by first resuspending it in 55 μl of TEBD buffer (10 mM Tris, 1.5 mM EDTA, 0.5 mM bacitracin, 1 mM DTT; pH 7.4) for 5 min and then adding 55 μl of TEBDK buffer (TEBD plus 1.6 M KCl; pH 7.4) to achieve a final salt concentration of 0.8 M. This solution was incubated for 25 min with frequent mixing. Centrifugation at 37,000 x g for 5 min (4°C) separated salt-extracted receptor from Cellex and nuclei. Salt extraction was repeated and the two supernatants combined for the nuclear exchange assay. The pellets were frozen until assayed for DNA by the method of Burton[184] as modified by Giles and Myers[185].

Receptor assay

Methods for the estimation of ARc and ARn in the adult guinea pig have been validated in our laboratory[154]. Androgen receptors were quantitated by the *in vitro* binding of (^3H)1,2,4,5,6,7-dihydrotestosterone ([^3H]-DHT; New England Nuclear, Boston, MA). This ligand was chosen for its high specific activity (128.4 - 148.1 Ci/mmol). Validation studies were performed before quantifying AR in fetal guinea pig brain.

For determination of an apparent dissociation constant (Kd), cytosols were prepared from the combined hypothalamus-preoptic area-amygdala-septum (HPAS) of litters of fetuses at approximately 60-65 days of gestation. Salt-extracted nuclear extracts were prepared from the combined HPAS of litters of 6 day old neonatal guinea pigs injected with 1 mg/kg of testosterone propionate (TP) 4 hours prior to sacrifice. Aliquots of cytosolic or nuclear receptors (100 μl) were incubated with increasing concentrations of $[^3H]$-DHT (0.09-3.2 nM for cytosol; 0.04-5.8 nM for nuclear) in a total incubation volume of 150 μl. Parallel incubation tubes containing a 200-fold molar excess of radioinert R1881 (methyltrienolone; NEN, Boston, MA) were used to determine non-specific binding. Tracer binding to sex hormone binding globulin (SHBG) was considered unlikely as this protein has not been found in the guinea pig[186,187]. Incubations were carried out for 20 hours at 0-4°C. Preliminary studies indicated that binding equilibrium was reached by 20 hours with no increase in binding at 40 hrs.

The specificity of $[^3H]$-DHT binding was determined by incubating total and non-specific binding tubes (200 fold excess of R1881) with increasing amounts of radioinert steroids (T, DHT, progesterone [P], estradiol [E_2], and corticosterone [C]; Steraloids, Inc., Wilton, NH) and calculating the percentage displacement of specific [^{3}H]-DHT bound to androgen receptor. Cytosols used for specificity studies were obtained by combining the HPAS of fetuses from two litters at 60-65 days of gestation.

Single point analyses of AR numbers in the MBH, POA, AMYG, SEPT, FRCTX, and PIT of fetal and neonatal guinea pigs were carried out in the same manner and incubated 20 hours at 0-4°C with either 3.2 nM [3H]-DHT (cytosolic) or 5.8 nM [3H]-DHT (nuclear).

Bound and free radioactivity were separated on Sephadex LH-20 (Sigma, St. Louis, MO) mini columns by a method published previously[182]. Specific binding was determined by subtracting non-specific binding from total binding. Bound radioactivity was counted in a Packard Tricarb scintillation spectrometer with 40% efficiency and a counting error of 5%. Data were reported as fmol bound/mg protein for ARc and fmol bound/mg DNA for ARn. The sensitivity of the assay was 0.01 fmol/tube, which corresponds to 2-4 specific dpm/tube.

Steroid RIA

Serum concentrations of T, DHT, P, estrone (E_1), and E_2 were determined by methods previously described[181]. We were unable to obtain sufficient amounts of blood for analysis from fetuses on day 30 of gestation. The intra and inter-assay coefficients of variation (percent) were as follows:

E_1: 8.9, <u>16.5</u>; E_2: 6.3, <u>13.7</u>; P: 8.3, <u>14.2</u>; T: 8.9, <u>12.4</u>; DHT: 9.7, <u>9.4</u>.

Statistics

Data from single point determinations and serum steroids were analyzed by a two-way analysis of variance. When no apparent sex differences were found, data from males and females were combined and analyzed by a one-way analysis of variance. If a significant F value was obtained, *post hoc* comparisons were determined by the method of Neuman-Keuls[188]. When differences between the sexes were demonstrated by a two-way analysis of variance using age and gender as variables, comparisons were made within individual ages using a Student's t test[188].

RESULTS

Steroid hormones in the maternal and fetal circulation

In the maternal circulation all the steroids examined varied significantly compared to non-pregnant values [E_1: F(4,45)=18.04, $p<.001$; E_2: F(4,45)=12.06, $p<.001$; P: F(4,37)=13.19, $p<.001$; T: F(4,48)=6.41, $p<.001$; DHT: F(4,48)=3.03, $p<.05$]. Estradiol, T, and DHT tended to rise throughout gestation (Fig.1; data from day 34 [n=2] were not used in the analysis). Maternal T levels differed significantly from those of non-pregnant animals only in late gestation (day 59; $p<.01$) while E_1 and P were highest during mid-gestation with peaks at day 40 ($p<.05$ compared to all other times), and declined by day 59 of gestation. A two-way analysis of variance showed no sex differences in fetal serum levels of E_1, E_2, or P so the data from males and females were combined for analysis although they are plotted separately in Figure 2. The one-way analysis of variance revealed that the estrogen levels (both E_1 and E_2) varied with gestational age [E_1: $F(4,132)=30.33$, $p<.001$; E_2: $F(4,131)=8.10$, $p<.001$]. Both E_1 and E_2 were significantly higher at day 40 of gestation than at any other age. Progesterone levels also varied with age [$F(4,131)=8.85$, $p<.001$], being high before parturition but dropping at least 2.5-fold after birth ($p<.05$, pre- vs. postnatal).

Serum DHT levels showed a significant variation with sex [$F(1,124)=6.09$, $p<.001$]. In general, male levels tended to be greater than those of females of the same age, but this was only significant after parturition ($p<.05$ at postnatal day 6, $p<.01$ at postnatal day 120). Testosterone levels varied significantly with both age and sex [age: $F(4,122)=92.06$, $p<.001$; sex: $F(1,122)=204.28$, $p<.001$] and were higher in males than in females ($p<.01$) at every age studied. The T levels at both 6 and 120 days postpartum were greater than those of males examined prenatally ($p<.01$).

Binding kinetics

Saturation analysis of cytosols from fetal HPAS and PIT showed a single high affinity AR (Fig. 3). The apparent K_d for the ARc calculated by Scatchard analysis was 1.9±1.1 (mean ± SEM, n=3) x 10^{-10} M in the HPAS and 6.7±4.8 x 10^{-10} M in the PIT (Fig. 4). Nuclear AR in the HPAS from neonates pretreated with TP had an apparent K_d of 3.4±3.2 (mean ± SEM, n=3) x 10^{-10} M (Fig. 5).

Competition studies in fetal and maternal HPAS were performed using radioinert T, DHT, P, E_2, and C (Fig. 6). DHT and T were the most effective competitors of $[^3H]$-DHT binding to ARc, while P, E_2 and C caused little displacement.

Ontogeny of ARc

Cytosolic AR binding was measured in the guinea pig brain and PIT at selected times in fetal, early postnatal, and adult life (Fig. 7). A two-way analysis of variance (age and gender as variable) revealed no sex differences in the number of ARc present in any tissue; therefore, data from males and females were combined for future analyses. The changes in receptor numbers with development showed a tendency to increase with age. Androgen receptor content in the fetal POA, SEPT, and MBH showed significant changes with age [POA: $F(4,58)=10.44$, $p<.001$; SEPT: $F(4,58)=11.09$, $p<.001$; MBH: $F(4,58)=9.08$, $p<.001$] with lowest levels early in gestation and a significant increase between day 50 and 59 (p<.01). Levels of ARc in FRCTX were low and showed little change throughout gestation (<1.0 fmol/mg protein). Low levels were also found in the AMYG compared to other brain areas that we studied. The increases in number of ARc in the AMYG and PIT were not significant until postnatal day 120 (p<.01).

Ontogeny of ARn

A two-way analysis of variance (age and gender are variables) showed sex differences in the numbers of ARn found in the POA, MBH, AMYG, and PIT of fetal and neonatal guinea pigs (Fig. 8); [POA: F(1,52)=4.6, p<.05; MBH: F(1,50)=7.39, p<.01; AMYG: F(1,52)=9.10, p<.01; PIT: F(1,50)=4.57, p<.05]. Sex differences in ARn content were most obvious in MBH where male levels were significantly higher (p<.05) than female levels on prenatal days 40, 50 and 59 and postnatal day 120. Male fetuses also contained higher levels of ARn than females in POA on day 59, SEPT and PIT on day 50, and AMYG on day 40 of gestation. No differences between tissues were found except on prenatal day 59 when ARn in the male MBH was significantly higher than in the SEPT, FRCTX, and PIT (p<.05). Nuclear binding in any given tissue showed an increasing trend with age but the changes were not statistically significant.

DISCUSSION

We have described a single, saturable, high affinity receptor specific for androgen in the brain and anterior pituitary of fetal guinea pigs throughout gestation. We have also presented a quantitative ontogenic profile of both ARc and ARn in these tissues, and reported on the changes in serum steroid levels during development.

The binding kinetics of [^{3}H]DHT in the fetal guinea pig brain and anterior pituitary are similar to those previously reported in the adult male[154], the fetal rhesus monkey[140], and the neonatal rat[189]. This receptor possesses a high affinity for T and DHT. Estradiol and P are only weak competitors, which confirms previous reports[154,190].

Although there were no sex differences in ARc numbers during gestation, there were tissue differences. We found ARc in the HPOA on day 30 of gestation which is the earliest time we studied. Since the HPOA is the main area that mediates reproductive behavior in adulthood[86,191,192], the demonstration of AR in this area during the critical period for sexual development indicates that the biochemical machinery for androgen action is in place in this tissue early in development. Cytosolic AR numbers remain elevated in the MBH and in the PIT throughout the critical period (through day 50 in these experiments). From day 59 through adulthood, ARc levels were higher in POA, SEPT, MBH, and PIT compared to AMYG and FRCTX. This distribution is similar to that obtained in adult male rhesus monkey brain using uptake of $[^3H]DHT$[193].

In addition to inter-tissue differences there were changes in ARc number in individual tissues throughout development. In POA, SEPT, and MBH there was a dramatic increase in ARc number in late gestation (day 59) that was maintained in the postnatal period. In the AMYG and PIT, however, there was no significant increase until adulthood. This tendency of ARc numbers to increase throughout development is similar to that seen in the rat[139], mouse[142] and monkey although we found no early peak in cortical ARc as reported in the monkey by our laboratory[140].

There were sex differences in the number of ARn in some tissues during development, most noticeably the MBH, where males consistently had more ARn than females. Postnatal day 6 proved to be the exception, however. On this day statistical significance was not found due to the high variance between samples. Adult males tended to have more ARn than females in all tissues studied. There were also apparent prenatal sex differences in the POA (day 59), SEPT (day 50), AMYG (day 40), and PIT (day 50). These data indicate that the AR in the fetal guinea pig brain can respond to endogenous androgen during the critical period for sexual differentiation. Following androgen binding, the AR is transformed to a species which is tightly bound within the nucleus and can affect transcription of the genome.

Testosterone levels in male fetuses were higher than in female fetuses at all times studied and increased markedly in the males from prenatal to neonatal and neonatal to adult ages. These data demonstrate the importance of the fetal testes for sexual development in this species, viz., for the production of T that will organize (androgenize) undifferentiated tissues. This hormone is produced during the time that others have observed effects of exogenous T on sexual development of the CNS of the guinea pig[36,38]. These data are similar to those reported in fetal guinea pigs[52,53], rhesus monkeys[194], and rabbits[195] with increased androgen production in the male during the time of sexual differentiation. Testosterone in fetal male guinea pigs is secreted by the testes[52,196-198] and the rise in maternal T in late gestation is most likely of fetal origin.

No sex differences were seen in fetal serum levels of P, E_1, or E_2 at any time studied and no sex differences in DHT levels were apparent until after birth at which time male serum contained more DHT than female serum. This contrasts with data from Buhl et al.[53] who reported significantly higher levels of DHT in males than in females on days 35 and 45 of gestation. The fact that sera were pooled in this study and were obtained at different gestational ages may explain the discrepancies.

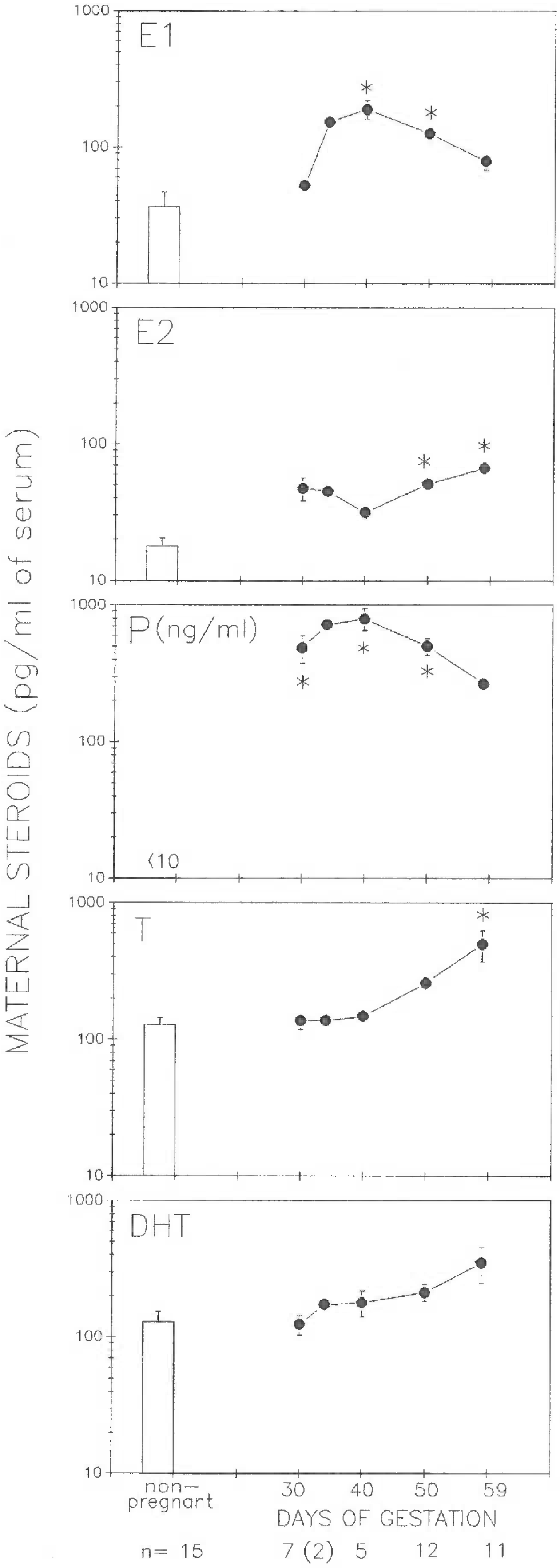
During development there are significant changes in serum E_1 and E_2 concentrations in the fetal circulation.

These hormones are significantly higher on day 40 compared to other times that we studied. Also, P is elevated in sera of prenatal compared to postnatal guinea pigs. The elevated amount of E_2 that is found in the fetal circulation of both sexes probably arises from placental biosynthesis or by conversion from E_1 by fetal 17ß-hydroxysteroid dehydrogenase. An ovarian origin for this hormone seems unlikely, since the fetal ovaries do not contain antral follicles (unpublished data from our laboratory) which are the usual source of ovarian estrogens in the adult female. The fetal ovary is relatively quiescent with regard to steroid biosynthesis but Sholl and Goy[51] reported aromatase activity in the fetal guinea pig ovary prior to formation of rudimentary follicles. This activity would require endogenous substrate, however, before estrogens could be formed. Estrone and P in the fetal circulation peak on day 40 and parallel the steroid concentrations in the mother's circulation. The source of these hormones at this time in gestation is unknown, but may be derived from the placenta[199]. Nelson[200] concluded that placental output of hormones by day 40 of gestation was sufficient to maintain pregnancy in the guinea pig since females did not abort after ovariectomy on day 40 of pregnancy.

We demonstrated the presence of a specific, high-affinity AR in the brain and anterior pituitary of the fetal guinea pig throughout the period of sexual differentiation and found higher levels of nuclear AR in males versus females in the MBH during that time which parallels the high T levels in the male. This availability and responsiveness of AR to T during the critical period provides additional evidence that the AR is part of the cellular mechanism that mediates sexual differentiation of the fetal guinea pig brain by androgen.

Figure 1. Steroid hormone concentrations in the systemic circulation of non-pregnant and pregnant guinea pigs. Data are presented as mean ± SEM. * indicates means differ significantly ($p<.05$) from the non-pregnant value (bar). Steroid values are plotted on a log scale. Abbreviations: E_1 (estrone), E_2 (estradiol-17ß), P (progesterone), T (testosterone), DHT (dihydrotestosterone), n (number of animals at each time period).

Fig. 1



MATERNAL STEROIDS (pg/ml of serum)
E1
E2
P(ng/ml)
<10
T
DHT
1000
100
10
*
non–
pregnant
30
40
50
59
DAYS OF GESTATION
n= 15
7 (2)
5
12
11

Figure 2. Steroid hormone concentrations in the systemic circulation of developing guinea pigs (mean ± SEM). Means with dissimilar letters differ significantly with age ($p<.05$). + indicates means differ significantly between males (o) and females (●) at the same age ($p<.05$). Steroid values are plotted on a log scale. See legend to Figure 1 for abbreviations.

Fig. 2

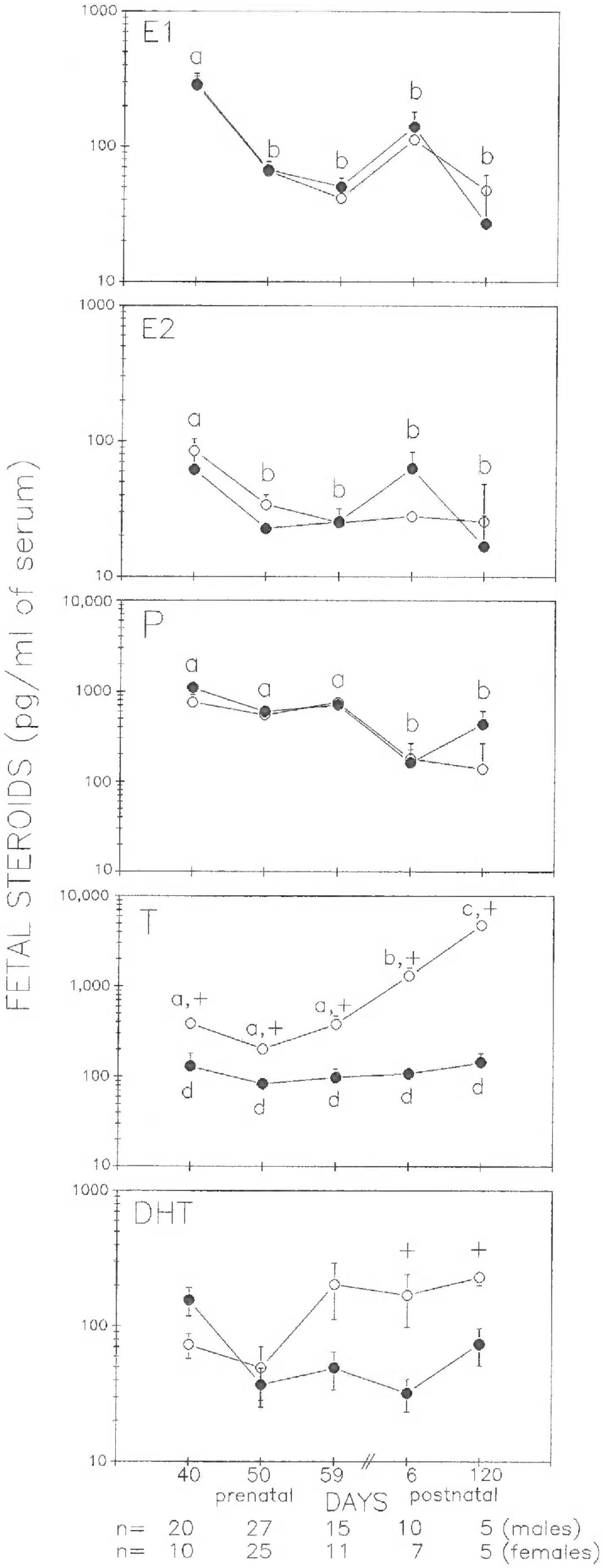
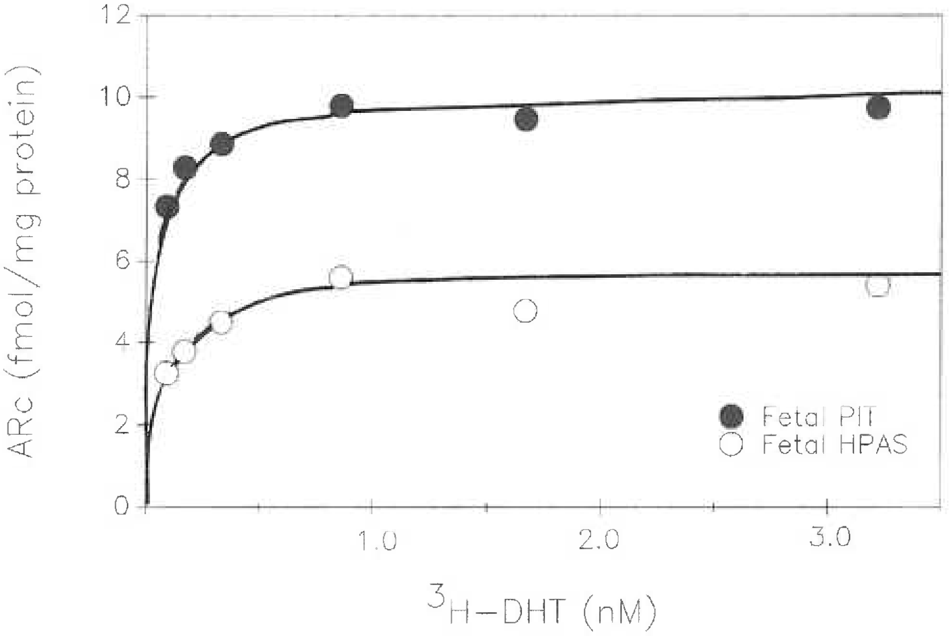

FETAL STEROIDS (pg/ml of serum)
E1
1000
100
10
a
b
b
b
b
E2
1000
100
10
a
b
b
b
b
P
10,000
1000
100
10
a
a
a
b
b
T
10,000
1,000
100
10
a,+
a,+
a,+
b,+
c,+
d
d
d
d
d
DHT
1000
100
10
+
+
40
50
59
6
120
prenatal
DAYS
postnatal
n= 20 27 15 10 5 (males)
n= 10 25 11 7 5 (females)

Figure 3. Saturation curves for the in vitro binding of $[^3H]$-DHT to cytosolic androgen receptors (ARc) purified from pooled fetal guinea pig pituitary (PIT,●) and combined hypothalamus-preoptic area-amygdala-septum (HPAS,o).

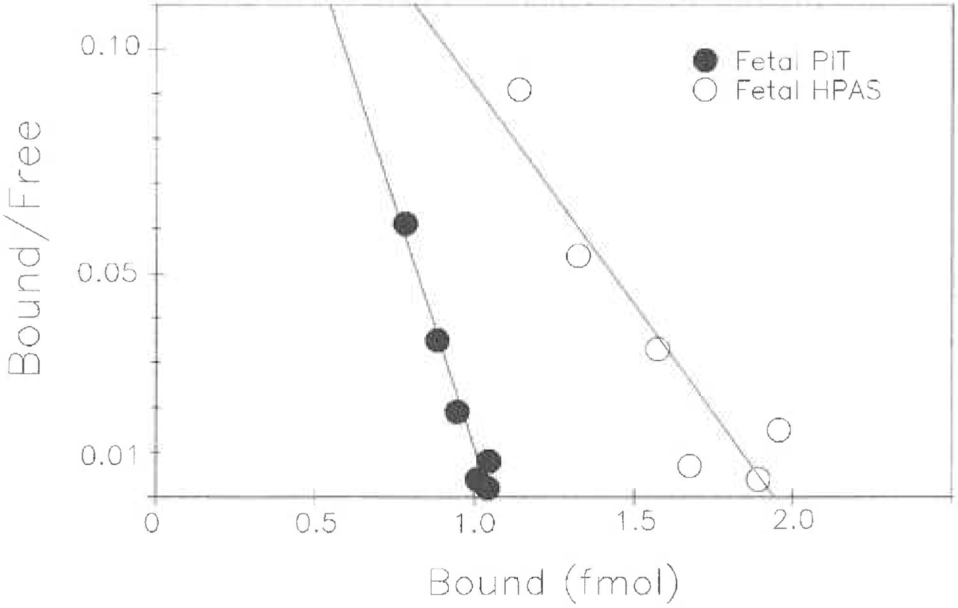
Figure 4. Scatchard analyses of the saturation curves shown in Figure 3.

Fig. 3



ARc (fmol/mg protein)
0
2
4
6
8
10
12
1.0
2.0
3.0
^{3}H–DHT (nM)
● Fetal PIT
○ Fetal HPAS

Fig. 4

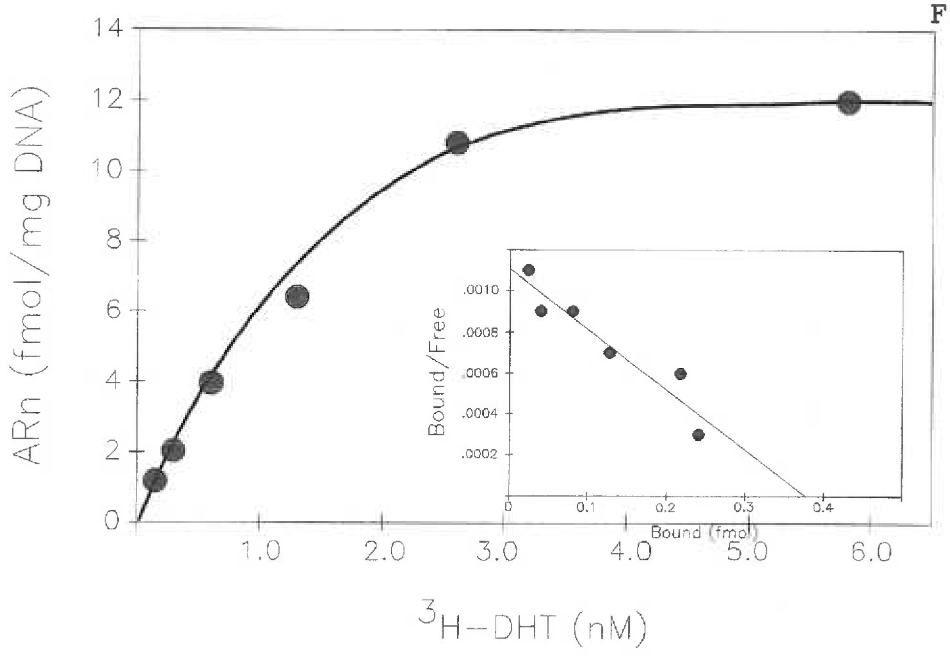


Bound/Free
0.10
0.05
0.01
0
0.5
1.0
1.5
2.0
Bound (fmol)
● Fetal PIT
○ Fetal HPAS

Figure 5. Saturation curve for the in vitro binding of [^{3}H]-DHT to nuclear androgen receptors (ARn) prepared from the pooled HPAS of 6 day old guinea pigs treated with testosterone propionate for four hours. Insert: Scatchard analysis of the data.

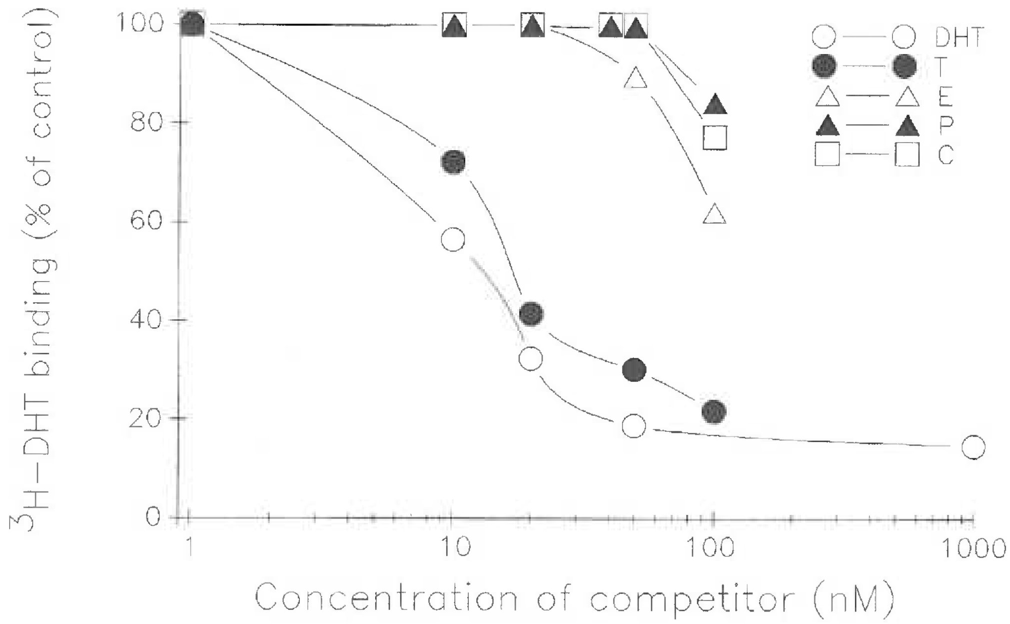
Figure 6. Competition studies showing the relative binding of [^{3}H]-DHT (3.0 nM) to purified cytosol from HPAS of two litters of fetuses at day 60-65 of gestation in the presence of an excess of dihydrotestosterone (DHT), testosterone (T), estradiol (E), progesterone (P), or corticosterone (C).

Fig. 5



14
12
10
8
6
4
2
0
ARn (fmol/mg DNA)
1.0
2.0
3.0
4.0
5.0
6.0
^{3}H--DHT (nM)
Bound/Free
.0010
.0008
.0006
.0004
.0002
0
0.1
0.2
0.3
0.4
Bound (fmol)

Fig. 6



100
80
60
40
20
0
^{3}H–DHT binding (% of control)
1
10
100
1000
Concentration of competitor (nM)
○—○ DHT
●—● T
△—△ E
▲—▲ P
□—□ C

Figure 7. Cytosolic androgen receptors in the preoptic area (POA), septum (SEPT), medial-basal hypothalamus (MBH), amygdala (AMYG), frontal cortex (FRCTX), and anterior pituitary (PIT) on days 40, 50, and 59 of gestation and postpartum days 6 and 120. Each bar represents the mean ± SEM. Means for males (open bars) and females (hatched bars) were not different, so data were combined for analysis. Means with dissimilar letters differ significantly ($p<.05$) from other ages for that tissue. POA and MBH at day 30 were taken as a single block (HPOA) and is shown here in relationship to both POA and MBH at later ages; tissues at day 30 were combined without regard to fetal sex (solid bars).

Fig. 7

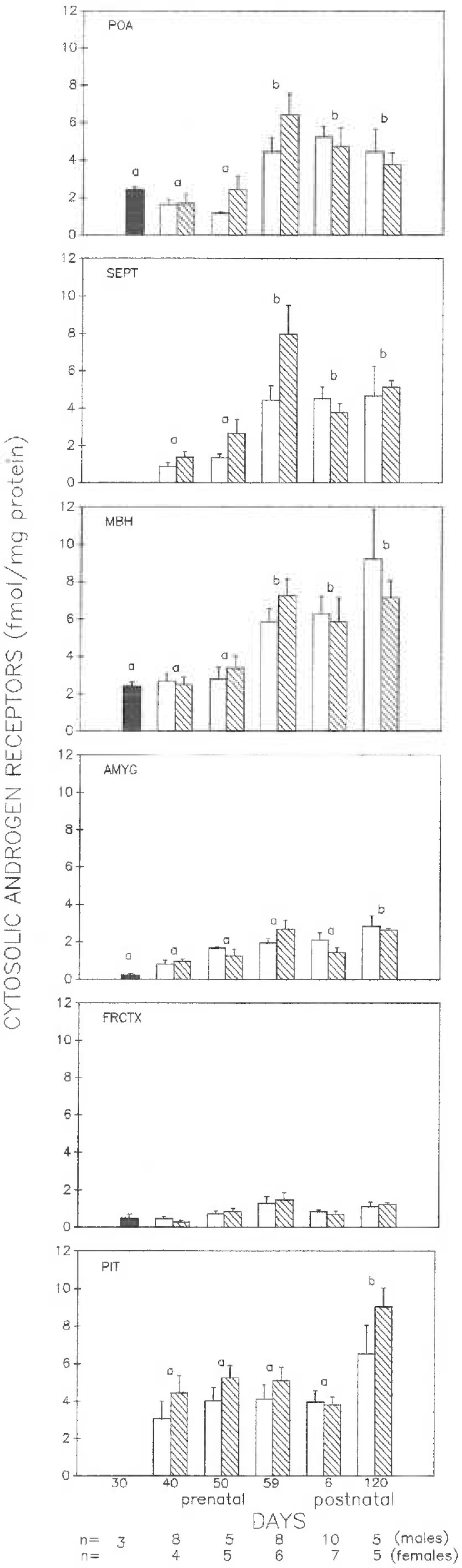
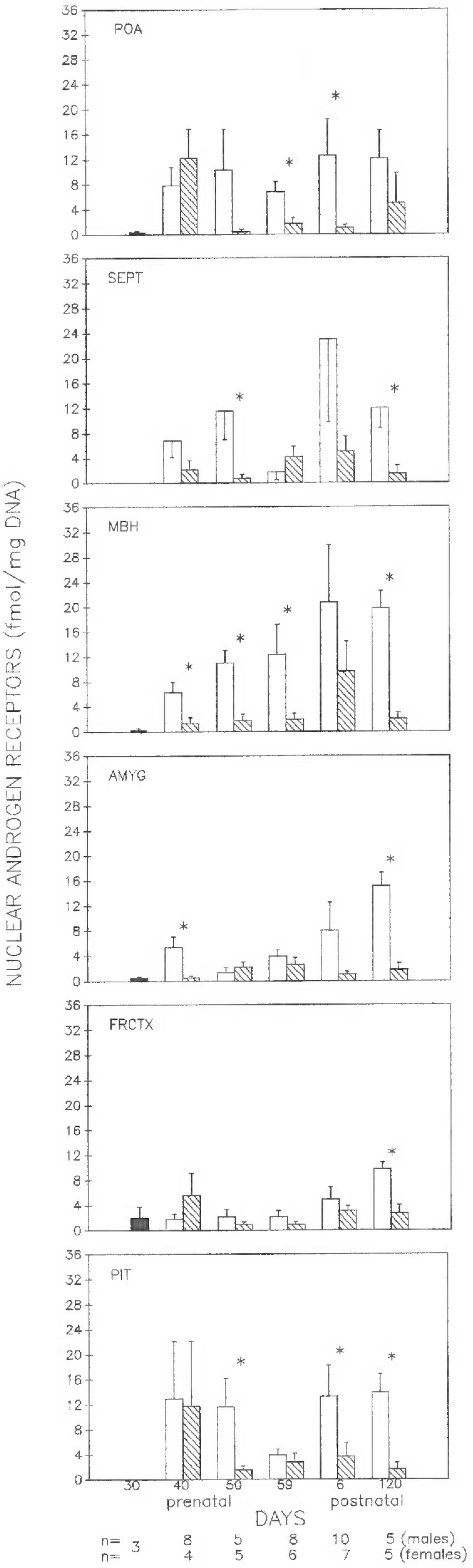

CYTOSOLIC ANDROGEN RECEPTORS (fmol/mg protein)
POA
SEPT
MBH
AMYG
FRCTX
PIT
0
2
4
6
8
10
12
a
b
30
40
50
59
6
120
prenatal
postnatal
DAYS
n= 3 8 5 8 10 5 (males)
n= 4 5 6 7 5 (females)

Figure 8. Nuclear androgen receptors in the POA, SEPT, MBH, AMYG, FRCTX, and PIT on days 40, 50, and 59 of gestation and days 6 and 120 postpartum. Each bar represents mean ± SEM. * indicates means for males (open bars) and females (hatched bars) differ significantly ($p<.05$) at that age. Tissues at day 30 of gestation were prepared as in Figure 7 (solid bars).

Fig. 8


POA
SEPT
MBH
AMYG
FRCTX
PIT
0
4
8
12
16
20
24
28
32
36
*
NUCLEAR ANDROGEN RECEPTORS (fmol/mg DNA)
30
40
50
59
6
120
prenatal
postnatal
DAYS
n= 3 8 5 8 10 5 (males)
n= 4 5 6 7 5 (females)

CHAPTER IV

EFFECTS OF EXOGENOUS STEROIDS ON ANDROGEN RECEPTORS IN FETAL GUINEA PIG BRAIN

ABSTRACT

We treated pregnant guinea pigs (GP) on day 50 of gestation with 10 mg testosterone propionate (TP), obtaining fetuses 2, 4, 8 or 18 h later as well as after 5 days of treatment. In a second group of pregnant GP, dihydrotestosterone propionate (DHTP), estradiol benzoate (E_2B), progesterone (P), or cortisol was given 2 h before obtaining fetuses. Although TP treatment elevated fetal serum T ($p<.05$), brain cytosolic androgen receptor (ARc) content was unchanged in fetuses of either sex. In female fetuses, nuclear androgen receptors (ARn) increased 10-fold in medial-basal hypothalamus (MBH) and preoptic area (POA) at 2 and 4 h (respectively) after treatment while fetal male ARn content was unchanged. Maternal injection of other steroids (E_2B, P or cortisol, but not DHTP) significantly increased these hormones in the fetus 2 h later ($p<.05$). Only androgens affected fetal AR content. While TP increased ARn in female MBH, DHTP decreased ARc in fetal anterior pituitary of both sexes. In this latter case, a metabolite of DHT may mediate the effects.

We conclude that T crosses the GP placenta and activates ARn in POA and MBH of female fetuses; male ARn

appear to be maximally occupied by endogenous T. Steroids of other classes do not induce AR responses in fetal GP brain. These AR changes may represent an initial cellular mechanism in brain sexual differentiation.

INTRODUCTION

Male sexual differentiation of the guinea pig brain, like that of other mammals, is androgen-dependent[36,93,94] and presumably mediated through androgen receptors (AR). Androgenization of the fetal guinea pig brain takes place during a period of maximal susceptibility to androgens between days 30 and 55 of gestation[38,77]. Exposure of female fetuses to androgens during this period results in their exhibition, as adults, of increased male behavior when exposed to testosterone (T) and to decreased lordosis behavior after estrogen-progesterone priming[36,93]. Local aromatization of T to estrogen within the brain is necessary for masculinization in many species including rats and mice[178,179,201]. However, in the guinea pig and primate 5α-dihydrotestosterone (DHT), a non-aromatizable androgen, is adequate to androgenize the female brain so that the adult female will respond to a hormonal challenge with typical male behavior[77,93].

Androgen receptors are present in the fetal guinea pig brain during the "critical period" of sensitivity to prenatal androgens[202]. After tissue fractionation these receptors are found in both the cytosolic (ARc) and nuclear (ARn) fractions. The current model for steroid hormone action places all receptors in the nucleus of the cell; thus the ARc corresponds to unoccupied receptors loosely bound to nuclear components and the ARn corresponds to steroid-occupied receptors tightly bound within the nucleus[203-205]. Testosterone (T) treatment increases ARn in the adult brain, apparently an activational step necessary for hormone action. Androgens interact with DNA through ARn to produce specific androgen effects[206,207]. Little is known about AR dynamics in developing neural tissue. Although receptors capable of binding androgens have been found in the fetal guinea pig brain, little information is available concerning their maturity or functional capacity: i.e., can the ARc found in the fetal brain during the critical period be similarly activated to the nuclear form[208-210]?

In order to examine this question, we investigated the ability of both short-term and long-term treatment with testosterone propionate to activate the ARc and studied the time course of this activation in the brain and anterior pituitary of pregnant guinea pigs and their fetuses. In addition, we evaluated the specificity of activation by investigating the action of different classes of steroid hormones -androgens, estrogens, progestins and corticoids- on AR content and dynamics in the fetal and maternal brain and anterior pituitary.

MATERIALS AND METHODS

Steroids

Testosterone propionate (TP), dihydrotestosterone propionate (DHTP), estradiol benzoate (E_2B), progesterone (P) and cortisol [Steraloids, Inc., Wilton, NH] were dissolved in sesame oil (Sigma Chemical Co., St. Louis, MO) to a final concentration of 20 mg/ml.

Animals

Female Duncan-Hartley guinea pigs (Simonsen, Gilroy, CA) were housed (14 h light:10 h dark) with males overnight on the first day of vaginal opening, which was designated day 0 of gestation. Females that did not open again in 14-18 days were assumed to be pregnant; pregnancy was confirmed by palpation after day 30 of gestation. Nonpregnant females were bred again.

For the time course study, pregnant guinea pigs were killed by decapitation (4-8 h after light on) on day 50 of gestation after being injected subcutaneously (sc) with 0.5 ml of TP solution (10 mg of TP) either 2, 4, 8 or 18 h prior to being killed. A second group of animals was injected daily for five days (days 46-50 of gestation) with 10 mg of TP sc; the last injection was given 2 hours prior to decapitation on day 50. Animals used to test for specificity of hormone action were injected with 10 mg sc of either dihydrotestosterone propionate (DHTP), estradiol benzoate (E_2B), progesterone (P), cortisol or sesame oil alone 2 h before decapitation on day 50.

Tissue Preparation

Brain tissues, pituitaries, and trunk blood were collected and brain dissections carried out as reported previously[202]. The anterior pituitary and five brain areas: the medial-basal hypothalamus (MBH), preoptic area (POA), amygdala (AMYG), septum (SEPT) and frontal cortex (FRCTX) were analyzed for androgen receptor (AR) content. When two fetuses of the same sex were available in a given treatment group, their tissues were combined. All procedures were carried out on ice.

Dissected tissues were homogenized and the cytosolic and nuclear fractions were initially separated by centrifugation at 1000 x g. The cytosolic fraction was purified further by ultracentrifugation at 106,000 x g. The pellet from the low speed centrifugation was washed by suspension in a low sucrose (0.32 M) buffer and centrifuged at 1000 x g. The pellet was then resuspended in a Cellex 410 (Bio-Rad Labs., Richmond, CA) solution (120 mg/500 μl) and a high sucrose buffer (2.4 M) was added. Centrifugation at 55,000 x g yielded a purified nuclear pellet. Nuclear AR was extracted from the purified nuclear pellet by first resuspending it in 55 μl of TEBD buffer (10 mM Tris, 1.5 mM EDTA, 0.5 mM bacitracin, 1 mM dithiothreitol; pH 7.4) for 5 min and then adding 55 μl TEBDK buffer (TEBD plus 1.6 M KCl; pH 7.4) to achieve a final salt concentration of 0.8 M. This solution was centrifuged at 37,000 x g after a total incubation time of 30 minutes. The supernatants from the salt extraction were used to measure nuclear androgen receptors (ARn) and the pellets were frozen until they were assayed for DNA by the method of Burton[184] using the modifications of Giles and Myers[185].

Receptor Assays

Methods for estimating ARc and ARn in the adult[154] and fetal[202] guinea pig have been validated in our laboratory. Androgen receptors were quantitated by the _in vitro_ binding of [1,2,4,5,6,7-^{3}H]-dihydrotestosterone ([^{3}H]DHT; New England Nuclear, [NEN] Boston, MA); its high specific activity (148.1 Ci/mMol) made this the ligand of choice. One hundred microliter aliquots of either purified cytosolic or nuclear solutions were incubated with 3.2 nM (ARc) or 5.6 nM (ARn) [^{3}H]DHT for 20 h at 0-4°C. Nonspecific binding was determined by incubating a duplicate set of tubes with the addition of a 200-fold molar excess of radioinert R1881 (methyltrienelone, NEN). Bound and free radioactivity were separated by passage through a Sephadex LH20 mini-column[182]. Specific binding was that remaining after subtraction of nonspecific binding from total binding. Bound radioactivity was counted in a Packard Tricarb scintillation spectrometer model 640 (United Technologies Packard, Downers Grove, IL) with 40% efficiency and a counting error of 5%. AR were quantitated as fmol/mg protein for ARc and fmol/mg DNA for ARn. Protein concentrations in cytosolic fractions were determined by the method of Lowry[183].

Steroid RIA

Serum concentrations of T, DHT, estrone (E_1), estradiol (E_2), P, cortisol and androstenedione (Δ_4) were determined by methods described previously[181]. The intra- and interassay coefficients of variation (percent) were: E_1, 8.2 and 16.5; E_2, 6.3 and 13.7; P, 8.2 and 14.2; T, 10.5 and 12.4; DHT, 10.8 and 9.4; cortisol, 4.0 and 9.0; Δ_4,11.7 and 16.3, respectively.

Serum steroid concentrations for untreated and oil treated guinea pigs on day 50 of gestation (maternal and fetal) did not differ significantly by an analysis of variance so these values were combined. Cortisol and Δ_4 were not measured in untreated control animals.

Statistics

Maternal serum steroid concentrations and AR content were analyzed by one-way analysis of variance (ANOVA)[188]. If a significant F value was obtained, post hoc comparisons were made using the Dunnett's Multiple Range test[211]. Fetal male and female controls were first compared by Student's T test. If the control values were different, then one-way ANOVA was performed on male and female data separately, followed by a Dunnett's test when applicable. If male and female controls were not different, then data were analyzed by two-way ANOVA, with sex and treatment as variables. When there were no apparent sex differences after the two-way ANOVA, data for males and females were combined and analyzed using one-way ANOVA and Dunnett's Multiple Range test when appropriate. If the two-way ANOVA indicated a sex difference, one-way ANOVA was performed on male and female data separately for any treatment. When variances were found to be unequal by the F-Maximum Test for Homogeneity of Variances[211], data were log transformed prior to analysis.

RESULTS

TIME COURSE FOR TP ACTIVATION OF THE ANDROGEN RECEPTOR

Steroid Hormones In Sera

Maternal Serum Concentrations

Changes in systemic serum steroid hormone concentrations of pregnant guinea pigs treated with TP are shown in Figure 9. Progesterone and E_1 concentrations did not change after treatment (Fig. 20). Significant changes in E_2 were detected by one-way ANOVA ($F[5,35]=2.75$; $p<.05$), but the *post hoc* tests between times after treatment were not significant. All of the androgens measured (T, DHT, Δ_4)

were significantly elevated in the systemic circulation of the mother after TP treatment (p<01).

Fetal Serum Concentrations

In the fetus, TP treatment had no effect on serum levels of E_1, P (Fig. 21) or E_2 (Fig. 10); androgen levels in the fetus, however, were significantly increased by TP administration (Fig. 10). Control values of T and Δ_4 were different for males and females, so the data for each sex were analyzed separately. Since no sex differences were observed by a t-test for the other hormones, data from males and females were combined. Serum T concentrations increased at 2 h (p<.01), 8 h (p<.05), or after 5 days (p<.01) of TP treatment in the male, and at 2 h (p<.01) after TP treatment in the female; Δ_4 in the fetus was increased at 2 h (p<.05), 8 h and 5 days (p<.01) in the male and at 2 h (p<.01) and after 5 days (p<.05) in the female. Fetal DHT was elevated at the 2, 4 and 8 hour periods and after 5 days of daily TP injection (p<.01).

ANDROGEN RECEPTORS

Maternal Tissues

TP injections had no effect on maternal levels of ARc in POA (Fig. 11), SEPT, or FRCTX (Fig. 22). The AMYG, however, showed a significant decrease in ARc at 2 h and after 5 days of treatment (p<.05, Fig. 22). The most dramatic changes occurred in the MBH and PIT where ARc levels were depressed at all time periods except for 18 h after TP injection ($p<.05$, Fig. 11).

Nuclear AR content also varied in response to TP, except in AMYG and FRCTX (Fig. 22). In SEPT, one-way ANOVA indicated an effect of treatment ($F[5,27]=3.17$; $p<.02$), but significant effects were not demonstrated by the Multiple Range Test (Fig. 22). Preoptic area ARn levels were elevated at 2, 4 and 8 h after treatment ($p<0.05$) and at 18 h and after 5 days of treatment ($p<0.01$). In the MBH, TP treatment produced elevated amounts of ARn but only at the 4 hour ($p<.05$) and 5 day periods ($p<.01$). Nuclear AR levels in PIT were elevated at all times ($p<0.05$ at 4 and 18 h and $p<0.01$ at 8 h and 5 days) except the 2 hour period.

Fetal Tissues

The effects of maternal TP injection on brain ARc content are shown in Figure 12. Although other brain areas (SEPT, FRCTX and AMYG) were analyzed, no effect of treatment was demonstrated (Fig. 23). One-way ANOVA indicated a significant effect of TP treatment on ARc only in the fetal female MBH ($F[5,32]=2.72$; $p<.05$) (Fig. 12), but specific changes were not shown by the Multiple Range Test.

There were also no effects of TP on ARn in the fetal male (Fig. 12). In female fetuses there were significant increases in ARn in the POA at 2 ($p<.05$), 4 and 18 h ($p<.01$)

and also in the MBH at 2 h ($p<.01$), 4 h and after 5 days of treatment ($p<.05$, Fig. 12). No changes in ARn content of fetal AMYG, SEPT or FRCTX were observed (Fig. 23).

SPECIFICITY OF STEROID HORMONES IN AR ACTIVATION

STEROID HORMONES IN SERA

Maternal

Two hours after s.c. injection of the hormones used in this study (TP, DHTP, E2B, P or cortisol) the quantities of these hormones were significantly elevated over control values in the maternal sera, ($p<.001$ for all treatment groups, Fig. 13). Data for TP-treated animals were taken from the 2 h values reported in the previous section and were not repeated in a separate experiment. The selection of a two hour time period to test the specificity of steroid binding was based on these data. After TP treatment, DHT ($p<.05$) as well as T and Δ_4 ($p<.01$; Fig. 13 and Table 1) levels were elevated in the maternal circulation compared to controls. Estradiol benzoate administration raised serum levels of both E_2 and E_1 ($p<.01$; Fig. 13 and Table 1).

Fetal

Similarly, serum concentrations of T, E_2, P and cortisol were elevated in fetuses obtained from mothers injected with that particular hormone ($p<.001$ for all treatment groups, Fig. 14). Maternal injection of DHTP, however, had no effect on fetal serum hormones. Control values of Δ_4 (Table 1) and T (Fig. 14) were greater in males than in females, so data for these hormones were analyzed separately for each sex. Concentrations of other hormones in the systemic circulation of the fetuses analyzed by a two-way ANOVA (sex and treatment as variables) revealed sex differences only between the concentrations of progesterone, which were approximately twice as high in females as compared to males (Fig. 14); therefore, data for males and females were combined (except for progesterone) and analyzed by a one-way ANOVA. Unexpectedly, TP treatment of the mother produced significant elevations in fetal cortisol as well as fetal androgens (T, DHT, and Δ_4) ($P<.01$ for all treatment groups). Estradiol benzoate treatment resulted in significant elevations in both E_2 and E_1 ($p<.01$ for both) in the systemic circulation of the fetus (Fig. 14 and Table 1). In fetuses whose mothers were injected with P, the levels of P in female serum were significantly greater than in any other group ($p<.001$); however, P levels in male fetuses treated with P were greater than all other groups except for cortisol-treated female fetuses ($p<.05$). Also, maternal P injection led to increased Δ_4 in male fetuses ($p<.05$; see Table 1).

EFFECTS OF DIFFERENT CLASSES OF STEROIDS ON AR DYNAMICS

Maternal Tissues

Cytosolic AR content in MBH and PIT of pregnant guinea pigs treated with TP or DHTP two hours before sacrifice was significantly lower than in tissues from control animals ($p<.05$, Fig. 15). Treatment with other classes of steroids had no effect on maternal ARc. Although a significant F value ($F[5,28]=4.30$, $p<.001$) was obtained by a one-way ANOVA for ARc in maternal SEPT (Fig. 15) and AMYG ($F[5,28]=2.72$, $p<.05$, Fig. 24), there were no specific differences found by the Multiple Range Test.

Treatment of pregnant animals with TP for 2 h increased ARn in maternal POA, MBH and PIT ($p<.01$ for all treatment groups), but DHTP treatment was effective only in maternal SEPT ($p<.01$, Fig. 15).

Fetal Tissues

In fetal tissues no sex differences were observed in ARc content, therefore, data for males and females were combined for statistical analysis. No significant effects of maternal steroid treatment on ARc content in the fetal brain were found. In the fetal PIT, however, maternal DHTP treatment resulted in a significant decrease in ARc compared to controls ($p<.001$, Fig. 16).

In fetal tissues sex differences in control values of ARn were observed in the POA, SEPT, MBH and AMYG (Fig. 16

and Fig. 25); data for males and females were combined in FRCTX and PIT for statistical analysis. Treatment effects on ARn content were found only in the MBH. TP administered to the mother resulted in a significant elevation in ARn content in the MBH of the female fetus two hours after maternal treatment ($p<.01$, Fig. 16).

DISCUSSION

We demonstrated that steroids of various classes administered to pregnant guinea pigs cross the placenta and are found in the fetal circulation within two hours. Only androgens administered in this fashion affect androgen receptor dynamics in maternal and fetal brains.

Testosterone propionate administration to pregnant guinea pigs results in increases in serum T and its active metabolite DHT in both maternal and fetal circulation within 2 h. Resko and workers[104] found that T reached maximum amounts in plasma of castrated adult guinea pigs one h after injection but two h samples were not analyzed. In these animals T concentrated in hypothalamic and pituitary tissues providing early evidence for the presence of AR in these tissues.

All of the steroids that we administered (except DHT) reached the circulation of the fetus in a non-metabolized form after injection into the mother. Although maternal injection experiments during fetal development have been performed for many years[36,38,45], these data finally describe the amount of systemic androgen that reaches the developing anlagen of the central nervous system and tissues of the reproductive system in this species. Elevated concentrations of Δ_4 and E_1 in the fetal and maternal circulation after injecting T and E_2 into the mother indicate that 17ß-hydroxysteroid dehydrogenases (17ß-HSD) are active in converting T and E_2 to less effective compounds.

Maternal injection with P leads to an unequal increase in the levels of P in the male and female fetal circulation; male P levels approximately double over control while female levels are approximately four times higher and also significantly greater than the levels seen in the male. There appears to be a fundamental difference in either the manner or rate in which male and female fetuses and/or their placentas metabolize P. Secondly, male fetuses exposed to exogenous P <u>in</u> <u>utero</u> have elevated serum Δ_4 concentrations in their systemic circulation. This latter observation suggests that P stimulates 17ß-HSD activity in the placenta and/or the fetus. The difference in activity between males and females compared to their control, could reside in the greater quantities of substrate (T) that are available for conversion in males compared to females. The 17ß-HSD's are controlled by P in the human uterus and vary with the stages of the ovarian cycle[212]. Similar observations have been made on uteri[213] and pituitaries[214] of adult rhesus monkeys. The fetal monkey liver is also highly active[215].

Interestingly, there is a significant increase in fetal cortisol in both males and females after TP administration. In rats, maternal prenatal stress elevates plasma T in the fetal male on day 17 of gestation but inhibits its production on day 18 of gestation[216]. Male offspring of these pregnancies display a lower level of masculine behavior in adulthood compared to males born to unstressed females[217]. Our data indicate that the elevations in fetal testosterone are coupled to elevated levels of fetal cortisol in the guinea pig. The mechanism whereby these two secretory events are coupled has yet to be determined.

Despite the high levels of androgen in the circulation, no significant elevations of serum estrogen were found in sera of either the mother or the fetus. These data support the proposal that estrogens formed locally, rather than those of systemic origins, may be responsible for masculinization of the rodent brain[178]. Even though no sex differences in aromatase enzyme activity are found in fetal guinea pig brain[218], in situ estrogen production could be regulated by the availability of substrate T, for which there is a sex difference. However, while elevated levels of T enhance aromatase activity in adult rat and guinea pig brain[219,220], this does not seem to be the case for the fetal guinea pig[218]. Hines and workers[92], using an anti-estrogen, tamoxifen, demonstrated estrogen effects on fetal masculinization and defeminization of GP sexual behavior; however, they observed no changes in lordosis behavior or anatomy of the sexually dimorphic nucleus of the POA in the adult female. In earlier work, Hines and Goy[91] were unable to demonstrate any direct effects of fetal E_2B treatment on adult female sexual behavior although the synthetic estrogen, diethylstilbestrol (DES), was capable of masculinizing and defeminizing the GP fetus.

The role of aromatization of T versus its 5α-reduction in sexual development of the guinea pig is gradually unfolding. Inhibition of 5α-reductase activity in the developing GP fetus prevents normal development of the external genitalia but affects neither adult male sexual behavior of treated animals[90] nor patterns of gonadotropin secretion in response to exogenous estrogen treatment in adult males. The principal effect of this treatment occurred in females in which hypothalamic centers that control LH release seemed to be more sensitive to estrogen, i.e. "more feminized" when 5α-reductase activity is blocked in fetal life[90].

Previous data from our laboratory documented the presence of AR in pregnant females and fetuses of both sexes[202]. Testosterone administration to pregnant guinea pigs leads to a decrease in ARc and an increase in ARn within two hours in the maternal POA, MBH and PIT. In the adult rat, T treatment elevates ARn in the HPAS (combined hypothalamus, preoptic area, amygdala and septum) within thirty minutes[221].

Two hours after maternal TP injection, ARn increases in fetal POA and MBH of females only. These same areas appear to mediate the control of reproductive behavior in the adult animal[86,192]. The level of ARn found in the female brain after TP treatment approximates the level found in control male fetuses. The inability of elevated T levels in the male to affect the quantity of brain ARn indicates that these AR are already maximally activated by endogenous levels of T.

Another hormonal variable that may be important for understanding androgen action in sexual differentiation is P. In intact adult male GP, exogenous P blocks androgen-induced reproductive behaviors and lowers ARn in the POA and MBH[154]. These effects of P seem to occur through a P receptor-mediated mechanism rather then by competition of P with androgen for the AR[155]. The apparent inconsistencies that we found in the capacity of T and DHT to activate AR may be due to the high levels of endogenous P that are found in both the maternal and fetal circulation during development. We know little about the distribution of P receptors in various brain areas of the fetus, knowledge of which may be important for understanding potential interactions between endogenous progestins and androgens.

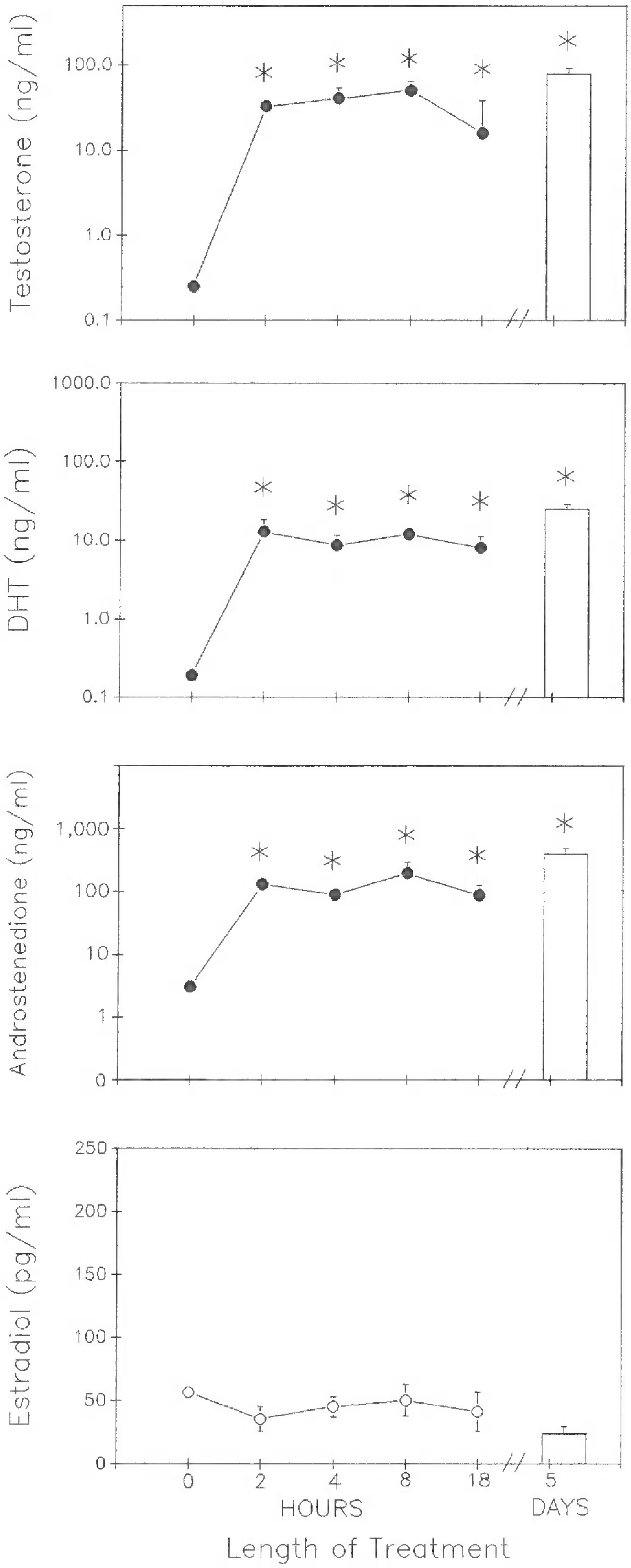
The results from pregnant animals treated with DHTP are inconsistent. Only in the maternal SEPT were elevated levels of DHT associated with elevated amounts of ARn. No effects of DHT were observed on the distribution of AR in the fetal brain, but DHT did affect ARc in the fetal pituitary gland. However, maternal injection of DHTP did not raise fetal DHT levels significantly above control values at two hours. Also, since we did not study the time course for the appearance of DHT in fetal serum, two hours may not represent the ideal time to study DHT effects on fetal AR. Alternatively, even though DHT treatment of the mother did not elevate DHT concentrations in fetal sera, the biological effect that was observed in the pituitary may be mediated by an active metabolite and not by DHT per se.

It has been proposed that more than one type of AR with different affinities for T and DHT are found in the brain[155,189], and that these hormones play different roles in differentiation of the male reproductive tract[76,190]. Whether or not these proposed differences in AR affect the results that we observe or, indeed, if they exist in a species such as the guinea pig has yet to be determined.

In conclusion, we have described the changes in serum steroids and AR content of selected brain areas and PIT of pregnant guinea pigs and their fetuses at 50 days gestation after injection of androgens and representatives of other classes of steroid hormones. We conclude that TP administered to pregnant guinea pigs crosses the placenta and activates ARn in POA and MBH of female fetuses. Nuclear AR content in males is not changed due to maximum activation by endogenous T. Activation of AR is specific to androgens and not affected by other classes of steroid hormones. The competence of fetal AR to respond to androgens during the critical period and the fact that male AR are activated during this period provides further evidence that AR are involved in the cellular mechanism regulating androgen-mediated sexual differentiation of the fetal guinea pig brain.

Fig. 9. Steroid concentrations in sera from pregnant guinea pigs injected with TP either for 0, 2, 4, 8 or 18 hours (points) or daily for 5 days (bars) before decapitation (number of animals per group was 22, 4, 6, 3, 3, and 5 respectively). Points and bars represent means ± SEM. Plus (+) and asterisk (*) indicate means differ from control ($p<.05$ and $p<.01$, respectively).

Fig. 9



Testosterone (ng/ml)
100.0
10.0
1.0
0.1
*
DHT (ng/ml)
1000.0
100.0
10.0
1.0
0.1
Androstenedione (ng/ml)
1,000
100
10
1
0
Estradiol (pg/ml)
250
200
150
100
50
0
0
2
4
8
18
5
HOURS
DAYS
Length of Treatment

Fig. 10. Steroid concentrations in sera from fetal guinea pigs after injection of the mother with TP for either 0, 2, 4, 8 or 18 hours (points) or daily for 5 days (bars) before decapitation (number of samples per group at 0, 2, 4, 8, 18 h and 5 days was: [male] 39, 9, 7, 6, 6, 6 and [female] 35, 10, 14, 6, 5, 10). Points and bars represent means ± SEM. Open circles and bars represent male values while solid circles and bars represent female values (DHT and estradiol data for males and females were combined for STATISTICS, whereas testosterone and androstenedione data for males and females were analyzed separately; see text). Plus (+) and asterisk (*) indicate combined means (male and female) differ from control for DHT and male values differ from control for testosterone and androstenedione ($p<.05$ and $p<.01$, respectively). Double plus (++) or asterisk (**) denotes the same comparisons as mentioned above for female fetuses.

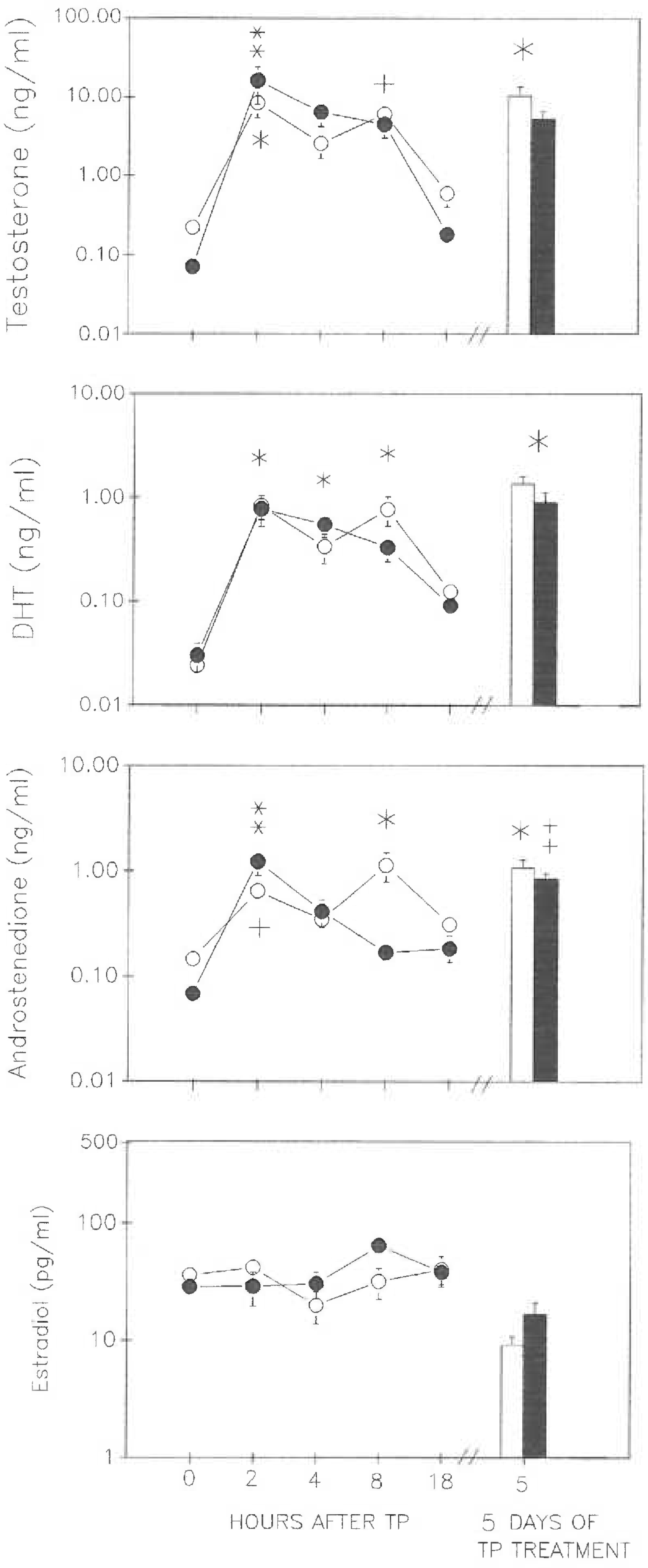

Testosterone (ng/ml)
100.00
10.00
1.00
0.10
0.01
DHT (ng/ml)
10.00
1.00
0.10
0.01
Androstenedione (ng/ml)
10.00
1.00
0.10
0.01
Estradiol (pg/ml)
500
100
10
1
0
2
4
8
18
5
HOURS AFTER TP
5 DAYS OF
TP TREATMENT

Fig. 10

Fig. 11. Cytosolic (ARc) and nuclear (ARn) androgen receptor content in various brain areas [preoptic area (POA), medial basal hypothalamus (MBH) and anterior pituitary (PIT) of pregnant guinea pigs injected with 10 mg of TP for 2, 4, 8, or 18 hours (points) or injected daily for 5 days (bars) before decapitation. Points and bars indicate means ± SEM. Plus (+) and asterisk (*) indicate means differ from control ($p<.05$ and $p<.01$, respectively). Number of samples per group at 0, 2, 4, 8, 18 hrs and 5 days was 12, 5, 5, 3, 3 and 5 animals, respectively.

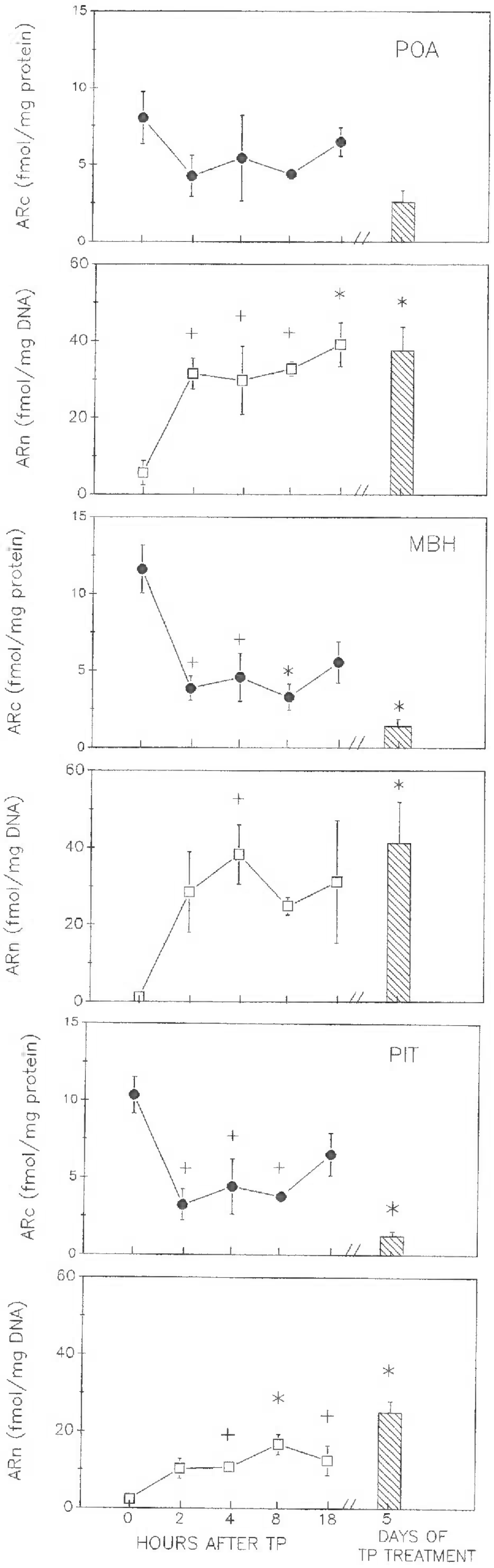

POA
ARc (fmol/mg protein)
ARn (fmol/mg DNA)
MBH
ARc (fmol/mg protein)
ARn (fmol/mg DNA)
PIT
ARc (fmol/mg protein)
ARn (fmol/mg DNA)
0
5
10
15
20
40
60
+
*
0
2
4
8
18
5
HOURS AFTER TP
DAYS OF
TP TREATMENT

Fig. 11

Fig. 12. Cytosolic (ARc) and nuclear (ARn) androgen receptor content in various brain areas (see Figure 11 legend for abbreviations) of fetal guinea pigs (50 ± 2 days gestation) whose mothers were injected with 10 mg of TP for 2, 4, 8 or 18 hours (points) or daily for 5 days (bars) before decapitation. Closed circles, open squares and cross-hatched bars indicate means ± SEM. Plus (+) and asterisk (*) indicate means differ significantly from control ($p<.05$ and $p<.01$, respectively). The number of samples per group at 0, 2, 4, 8, 18 hr and 5 days was: (male, ♂) 11, 4, 6, 4, 3 and 4 and (female, ♀) 11, 7, 8, 3, 3 and 6 respectively.

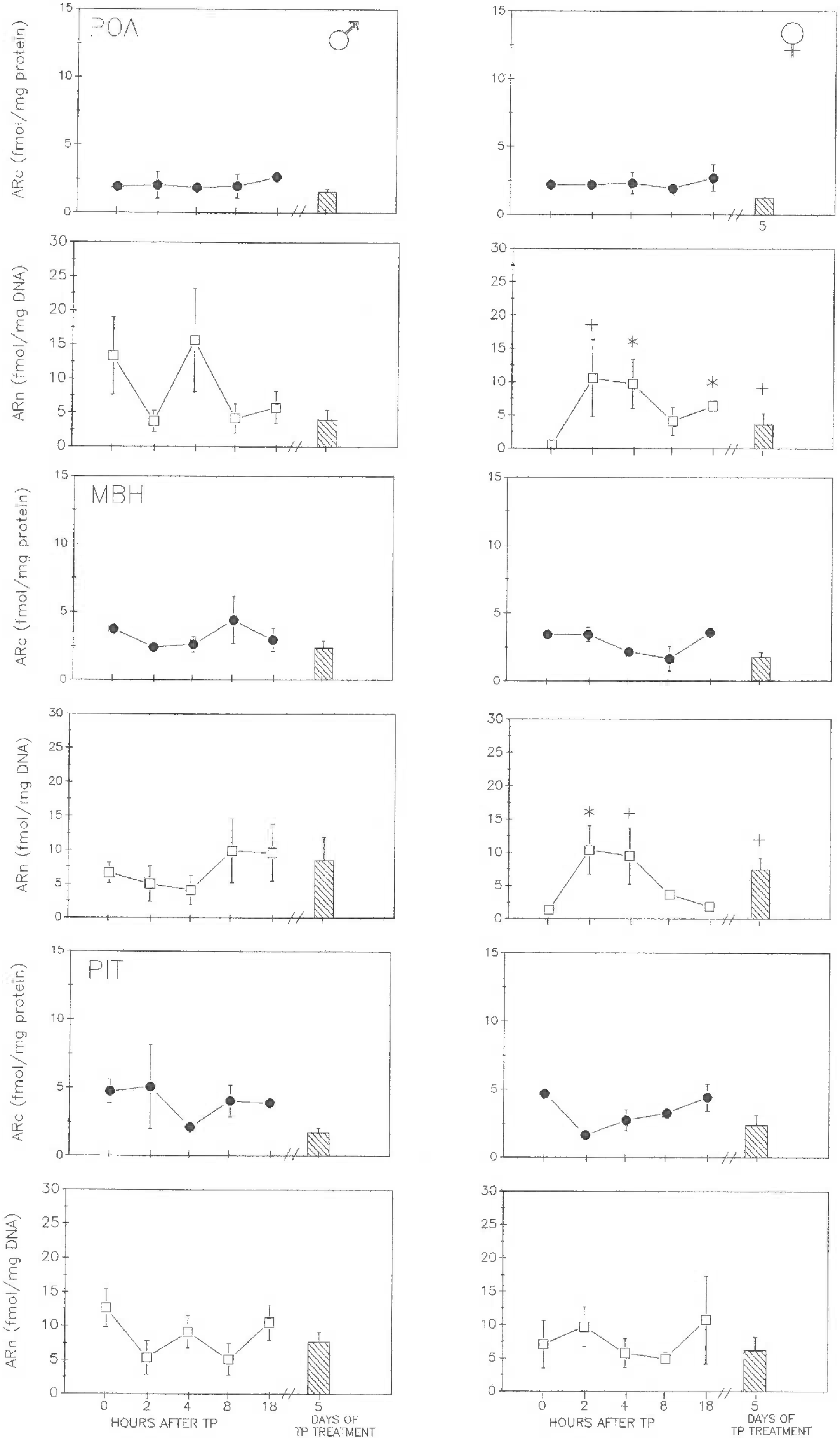

POA
♂
♀
MBH
PIT
ARc (fmol/mg protein)
ARn (fmol/mg DNA)
0
5
10
15
20
25
30
*
+
0
2
4
8
18
5
HOURS AFTER TP
DAYS OF
TP TREATMENT

Fig. 12

Fig. 13. Steroid hormone concentrations in the systemic circulation of pregnant guinea pigs (50 ± 2 days of gestation). Data are presented as mean ± SEM. Asterisk (*) and plus (+) indicate means differ significantly ($p<.01$ and $p<.05$, respectively) from control values (combined untreated and oil treated pregnant guinea pigs). Treatment regimens are identified by abbreviations on the abscissa. Testosterone propionate, dihydrotestosterone propionate, estradiol-benzoate, progesterone and cortisol are abbreviated T, DHT, E, P, and F, respectively. See Methods section for details. N indicates the number of animals in each group.

Fig. 13

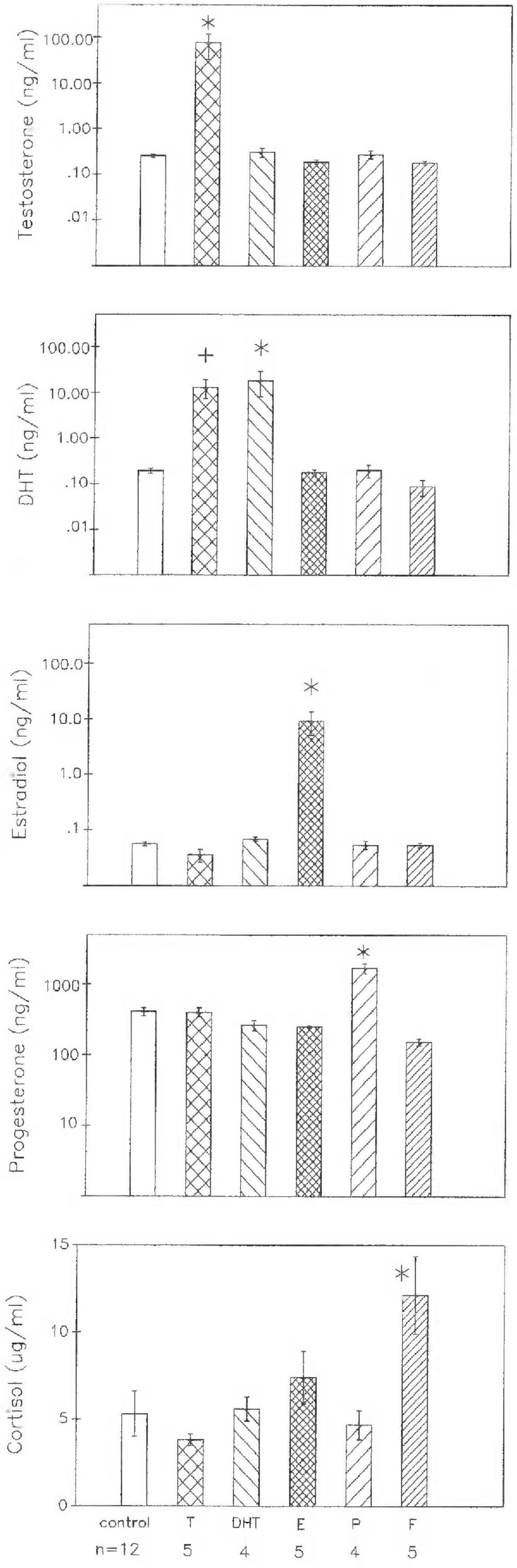

Testosterone (ng/ml)
100.00
10.00
1.00
.10
.01
*
DHT (ng/ml)
100.00
10.00
1.00
.10
.01
+
*
Estradiol (ng/ml)
100.0
10.0
1.0
.1
*
Progesterone (ng/ml)
1000
100
10
*
Cortisol (ug/ml)
15
10
5
0
*
control
T
DHT
E
P
F
n=12
5
4
5
4
5

Fig. 14. Steroid concentrations in sera of fetuses from pregnant guinea pigs injected with exogenous steroids. (See Fig. 13 legend for abbreviations). Open bars represent male fetuses and shaded bars represent females. (Data for males and females, except for T and P, were combined for statistics; see text). For these data asterisk (*) indicates combined means are different from control ($p<.01$). A single (*) and double (**) asterisk in panels that display the T and P data indicate that male and female values differ significantly from control male and female values, respectively ($p<.01$). A plus (+) indicates a $p<.05$ level of significance. Treatment regimens are indicated on the abscissa; see Methods section for details. N indicates the number of fetuses in each group.

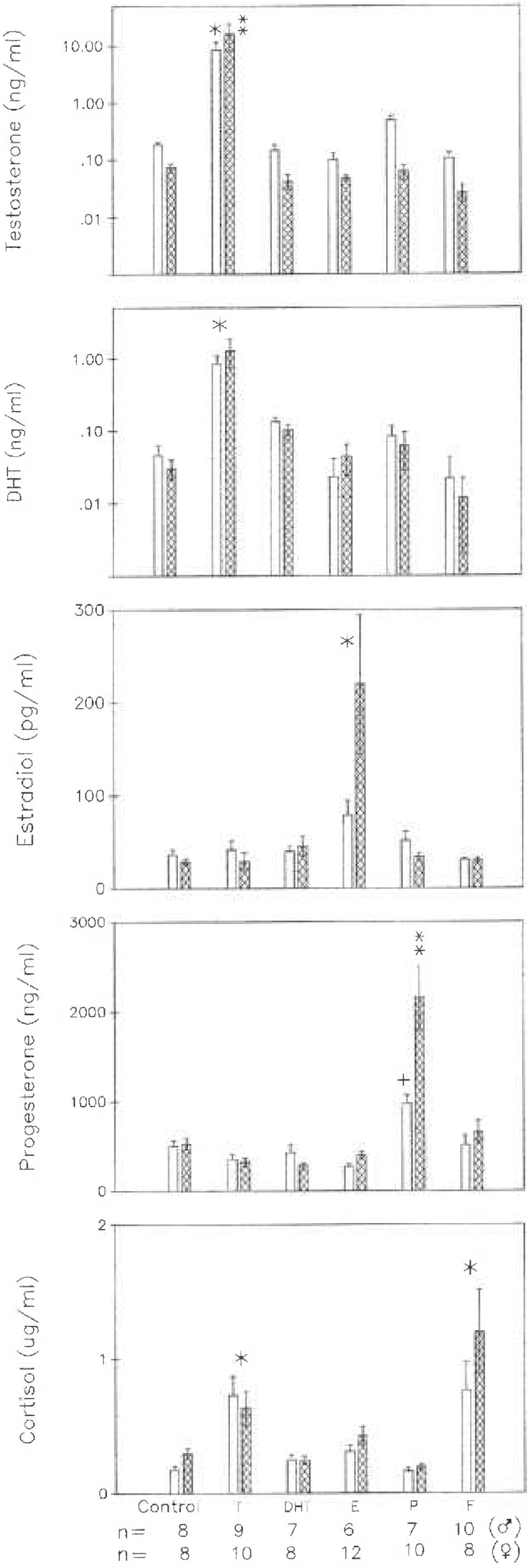
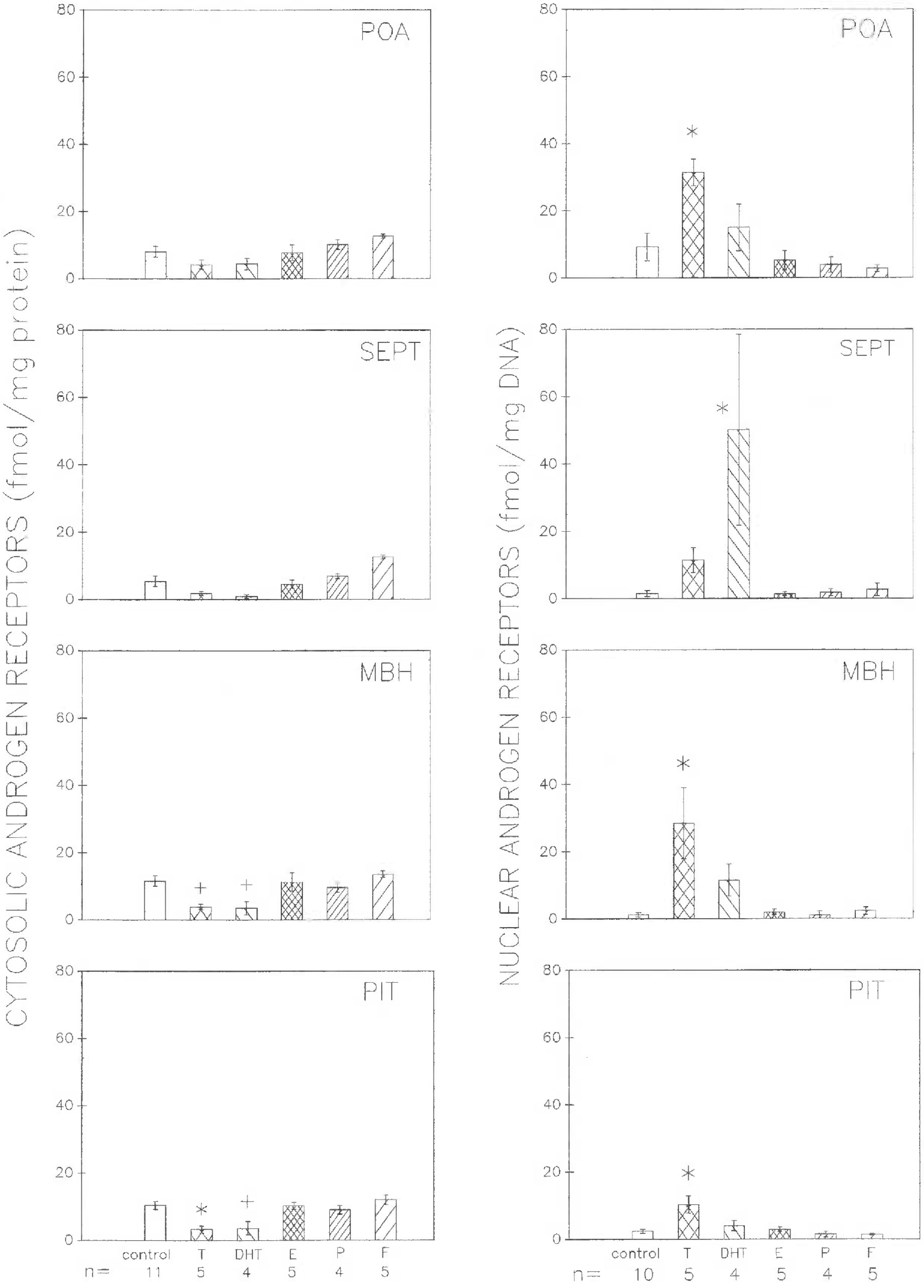

Testosterone (ng/ml)
10.00
1.00
.10
.01
DHT (ng/ml)
1.00
.10
.01
Estradiol (pg/ml)
300
200
100
0
Progesterone (ng/ml)
3000
2000
1000
0
Cortisol (ug/ml)
2
1
0
Control
T
DHT
E
P
F
n= 8 9 7 6 7 10 (♂)
n= 8 10 8 12 10 8 (♀)

Fig. 14

Fig. 15. Cytosolic and nuclear androgen receptors in the preoptic area (POA), septum (SEPT), medial-basal hypothalamus (MBH) and anterior pituitary (PIT) of pregnant guinea pigs injected on day 50 of gestation with exogenous steroids identified on the abscissa (see Fig. 13 legend for abbreviations). Bars represent mean ± SEM. Asterisk (*) and plus (+) indicate means are different from control ($p<.01$ and $p<.05$, respectively). See Methods section for details. N indicates the number of determinations in each group.

Fig. 15



CYTOSOLIC ANDROGEN RECEPTORS (fmol/mg protein)
NUCLEAR ANDROGEN RECEPTORS (fmol/mg DNA)
POA
SEPT
MBH
PIT
0
20
40
60
80
*
+
control
T
DHT
E
P
F
n=
11
5
4
5
4
5
10
5
4
5
4
5

Fig. 16. Cytosolic and nuclear androgen receptors in the preoptic area (POA), medial-basal hypothalamus (MBH) and anterior pituitary (PIT) of fetal guinea pigs whose mothers were injected with testosterone propionate (T), dihydrotestosterone propionate (DHT), estradiol benzoate (E), progesterone (P), or cortisol (F) on day 50 of gestation. Bars represent means ± SEM. Asterisk (*) and (plus +) indicate means for females differ from control females ($p<.05$ and $p<0.01$) respectively. Open bars represent males and shaded bars represent females. Treatment regimens are identified by abbreviations on the abscissa; see Methods section for details. N indicates the number of determinations in each group.

Fig. 16

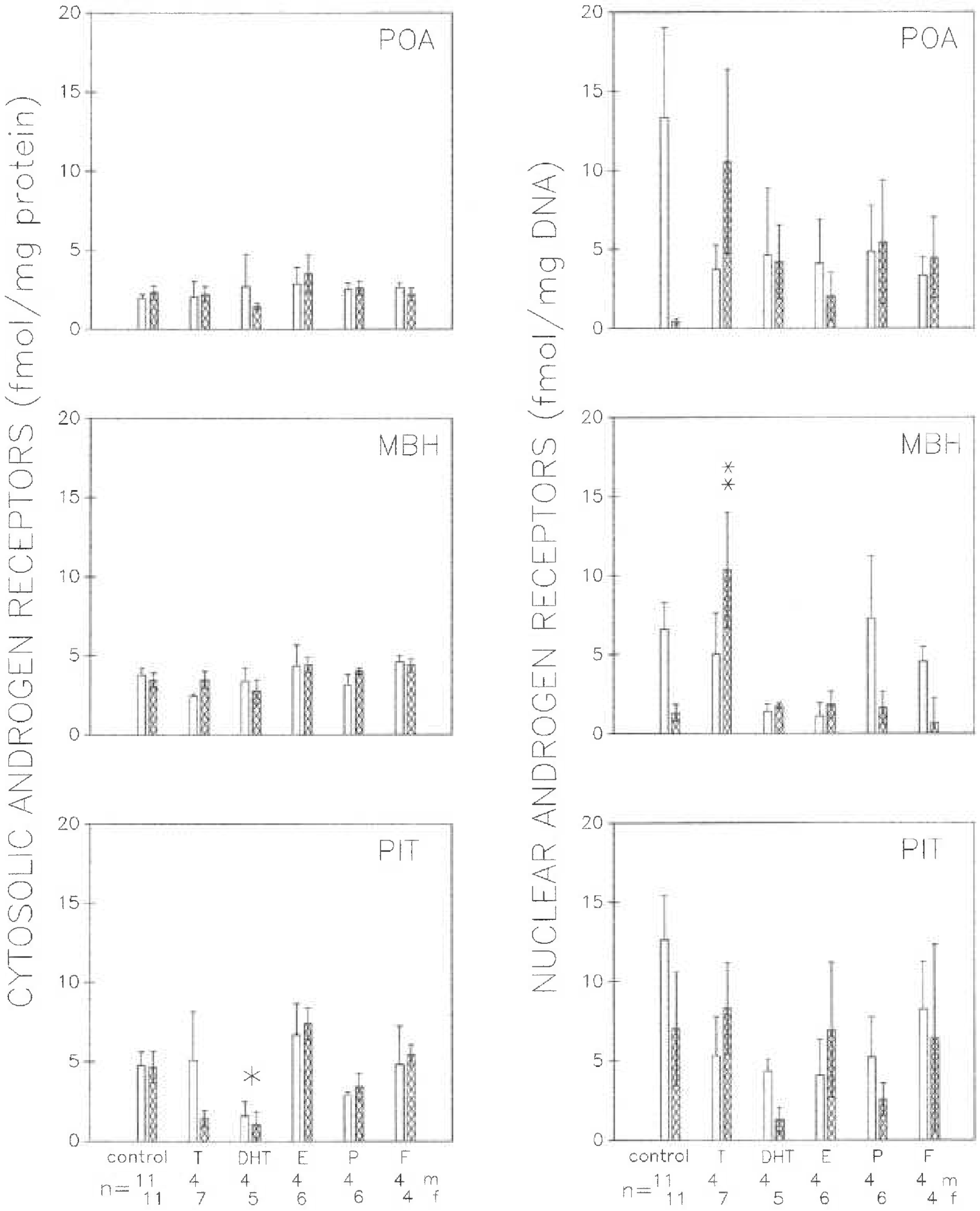

CYTOSOLIC ANDROGEN RECEPTORS (fmol/mg protein)
NUCLEAR ANDROGEN RECEPTORS (fmol/mg DNA)
POA
MBH
PIT
0
5
10
15
20
*
**
control
T
DHT
E
P
F
n= 11 4 4 4 4 4 m
11 7 5 6 6 4 f

TABLE 1: EFFECTS OF STEROID TREATMENT OF PREGNANT GUINEA PIGS ON ESTRONE AND ANDROSTENEDIONE CONCENTRATIONS IN THE MATERNAL AND FETAL COMPARTMENTS ON DAY 50 OF GESTATION.

		Steroid Treatments [a]				
	CONTROL	T	DHT	E	P	F
ESTRONE (pg/ml)						
Maternal	112±9 [b]	165±35	120±10	1723±795 [c]	115±18	108±7
	(22)	(5)	(4)	(5)	(4)	(5)
Fetal [d]	63±5	73±4	76±6	307±56 [c]	45±9	58±4
	(73)	(19)	(15)	(18)	(17)	(18)
ANDROSTENEDIONE (ng/ml)						
Maternal	3.00±.50	129.0±33.9 [c]	2.50±.50	3.50±.50	2.50±.60	2.10±.30
	(7)	(4)	(4)	(5)	(4)	(5)
Fetal ♂	0.13±.02 [e]	.64±.09 [c]	0.17±.06	0.21±.09	0.48±.13	0.05±.02
	(24)	(8)	(7)	(6)	(7)	(10)
♀	0.06±.01	1.23±.33 [c]	0.07±.03	0.17±.03	0.55±.31	0.01±.01
	(20)	(10)	(8)	(12)	(10)	(8)

a) Abbreviations: T = testosterone propionate; DHT = dihydrotestosterone propionate; E = estradiol benzoate; P = progesterone; F = cortisol. See text for dosage.

b) Mean ± SE, number in parenthesis ( ) equals the number of subjects in each group.

c) Mean differs from control, $p<0.01$.

d) Estrone data for male and female fetuses was combined for statistical analysis. See text.

e) Male control differs from female control, $p<.05$.

f) Mean differs from control, $p<0.05$

CHAPTER V

DISCUSSION AND CONCLUSIONS

Androgenization, or androgen-mediated masculinization, of the developing mammalian brain is accomplished through the actions of testosterone and/or its metabolites, which include estradiol and dihydrotestosterone. Estradiol is the principle effector in short gestation mammals such as rats, mice, and hamsters while androgens play a major role in sexual differentiation of long gestation mammals such as guinea pigs and primates. Steroid hormones produce their effects by binding to specific high affinity receptors. However, there is little information available about steroid receptors in the developing brain and much of that is contradictory.

Specific, high affinity androgen receptors have been reported in androgen target tissues including the central nervous system of adult animals. These receptors are distributed between cytosolic and nuclear fractions depending upon the physiologic state and gender of the subject. It is generally believed that androgen receptors are all nuclear and the cytosolic distribution is due to unoccupied nuclear receptors, loosely bound to the nuclear compartment, which detach during centrifugation. Also common to androgen receptors in both fetal and adult brains is the fact that endogenous androgen present in target tissues activates the receptor, thereby transforming it from a loosely bound (unoccupied) to a tightly bound (occupied) form.

If steroid-induced sexual differentiation is to occur, one would expect to find sex differences in the ratios of unoccupied/occupied androgen receptors in developing brains. This thesis was based on the hypothesis that if androgens are active in male sexual differentiation of the fetal guinea pig brain, androgen receptors must be present during a critical period during prenatal development, and that there should be differences in the quantity and possibly the distribution of these receptors in males and females. Also, if the ontogeny of androgen receptors in the guinea pig brain could be determined, the question of whether or not the expression of receptors in the brain marks the onset of the critical period for sexual differentiation might be answered.

Using in vitro binding techniques, we demonstrated the presence of specific, high affinity androgen receptors (both cytosolic and nuclear) in the fetal guinea pig brain and described their ontogeny. These receptors are present in the fetal brain throughout the critical period for differentiation, prenatal days 30 to 55. The quantity of nuclear androgen receptors differs between males and females. Cell nuclei from males contain greater amounts of receptor than those from females due to the higher levels of androgen secreted by the fetal testis compared to the fetal ovary. This specific dependence of the receptor on androgen is demonstrated in Chapter IV; in this part of the thesis we demonstrated that exogenous androgens activate cytosolic androgen receptors in the female brains so that they appear in the nuclear fraction. These data suggest that the biochemical machinery necessary for androgen action during the critical period is not merely present in the brain but is active, in as much as it responds to the high levels of androgen present in fetal males.

One of the reasons for initiating this work was to test the hypothesis that the level of androgen receptors in the HPOA determined the boundaries of the critical period. The data collected substantiated only part of this hypothesis. The beginning of the critical period is most likely regulated by the development of sufficient androgen receptors to mediate biological functions. The termination of the critical period cannot be explained in this way, however, since the end of the critical period occurs at a time when androgen receptor numbers are steadily increasing. Insensitivity to differentiation at this time is more likely related to the general maturity of target cells, either because regulatory mechanisms that interfere with androgenization are activated, or because areas of the genome that are unnecessary for cell function in adulthood are "switched off".

A key feature in male sexual differentiation is the development of the testes, which secrete testosterone during the critical period[48,49,52,53,181,195,222]. Sex differences in the concentration of this hormone provide substrate for metabolic conversion of testosterone to more active compounds such as estradiol-17ß. This process (aromatization) occurs in the brain of developing fetuses and may be important for differentiation of the brain in some rodent species[178]. Aromatase enzyme activity is found in the developing guinea pig brain but its role in sexual differentiation is not well understood at this time[218,223]. To investigate the possibility of estrogen action a little further, we found estrogen receptors in the fetal guinea pig brain (unpublished observations), indicating that the capability for estrogen binding is available during late gestation.

Since ". . . the physiologic consequences of testosterone action are the result of the combined effects of testosterone itself and those of its dihydrotestosterone and estrogen derivatives."[159], it is necessary to examine the role of DHT (a non-aromatizable androgen), as well as estrogen, in sexual differentiation. Studies in fetal guinea pigs whose mothers were given 5α-reductase inhibitors showed that while DHT is necessary for complete masculinization of the reproductive tract, no deficit was seen in male copulatory behavior[89,90]. In the brain, DHT appeared to play a "defeminizing" role in the normal females, decreasing baseline and surge quantities of LH compared to the treated females. On the other hand, female guinea pigs treated with 5α-DHT prenatally showed enhanced male sexual behavior (although testosterone was more effective than DHT alone)[93]. In fetal rhesus monkeys, 5α-reductase is abundant in the hypothalamus, preoptic area and amygdala, areas associated with sexual behavior[224]; although there is no sex difference in the quantities of 5α-reductase found in the brain, the greater availability of substrate in males could lead to increased levels of DHT in male brains, similar to the case we discussed for estrogen in Chapter IV. Human males with 5α-reductase deficiency assume a male gender role without prenatal exposure to DHT[171,173]. There remains much to sort out in defining the role of DHT in sexual differentiation of the brain.

Exogenous progesterone has been shown to prevent androgenization by testosterone in neonatal rats[225,226] and to suppress male sexual behavior in the adult guinea pig[227]. Progesterone appears to inhibit either transformation of the androgen receptor or its retention in the cell nucleus in the male guinea pig brain[154,155,228]. It has been hypothesized that this effect of progesterone is mediated through the progesterone receptor rather than by a direct effect on androgen receptors[155]. The ontogeny of cytosolic progesterone receptors in neonatal rat brain has been described[229]. Although we found progesterone receptors in the brains of fetal guinea pigs during late gestation (unpublished observations), we observed no effects of exogenous progesterone on androgen receptors in the fetal guinea pig brain after two hours of treatment on day 50.

Recent studies suggest that certain sexual dimorphisms in brain structure occur independently of steroid hormone action. Transient sex differences in the size of dopaminergic neurons in fetal rats arise before the testes begin to secrete testosterone, and these neurons do not respond to androgens in vitro[210]; these data suggest that some sexual dimorphisms may be under primary genetic, rather than hormonal, control.

Of further interest is the relationship between testosterone and cortisol in the fetus. While it has been demonstrated that cortisol can increase testosterone production by cultured Leydig cells in the immature pig[230], the mechanism whereby testosterone increases cortisol levels in the fetal guinea pig (shown in Chapter IV) is unclear. Also, is this relationship unique to the fetus and/or the guinea pig?

Ultimately, brain development and differentiation is a complex symphony of events, which "seems to be more complex than has generally been taken for granted"[61]. The role of androgens, and androgen receptors, in this multitude of events is just beginning to be understood.

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APPENDIX

ADDITIONAL TABLES AND FIGURES

This appendix contains data that were generated during the course of the experimental work which are either supportive of the reliability of the techniques used and/or of the methods of calculation used, or that were peripheral to the general theme of the major experiments.

INDEX

Table 2. THE EFFECT OF AGE ON WEIGHT IN FETAL GUINEA PIGS.

Average litter 3.6 ± 0.9 (range 2–6, n = 119)

Gestational age (days)	(Grams ± SD)*			
	Male		Female	
30	1.49 ± 0.18 (n = 17)**			
34	3.0 ± 0.47	(6)	3.52 ± 0.50	(4)
40	11.07 ± 2.28	(14)	9.24 ± 2.56	(9)
49	35.17 ± 4.31	(9)	31.89 ± 3.85	(5)
50	36.92 ± 3.02	(12)	35.05 ± 4.64	(11)
51	42.34 ± 3.56	(9)	40.32 ± 3.09	(11)
60	62.39 ± 9.77	(9)	62.15 ± 7.17	(11)

* mean ± S.D. (n)

** n = number of fetuses/mean

Fig. 17. Time course of androgen binding in fetal brain and pituitary. Combined POA, MBH, AMYG, and SEPT (HPAS; open circles), combined POA and MBH (HPOA; closed circles), or anterior pituitary (open triangles) from late gestation fetal guinea pigs was incubated at 4°C for 1, 2, 4, 8, 12, 20, 46, or 48 hours to determine at which time maximum binding was recovered.

Fig. 17

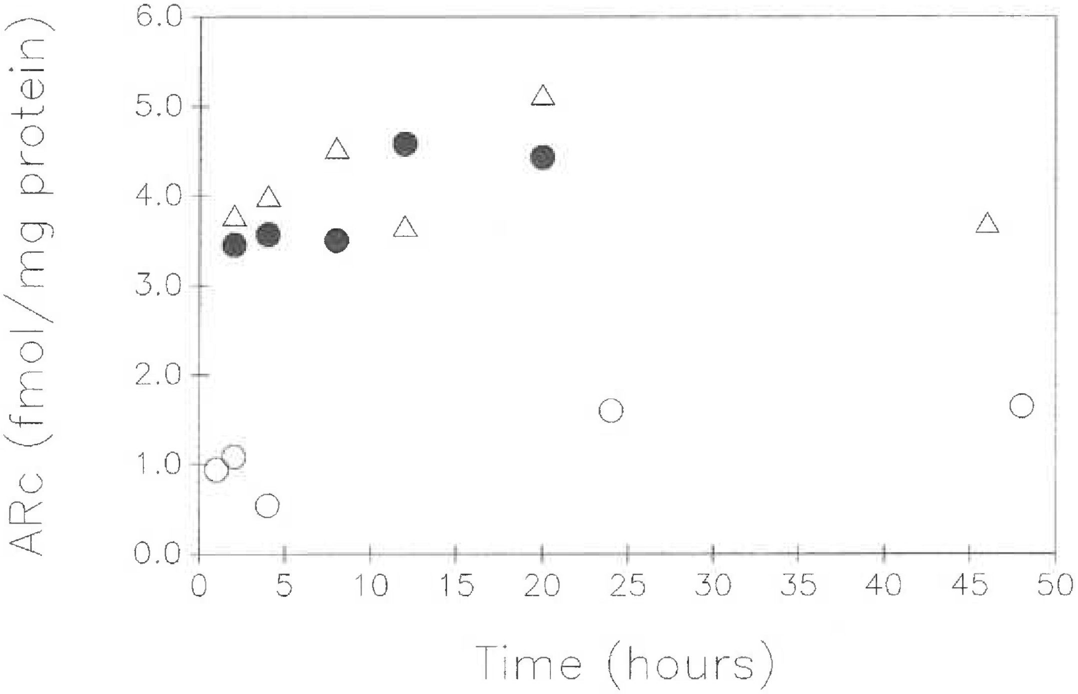
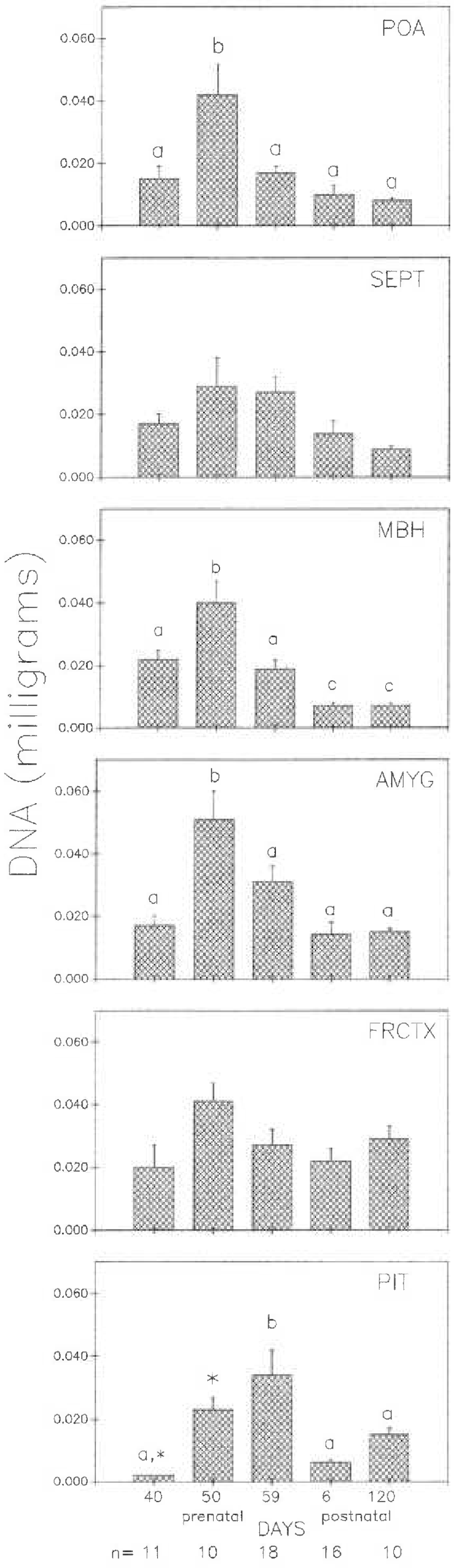

ARc (fmol/mg protein)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
5
10
15
20
25
30
35
40
45
50
Time (hours)

Fig. 18. Milligrams DNA vs. age. (For abbreviations see Figure 21).To investige whether changes in DNA with age might bias ARn data reported per mg DNA, we plotted DNA vs. age (days 40, 50, and 59 of gestation and postpartum days 6 and 120) in all tissues studied. No sex differences were found so data for males and females were combined; this indicates that calculation of nuclear androgen receptors (ARn) per mg DNA would not cause an artificial sex difference in the amount of ARn reported for males and females. The greatest amount of DNA was found at day 50 of gestation in POA, SEPT, AMYG, and MBH. Bars with different letters differ from each other significantly ($p<.05$). Also, bars each having an asterisk (*) differ from each other significantly ($p<.05$).

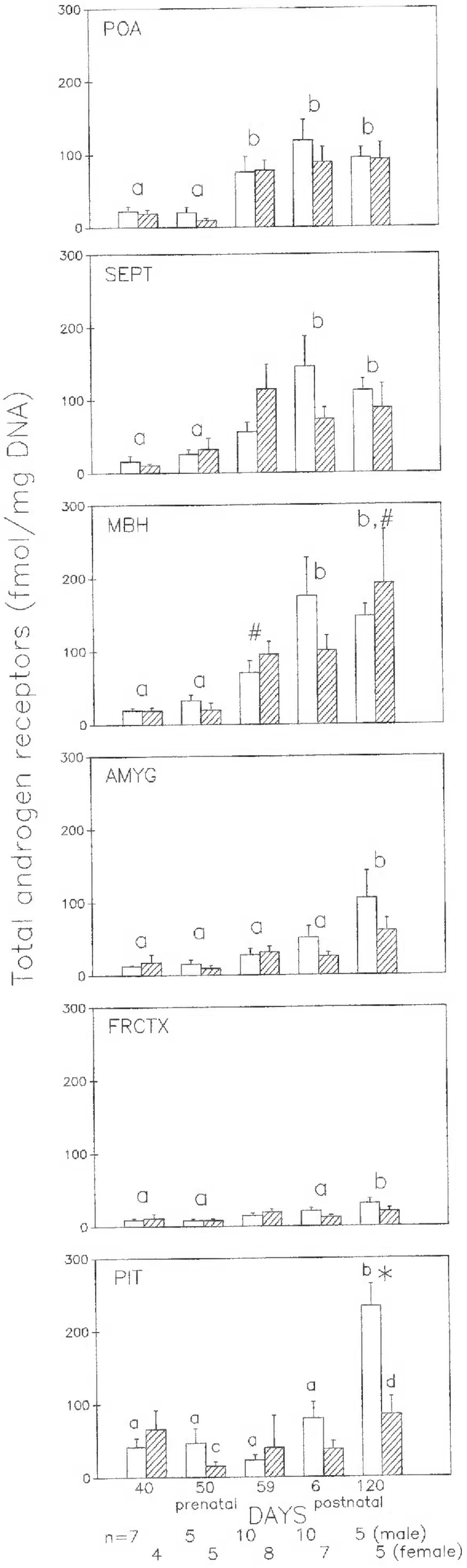
Fig. 18



DNA (milligrams)
POA
SEPT
MBH
AMYG
FRCTX
PIT
0.060
0.040
0.020
0.000
a
b
c
a,*
*
40
50
59
6
120
prenatal
postnatal
DAYS
n= 11
10
18
16
10

Fig. 19. Total androgen receptors (AR) in the preoptic area (POA), septum (SEPT), medial-basal hypothalamus (MBH), amygdala (AMYG), frontal cortex (FRCTX), and anterior pituitary (PIT) on days 40, 50, and 59 of gestation and postpartum days 6 and 120. Each bar represents the mean ± SEM. Means for males (open bars) and females (hatched bars) were only different in PIT, so data for other tissues was combined for statistical analysis. Bars with different letters differ significantly ($p<.05$) from each other (in PIT, letters apply to males or females only). Bars with a number sign (#) differ from each other also ($p<.05$). An asterisk (*) denotes a sex difference at that age ($p<.05$). Total receptor binding was calculated by combining the fmol of ^{3}H-DHT binding from the cytosolic and nuclear fractions and dividing by mg DNA for the sample. However, since the relative amount of binding in cytosol is so much greater than that in the nuclear fraction, this graph is hardly different from Figure 7. Reporting data as total AR could therefore trivialize large percent changes in ARn.

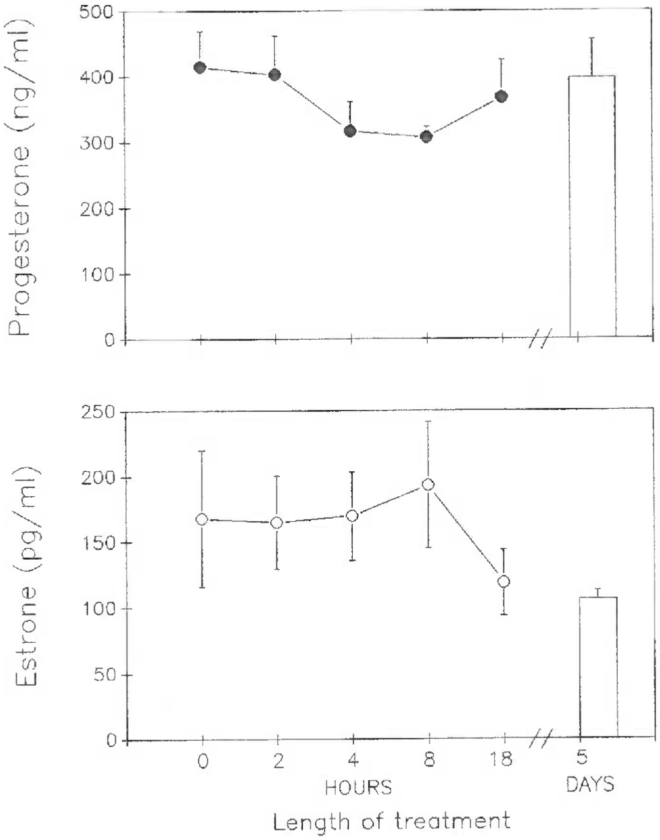
Fig. 19



Total androgen receptors (fmol/mg DNA)
POA
SEPT
MBH
AMYG
FRCTX
PIT
0
100
200
300
a
b
c
d
#
b,#
b *
40
50
59
6
120
prenatal
postnatal
DAYS
n=7
5
10
10
5 (male)
4
5
8
7
5 (female)

Fig. 20. Estrone and progesterone concentrations in sera from pregnant guinea pigs injected with TP either for 0, 2, 4, 8 or 18 hours (points) or daily for 5 days (bars) before decapitation (number of animals per group was 22, 4, 6, 3, 3, and 5 respectively).

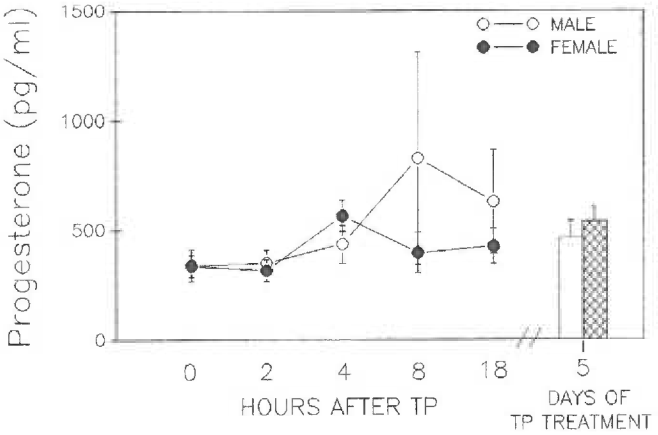
Fig. 20



Progesterone (ng/ml)
500
400
300
200
100
0
Estrone (pg/ml)
250
200
150
100
50
0
0
2
4
8
18
HOURS
5
DAYS
Length of treatment

Fig. 21. Estrone and progesterone concentrations in sera from fetal guinea pigs after injection of the mother with TP for either 0, 2, 4, 8 or 18 hours (points) or daily for 5 days (bars) before decapitation (number of samples per group at 0, 2, 4, 8, 18 h and 5 days was: [male] 39, 9, 7, 6, 6, 6 and [female] 35, 10, 14, 6, 5, 10). Points and bars represent means ± SEM. Open circles and bars represent male values while solid circles and bars represent female values.

Fig. 21


Progesterone (pg/ml)
1500
1000
500
0
○—○ MALE
●—● FEMALE
0
2
4
8
18
5
HOURS AFTER TP
DAYS OF
TP TREATMENT

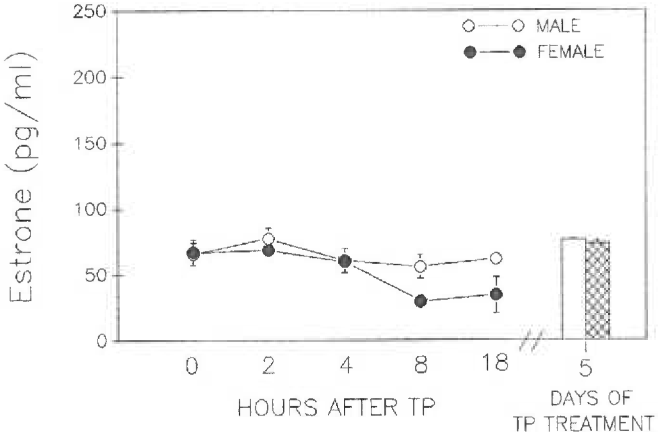
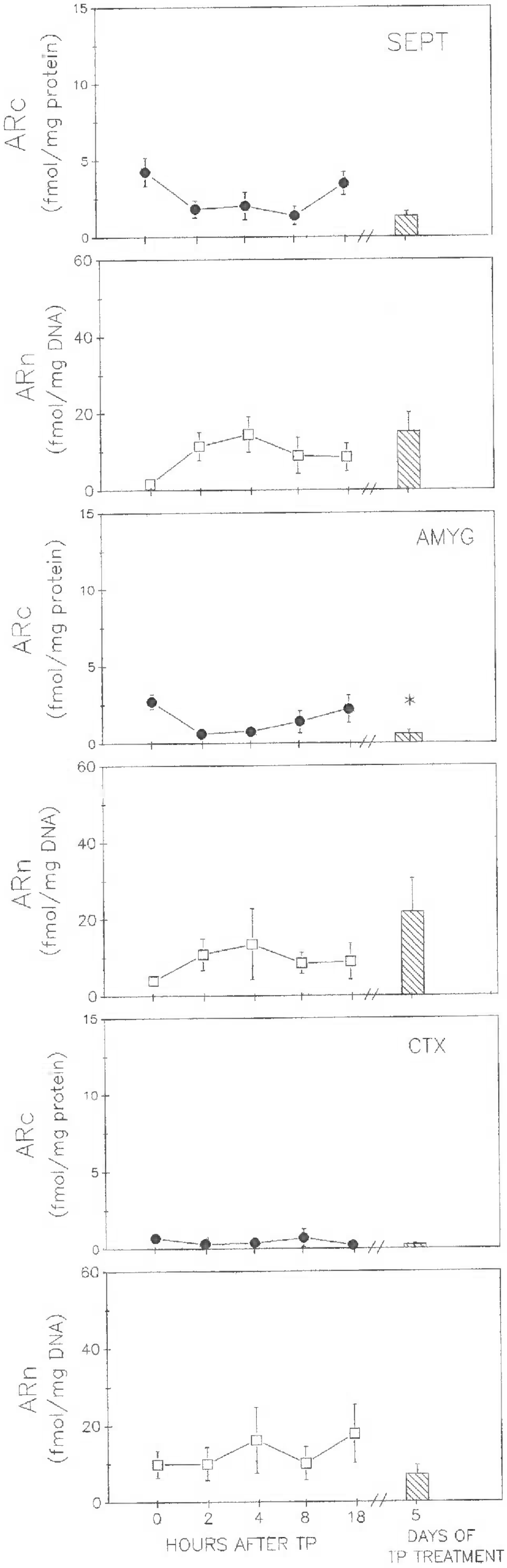

Estrone (pg/ml)
250
200
150
100
50
0
○—○ MALE
●—● FEMALE
0
2
4
8
18
5
HOURS AFTER TP
DAYS OF
TP TREATMENT

Fig. 22. Cytosolic (ARc) and nuclear (ARn) androgen receptor content in various brain areas [septum (SEPT), amygdala (AMYG) and frontal cortex (CTX)] of pregnant guinea pigs injected with 10 mg of TP for 2, 4, 8, or 18 hours (points) or injected daily for 5 days (bars) before decapitation. Points and bars indicate means ± SEM. Plus (+) and asterisk (*) indicate means differ from control ($p<.05$ and $p<.01$, respectively). Number of samples per group at 0, 2, 4, 8, 18 hrs and 5 days was 12, 5, 5, 3, 3 and 5 animals, respectively.



SEPT
ARc
(fmol/mg protein)
15
10
5
0
ARn
(fmol/mg DNA)
60
40
20
0
AMYG
ARc
(fmol/mg protein)
15
10
5
0
*
ARn
(fmol/mg DNA)
60
40
20
0
CTX
ARc
(fmol/mg protein)
15
10
5
0
ARn
(fmol/mg DNA)
60
40
20
0
0
2
4
8
18
//
5
HOURS AFTER TP
DAYS OF
TP TREATMENT

Fig. 22

Fig. 23. Cytosolic (ARc) and nuclear (ARn) androgen receptor content in various brain areas (see Figure 12 legend for abbreviations) of fetal guinea pigs (50 ± 2 days gestation) whose mothers were injected with 10 mg of TP for 2, 4, 8 or 18 hours (points) or daily for 5 days (bars) before decapitation. Closed circles ARc), open squares (ARn) and cross-hatched bars indicate means ± SEM. The number of samples per group at 0, 2, 4, 8, 18 hr and 5 days was: (male, ♂) 11, 4, 6, 4, 3 and 4 and (female, ♀) 11, 7, 8, 3, 3 and 6 respectively.

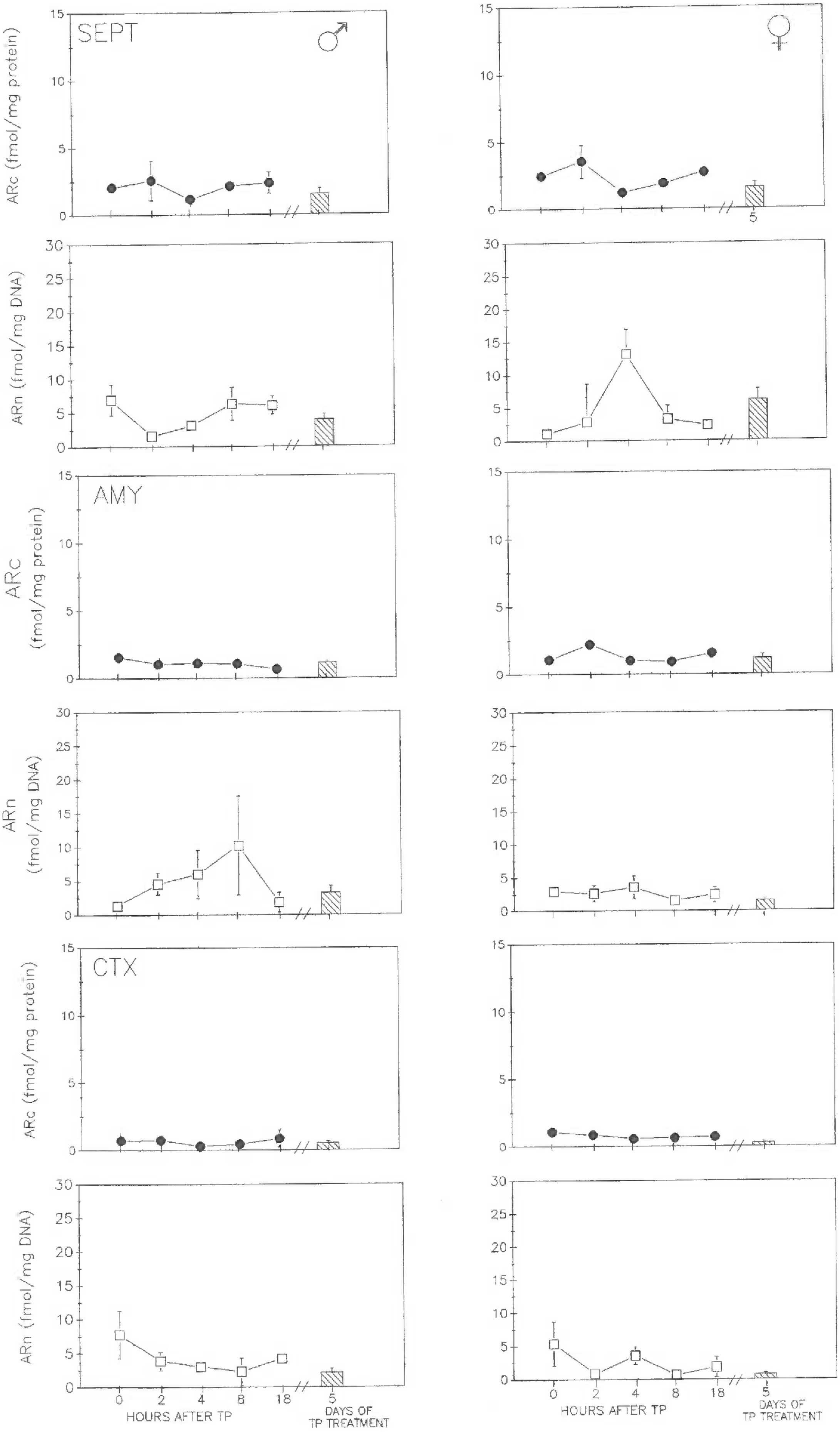
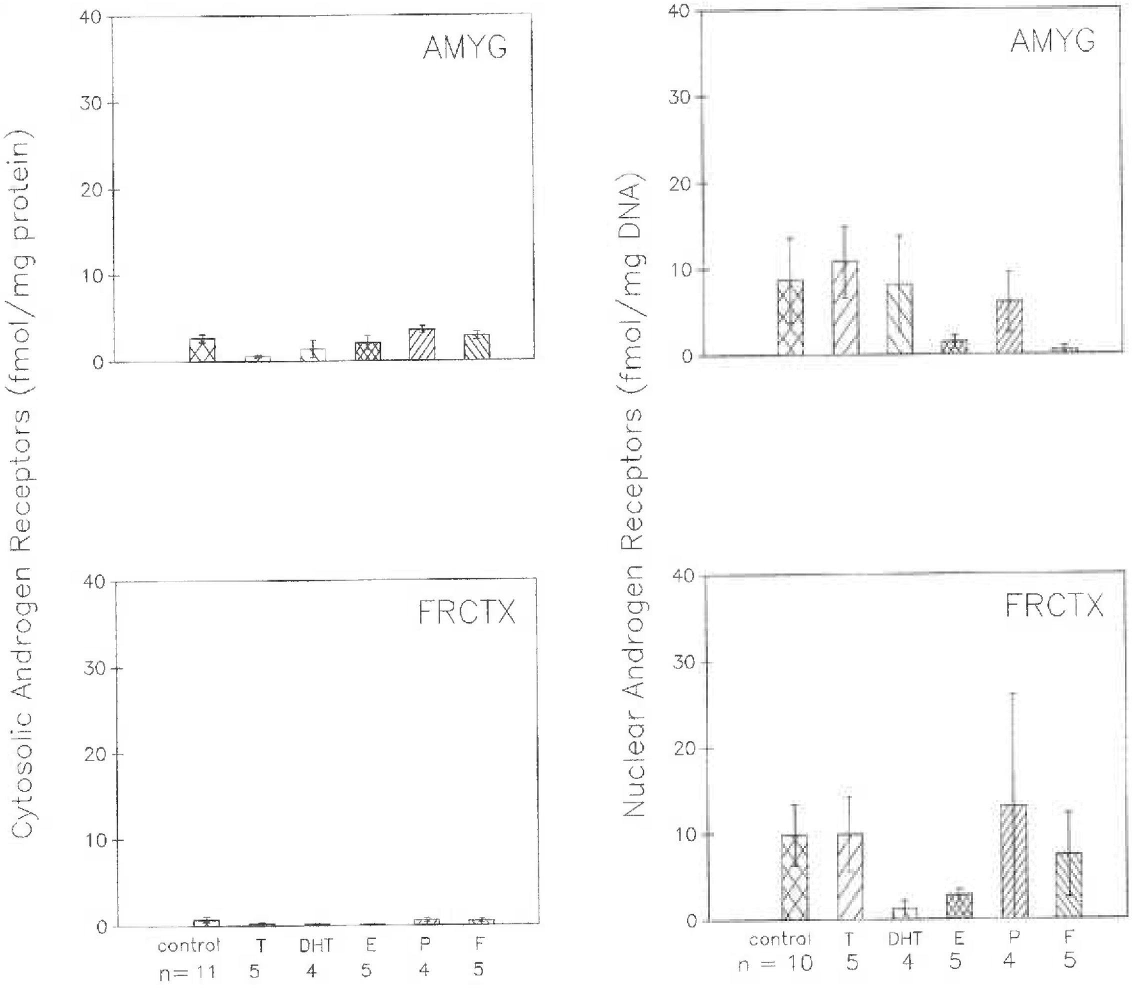

SEPT
♂
♀
ARc (fmol/mg protein)
ARn (fmol/mg DNA)
AMY
ARc (fmol/mg protein)
ARn (fmol/mg DNA)
CTX
ARc (fmol/mg protein)
ARn (fmol/mg DNA)
0
5
10
15
20
25
30
0
2
4
8
18
5
HOURS AFTER TP
DAYS OF TP TREATMENT

Fig. 23

Fig. 24. Cytosolic and nuclear androgen receptors in the amygdala (AMYG) and frontal cortex (FRCTX) of pregnant guinea pigs injected on day 50 of gestation with exogenous steroids identified on the abscissa (see Fig. 15 legend for abbrevations). Bars represent mean ± SEM. Asterisk (*) and plus (+) indicate means are different from control ($p<.01$ and $p<.05$, respectively). See Methods section for details. N indicates the number of determinations in each group.

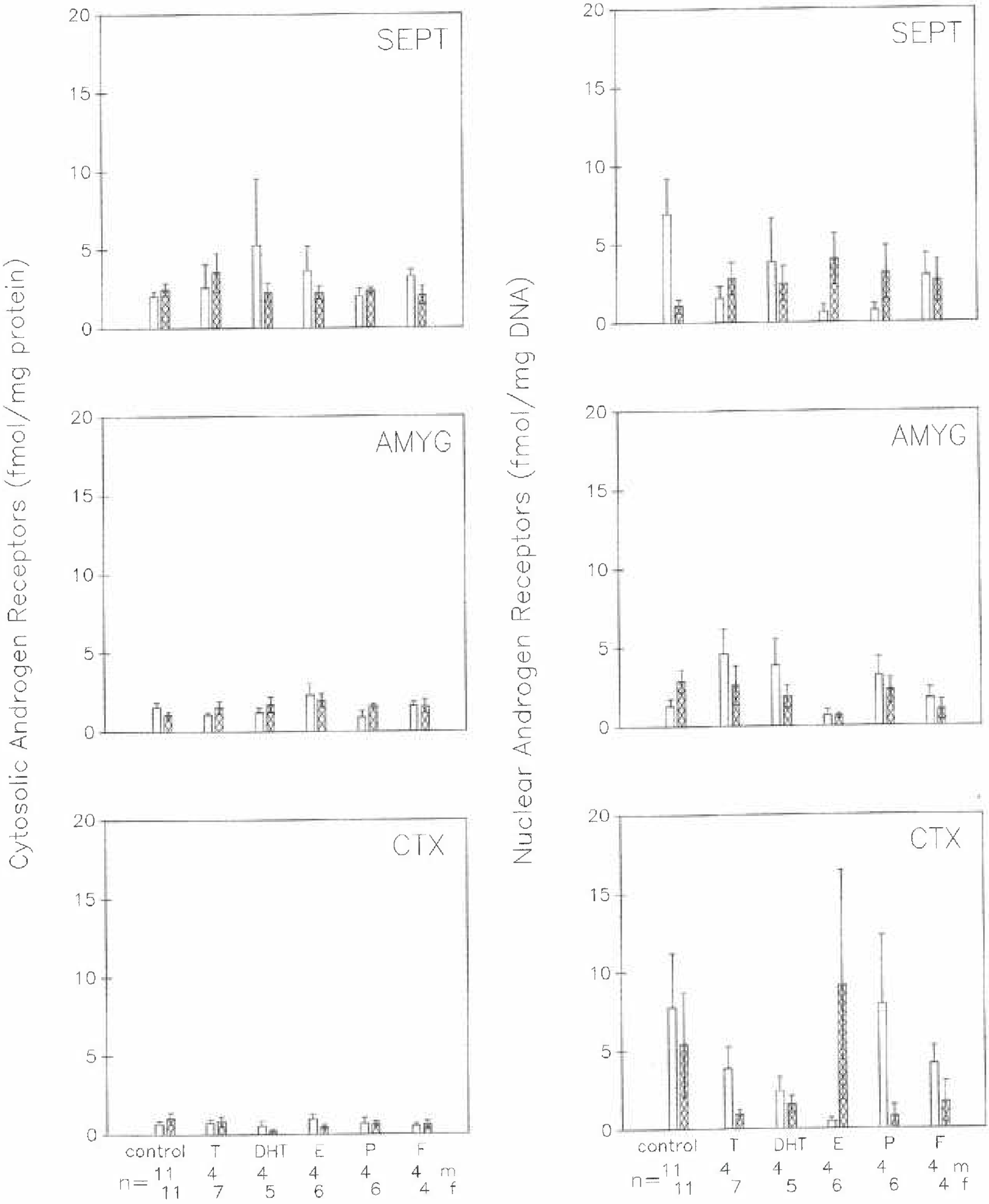
Fig. 24



Cytosolic Androgen Receptors (fmol/mg protein)
Nuclear Androgen Receptors (fmol/mg DNA)
AMYG
AMYG
FRCTX
FRCTX
40
30
20
10
0
control
T
DHT
E
P
F
n= 11
5
4
5
4
5
n = 10
5
4
5
4
5

Fig. 25. Cytosolic and nuclear androgen receptors in the septum (SEPT), amygdala (AMYG), and frontal cortex (CTX) of fetal guinea pigs whose mothers were injected with testosterone propionate (T), dihydrotestosterone propionate (DHT), estradiol benzoate (E), progesterone (P), or cortisol (F) on day 50 of gestation. Bars represent means ± SEM. Asterisk (*) and (plus +) indicate means for females differ from control females ($p<.05$ and $p<0.01$) respectively. Open bars represent males and shaded bars represent females. Treatment regimens are identified by abbreviations on the abscissa; see Methods section for details. N indicates the number of determinations in each group.

Fig. 25


Cytosolic Androgen Receptors (fmol/mg protein)
Nuclear Androgen Receptors (fmol/mg DNA)
SEPT
AMYG
CTX
20
15
10
5
0
control
T
DHT
E
P
F
n=
11 4 4 4 4 4 m
11 7 5 6 6 4 f