SEXUAL DIFFERENTIATION OF THE RAT HYPOTHALAMUS: AN EVALUATION OF SEXUAL BEHAVIOR AND HYPOTHALAMIC MORPHOLOGY FOLLOWING NEONATAL MANIPULATION

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ABSTRACT

SEXUAL DIFFERENTIATION OF THE RAT HYPOTHALAMUS:
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Morehead State University, 1984

Sexual differentiation of vertebrate reproductive and behavioral patterns is largely effected by hormones produced by the gonads. In many higher vertebrates, an integral part of this process is the induction of permanent, irreversible sex differences in central nervous function in response to gonadal hormones secreted early in development. The period of time during which the animal's central nervous system is susceptible to the differentiating effect of these gonadal hormones is of limited duration and is termed 'critical period'. The approximate time for critical period in the rat is the first week post-partum. It is believed that mammalian central nervous system development inherently follows a female pattern with critical period exposure to testicular hormones resulting in a repression of female traits (defeminization) and the induction of male traits (masculinization). In this study, neonate rats were subjected to a variety of treatments in order to further elucidate the effects of progesterone, clomiphene (a non-steroidal anti-estrogen) and flutamide (a non-steroidal anti-androgen), and to clarify the role of the androgen receptor in differentiation of the central nervous system. Animals were subjected to bilateral gonadectomy at 60 days of age, and two weeks later their ability to exhibit female sex behavior in response to hormonal priming was quantitated. Additionally, histological studies upon these animals' brains were performed in order to quantitate the

volume of the Sexually Dimorphic Nucleus of the Preoptic Area, a sexually dimorphic nucleus the volume of which has been shown to be steroidally mediated.

Progesterone was not found to effect defeminization of sex behavior. Clomiphene acted as an extremely potent estrogen agonist. Flutamide significantly attenuated testosterone propionate-induced defeminization of sex behavior, possibly by blocking androgen receptor system mediation of the enzyme aromatase. Brain morphology data did not achieve statistical significance.

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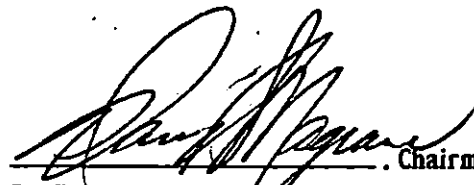

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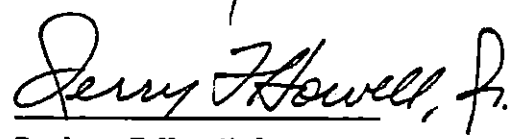

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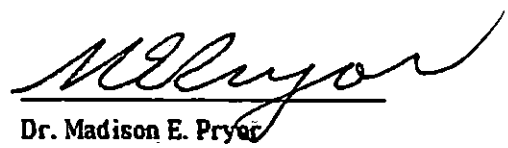

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INTRODUCTION

In many vertebrate species, an integral part of the reproductive process is the development of marked sex differences in the control of endocrine function and behavior by the central nervous system (CNS). Reproductive events, such as recognition of a suitable sex partner, mating, and the production and rearing of young are dependent upon normal sex differentiation of the CNS (1). Of extreme importance in the development of these sex differences are gonadal steroids (2).

Early endocrine research suggested that the type of sexual behavior displayed was dependent only upon the nature of the hormone administered. For example, it was believed that testicular hormones stimulated the expression of masculine behavior equally well in adult members of either sex, and that a similar situation existed in regard to the elicitation of feminine behavior with ovarian hormones (3). It is now accepted that permanent sex differences in morphology and function are imposed upon the CNS in many animals during their early developmental history. Depending on the sex of the animal, these differences result in heterogenous display of hormonally-stimulated behavior in the adult, and are largely mediated by gonadal hormones present during the perinatal period (1,2,4,5).

A general hypothesis has been formulated for the mechanism of CNS sexual differentiation which has much in common with the model for differentiation of the embryonic reproductive tract (6). CNS development inherently follows a pattern appropriate for the homogametic sex. In the heterogametic sex, hormones produced by the gonads results in differentiation away from this pattern. Thus, in mammals the intrinsic pattern is female, with

exposure to testicular hormones being necessary for differentiation toward male patterns of gonadotrophin secretion, behavior and CNS morphology (7). In contrast, the homogametic sex in birds is the male. Thus, the intrinsic pattern is male, with exposure to ovarian hormones being necessary for differentiation toward the female CNS phenotype (8).

In mammals, differentiation involves two processes: defeminization, which is the repression of feminine traits (i.e., cyclic gonadotrophin release, sexually receptive behavior in the presence of a male following estrogen-progesterone priming, etc.); and masculinization, which is the superimposition of a male trait upon the original homogametic (female) pattern (i.e., mounting of females, masculine pattern of brain morphology, etc.).

Critical Period

The period of time during which an animal's CNS is susceptible to the differentiating effect of gonadal hormones is of limited duration and is termed 'critical period' (see Table 1). Critical period has been delineated with some degree of precision in four placental mammals (rat, guinea pig, sheep, and rhesus monkey) by examination of neuroendocrine and behavior patterns following hormone treatments given at different times during their development (1). More approximate times for critical period are known for several other species (mouse, hamster, ferret, dog, and several species of birds) (1). Critical period for the rat is generally stated to be the first week post-partum, although this is a concept developed through the more obvious characteristics of reproductive function and behavior, and recent evidence suggests that it may be misleading to imply precise boundaries in time for critical period (1,5). For example, in the rat, while defeminization of cyclic gonadotrophin release and female sex behavior seems to be

Table 1. Time of Critical Period for Sexual Differentiation of the CNS in Several Species. Taken from MacLusky and Naftolin (1).

Animal	Gestation or Incubation (Days)	Critical Period (After Conception)
Rat	20-22	18-27 days
Mouse	19-20	Postnatal
Hamster	16	Postnatal
Guinea pig	63-70	30-37 days
Ferret	42	Postnatal
Dog	58-63	Prenatal + postnatal
Sheep	145-155	~30-90 days
Rhesus monkey	146-180	~40-60 days
Domestic chicken	22	Prehatching
Zebra finch	12-14	Posthatching

sensitive to steroidal influence primarily during the first few days post-partum, male sex behavior is highly sensitive to androgen treatment before birth (9). Additionally, since critical period may be lengthened under experimental conditions by increasing the dosages of hormones administered, Feder (5) argues that the term 'maximal susceptability period' is a more accurate reflection of biological reality and should be used instead of critical period.

The issue of critical period in the rat has been further clouded by research performed by Gladue and Clemens (10) and McPherson and Mahesh (11). Gladue and Clemens report that long-term prenatal exposure to the anti-androgen flutamide (days 10-22 post-conception) can attenuate the defeminization of sexual behavior in males. McPherson and Mahesh have shown that the female pattern of gonadotrophin release [i.e., a Luteinizing Hormone-Follicle Stimulating Hormone (LH-FSH) surge] may be induced by certain doses of progesterone in a male

rat that has been castrated at day 26 post-partum and subsequently estrogen primed. Current evidence suggests that this female pattern of gonadotrophin release cannot be induced in the adult male rat, implying that the critical period defeminization of gonadotrophin release has not yet been 'finalized' by day 26 post-partum. MacLusky and Naftolin suggest that while CNS function can be organized by a single exposure to gonadal steroids during critical period, differentiation is not always complete during this period, full development being partially dependent upon subsequent hormonal exposure (1).

Gonadal Hormones

The primary hormonal product of the developing testis is testosterone. Injection of testosterone essentially duplicates the androgenization effects produced by the presence of testes, suggesting that testosterone is the testicular factor responsible for sexual differentiation of the mammalian CNS (6,12,13,14). However, sexual differentiation cannot be viewed as a simple matter of testosterone being present in the male and absent in the female. In the rat, systematic radioimmunoassay has indicated that androgens circulate in the female as well as the male during critical period, though the relative amounts of androgen are controversial (1). Some earlier reports have indicated consistently higher testosterone values in the male during critical period (15,16), while others have reported that consistent sex differences in serum testosterone during critical period do not exist (17,18). More recent (1984) work by Rhoda, Corbier and Roffi (19), using specific radioimmunoassays, strongly suggests that there is a dramatic and transient testosterone increase in both serum and hypothalamus during the first two hours post-partum in the male that is not present in the

female. This surge does not occur in a gonadectomized male, indicating that this surge is linked to the presence of testes. This data is particularly pertinent in view of the theory that brief exposure to high levels of testosterone may sensitize the developing male CNS to subsequent lower levels of testosterone (20). Thus, after the testosterone surge in the male during the first few hours post-partum, similar testosterone concentrations in male and female could produce entirely different effects (20).

It has also been suggested that there may be gonadal hormones that serve to protect the female's hypothalamus from the effects of testosterone. It has been shown that, in the rat, the presence of ovaries tends to inhibit the defeminizing effects of critical period treatment of the animal with androgen (21). This, along with evidence that progesterone can exhibit some anti-androgenic effects has led some researchers to suggest that progesterone may be of importance as a protective mechanism for the female's hypothalamus in some species (1,2,4,9,22).

Mechanisms of Steroid Action

Steroid hormones are transported in the plasma bound to a variety of proteins in an inactive form (23). However, small amounts of free, unbound steroid may enter a cell through passive diffusion and bind to a receptor characterized by a high degree of affinity and specificity for the ligand (24). While it has long been believed that this receptor is a cytoplasmicly located protein which is translocated to the nucleus in a different form after steroid binding (25,26), recent evidence suggests that both forms of the steroid receptor may actually be localized in the nuclear compartment, with binding of steroid resulting in a change

in receptor affinity for nuclear elements rather than a nuclear translocation of the receptor. What has been termed the 'cytoplasmic' receptor is proposed to be an extraction artifact (27,28). This most recent evidence regarding the subject of translocation is incorporated into Figure 3.

Binding of hormone to receptor is followed by the hormone-receptor complex associating with chromatin (29) and subsequent mRNA synthesis and protein formation (30).

Neural Gonadal Steroid Receptors in the Critical Period Brain

In the neonatal rat, several areas within the hypothalamus, preoptic area, and amygdala have been found to concentrate testosterone or its metabolites (31). Neural estrogen, androgen, and progestin receptors in the rat are present in very low quantities prior to birth and increase dramatically in the immediate postnatal period. Estrogen receptors increase first, starting just before birth and continuing through day five or six post partum (32,33). Progesterone receptors begin to increase shortly after birth and rise in parallel with the estrogen receptor system (34). It should be noted that the progesterone receptor is not induced by estrogen until after critical period, which has led McEwen, among others, to hypothesize that the estrogen receptor is not "hooked up to normal cell function during critical period," since provision of estrogen to a cell which is estrogen receptor positive results in the production of progesterone receptor in the adult (35). Androgen receptors start to increase about one week post-partum (36).

Autoradiographic and biochemical studies indicate that androgen and estrogen receptors can bind within cell nuclei in the developing rat brain (1). There have been no similar reports on whether the progesterone receptor is also functional in terms of nuclear

binding.

Androgen Metabolites and the Aromatization Hypothesis

Figure 1 illustrates the various potential metabolic paths through which testosterone may be transformed and exert an influence upon a target tissue (37). The pathway that seems to be of primary importance is the conversion of testosterone to 17ß-estradiol via the enzyme aromatase, as illustrated in Figure 2.

Critical period exposure to estrogen produces results extremely similar to those resulting from exposure to testosterone. The postnatal injection of testosterone propionate or estrogen benzoate to the intact female (38,39,40,41) or the neonatally castrated male (42,43,44) suppresses feminine reproductive behavior in adulthood. The feminine pattern of cyclic gonadotrophin release may be changed to the tonic pattern of the male in like fashion (7), suggesting that estrogen may be the terminal effector hormone for many aspects of brain differentiation. It was shown in the early 1970's that 5-alpha dihydrotestosterone (which cannot be transformed into testosterone or aromatized to estrogen) does not exert the defeminization effect exhibited by testosterone and estrogen. The fact that DHT is the common terminal testosterone metabolite acting upon the androgen receptor and does not exert a defeminizing effect supports the concept that the aromatic conversion of testosterone to estrogen is of importance in defeminization (45). Other studies support the aromatase hypothesis. First, the defeminization effects of testosterone can be blocked with estrogen antagonists (46). Second, there is a dramatic rise in hypothalamic 17-ß estradiol during the first hour post-partum in the male rat. This increase coincides with a serum and hypothalamic increase of

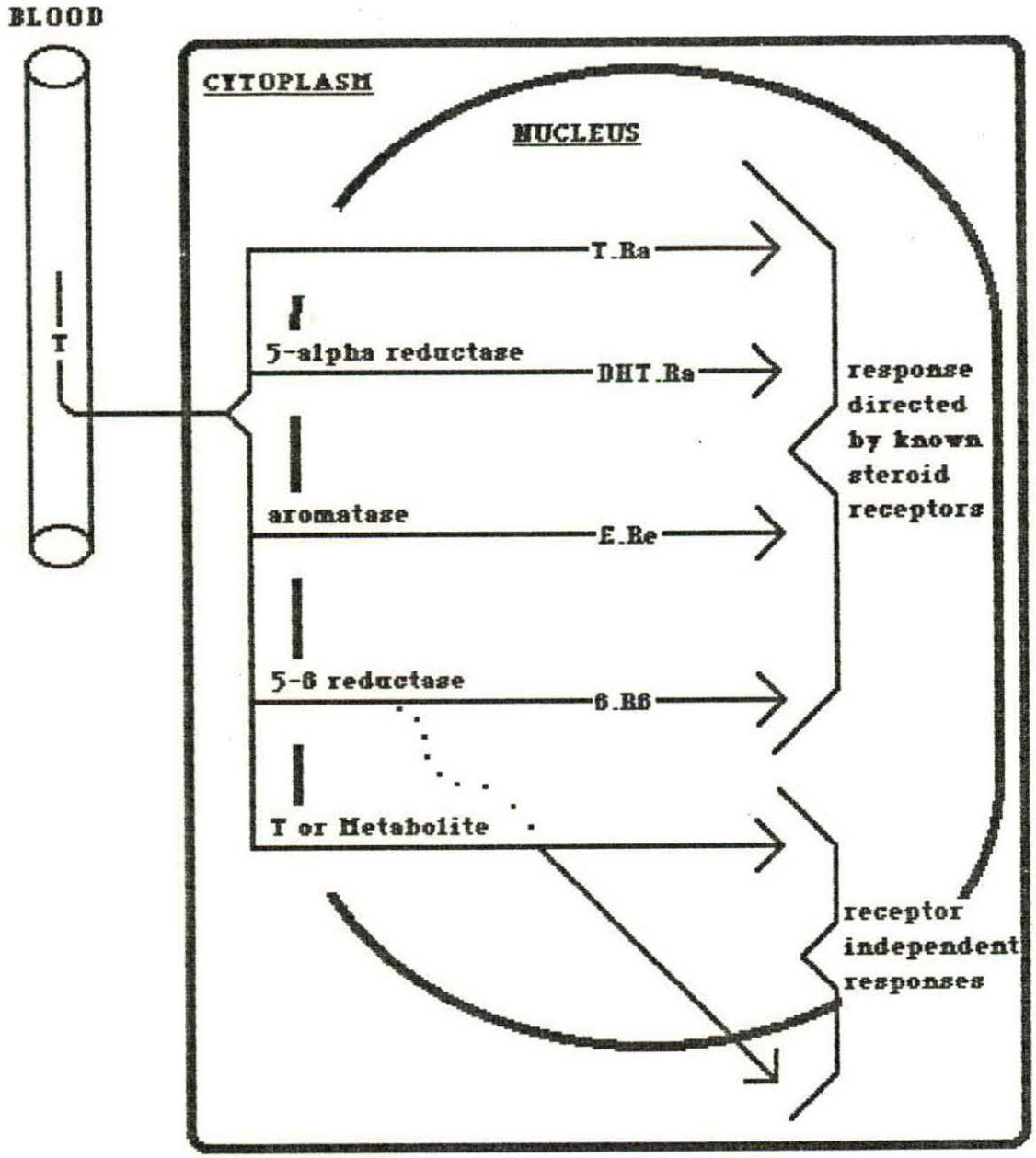

Figure 1. Testosterone (T) Metabolism and Receptor Binding. Ra, androgen receptor; DHT, dihydrotestosterone; Re, estrogen receptor; Rß, beta receptor. Taken from Bardin and Catteral (37).

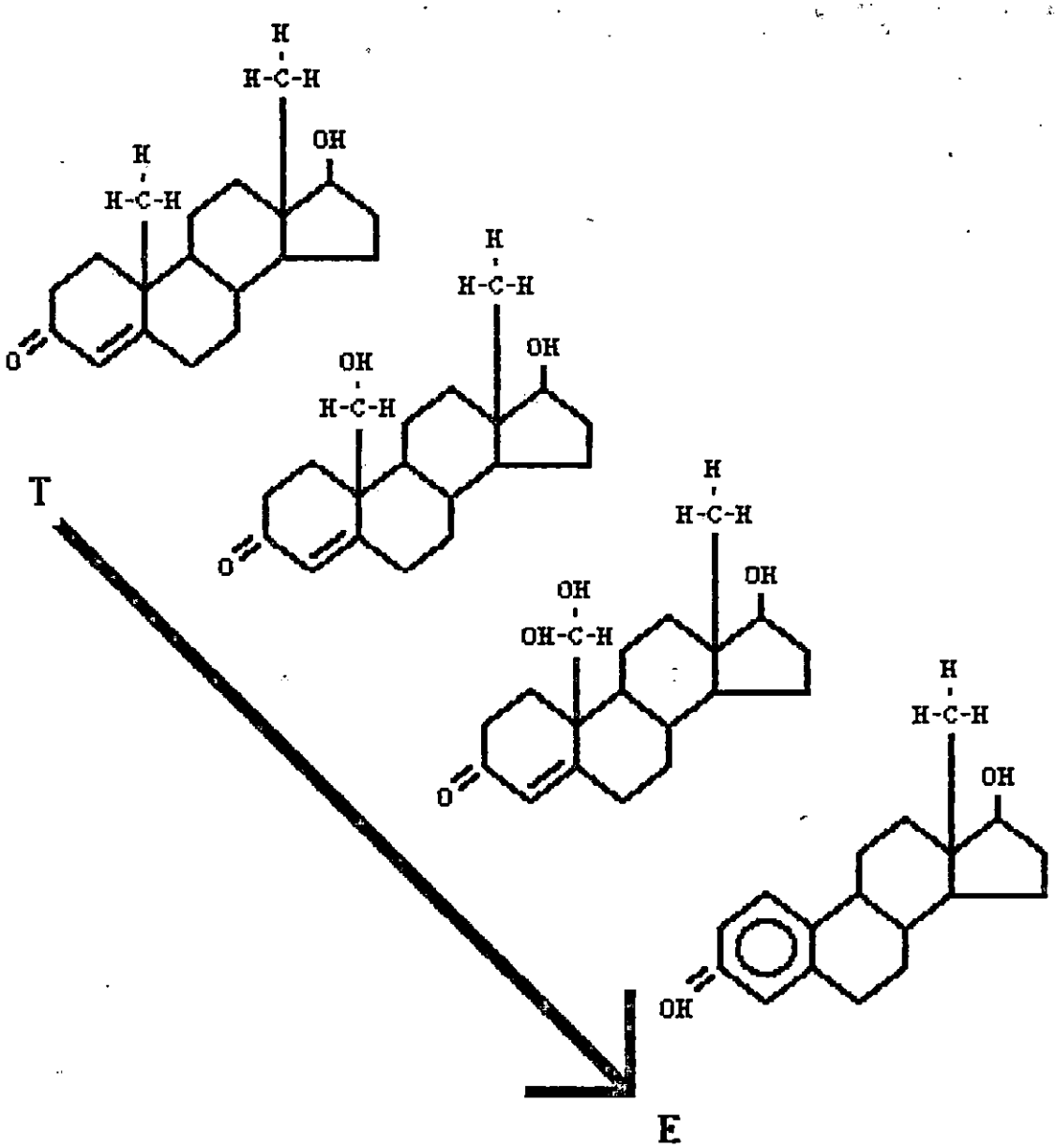

Figure 2. Aromatase Conversion of Testosterone to 17β-Estradiol. T, testosterone; E, 17β-estradiol. Intermediates formed are a 19-hydroxy-4-en-3-one and a 19, 19-dihydroxysteroid.

testosterone in the male and is absent in the female, indicating that it is linked to the presence of the testes and the testosterone surge (19). Third, autoradiographic studies show extremely similar patterns of labelled hypothalamic cells between neonate rats injected with tritiated estradiol or testosterone (31). Fourth, Toran-Allerand has shown that in vitro morphological changes produced in critical period hypothalamic cells by testosterone can be duplicated by estradiol (47). Fifth, evidence suggests that estradiol is, in terms of dosage administered, up to eight times as potent as testosterone in inducing certain aspects of androgenization (2).

Alpha-Fetoprotein and the Protection Hypothesis

Acceptance of the aromatase hypothesis directly implies the presence of a mechanism that serves to protect the genetic female's hypothalamus from being androgenized by endogenous estrogen from the placenta, maternal circulation, and the fetal endocrine system. In rats and mice the mechanism has been elucidated. In these two species, the developing yolk sac and fetal liver synthesize an estrogen-binding protein (alpha-fetoprotein, AFP), which circulates at high levels during the latter part of gestation through critical period and gradually disappears over the next few weeks of life (48). AFP binds and effectively sequesters much of the estrogen present in the neonate circulation, preventing its interaction with the neural substrate. Testosterone, however, is not bound by AFP and is thus free to enter the brain, where it is converted by neural aromatase to 17β-estradiol. Since this estrogen is now sequestered from the peripheral AFP protection mechanism, it may then interact with cellular estrogen receptors (Figure 3).

Several studies have provided evidence that confirms the effectiveness of the AFP

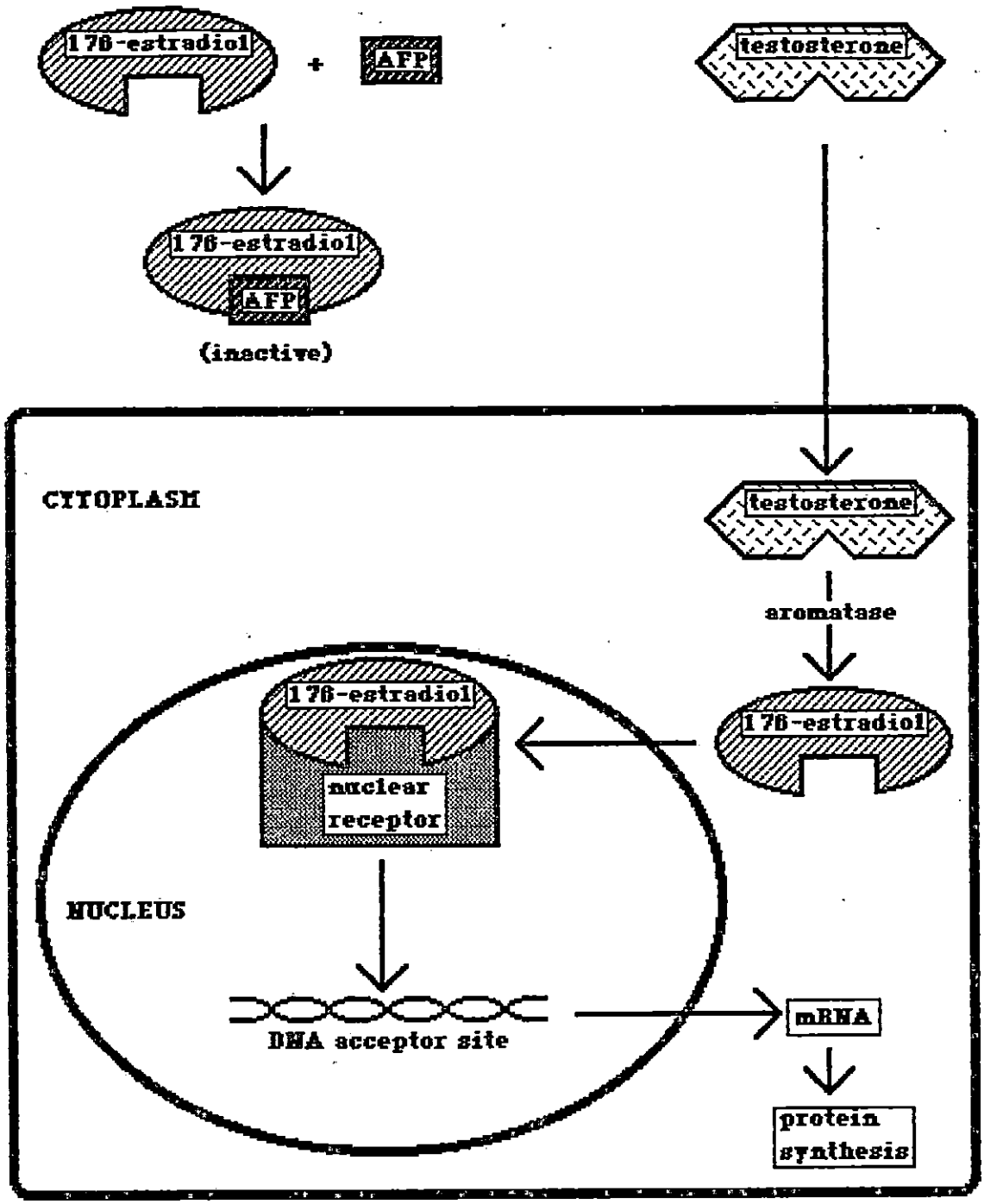

Figure 3. Alpha-Fetoprotein Protection Hypothesis. AFP, alpha-fetoprotein.

protection mechanism. First, the administration of AFP antibodies to neonate female rats mimics the effects of estradiol injections (49). Second, even though neonatal serum estradiol levels are high, free estradiol does not seem to be available to the tissues (50). Third, the synthetic estrogens Ru 2858 and diethylstilbestrol (DES) have considerably lower affinities for AFP than estradiol, and are more potent than estradiol in inducing differentiation of the female rat brain (51).

The AFP protection hypothesis may not yet be extended to animals other than the rat and mouse. While AFP is present in many higher vertebrates, there seem to be different variants of the molecule which have different estrogen binding properties. For example, in humans, only 0.1 percent of AFP in circulation actually binds estrogen (52), and it does not appear to be present at all in the guinea pig (53). It should be noted, however, that many elements of the aromatase hypothesis have been demonstrated in guinea pigs and primates. As in the rat, treatment of the guinea pig with estradiol as well as androgen can cause an androgenization of the hypothalamus (54), and aromatase has been shown to be present in the developing human brain (55). Thus, while in some species the AFP protection mechanism seems to be missing, this should not be construed as evidence indicating that the aromatase hypothesis does not apply, but rather that the protection mechanism is not known in these species. As was mentioned earlier, some investigators have proposed that progesterone may play a protective role in some species.

AFP has been found to be localized within developing nerve cells. It is interesting that areas that concentrate tritiated estradiol are devoid of this intracellular AFP, suggesting that AFP might also act as a local mediator of estrogen action (56).

Possible Cellular Events During Critical Period Sexual Differentiation

There are several potential primary cellular changes which may be evoked by hormonal stimulation of the CNS, including steroidally-mediated cell growth and cell death, re-initiation of mitotic activity, changes in and/or induction of cell migration, and changes in synaptic connection (4,57). Of these, McEwen suggests that models involving cell growth and death are the most attractive and likely explanations of the mechanisms involved in sexual differentiation of the brain and that morphological sex differences in the brain (see Table 2) may arise from a combination of these two cellular events (4). Cell growth and death could be mediated in one of, or a combination of, several specific ways. First, perinatal hormones could selectively induce the death of neurons directly or, conversely, cause a stabilization of these neurons. Second, simple hypertrophy could be induced, with resulting changes in cell size, spread of dendritic branching and, ultimately, neural circuitry. Third, hypertrophy could be coupled with cell survival: those cells successful in establishing sufficient connection with other cells would survive through the developmental period, while cells that do not would be preferentially eliminated (58). Gorski et al. (59) have shown that the Sexually Dimorphic Nucleus of the Preoptic Area (SDN-POA) in the rat hypothalamus has a gross volume in the male that is approximately four times that of the female, and that this size difference is mediated by the perinatal hormonal environment. Furthermore, this volume difference is due to an actual difference in the size and number of neurons in the male rat's SDN-POA. Jacobson has proposed that hormonally-induced hypertrophy may be of importance in the development of this nucleus. Thus, a group of neurons in the nucleus may be hormonally induced to hypertrophy in the male and thus survive, while these same neurons in the female's SDN-POA are eliminated (57). This

Table 2. Androgen-Dependent Sexual Dimorphism in the CNS. Data from MacLusky and Naftolin (1), except the Sexually Dimorphic Nucleus of the Preoptic Area (SDN-POA), taken from Gorski et al. (59).

Sex Difference	Region	Animal
Neuronal nuclear and nucleolar size	Preoptic area, amygdala, ventromedial hypothalamus	Rat
Synaptic vesicles and terminals	Arcuate	Rat
Synaptic organization	Preoptic area	Rat
Dendritic branching patterns	Preoptic area:	
	Suprachiasmatic nucleus	Hamster, rat
	Hippocampus (Ammon's Horn)	Mouse
Gross nuclear volume	Preoptic area:	
	SDN-POA	Rat
	Lumbar spinal cord	
	Nucleus robustus archistriatalis, nucleus hyperstriatum ventrale pars caudalis	Zebra finch

is a particularly interesting model, considering that a rat is born with many more neurons than it will possess as an adult, and that the loss of neurons is a normal part of post-partum brain development (2).

The hypothesis that steroidally-mediated hypertrophy may act as a mechanism of sexual differentiation of the brain is strongly supported by the in vitro studies performed on hypothalamic explants from neonate mice performed by Toran-Allerand (47). These studies revealed that treatment of critical period tissue with either testosterone or estradiol resulted in

a dramatic neurite outgrowth with extensive arborization of the new processes in a dose-dependent fashion. This hormonally-stimulated growth involves only small groups of neurons; not all cells in a given nucleus appear to be steroid-sensitive. Thus, these in vitro effects appear to be highly specific morphologically, with the most sensitive neurons located in the estrogen-receptor rich anterior preoptic and infundibular-premammillary regions.

Female Sex Behavior

Introduction of a normal, sexually receptive female into the home cage of a normal, sexually vigorous male rat results in the following behavioral events, usually occurring sequentially. The male investigates the female, particularly her anal-genital region. The receptive female displays several solicitory behaviors, the more obvious of which are rapid quivering of the ears and abrupt darting across the cage, usually with the male in pursuit. At intervals the male mounts the female, palpates her flanks with his forelegs, and exhibits pelvic thrusting as he attempts intromission. For the male to achieve intromission the female exhibits a characteristic behavioral response called lordosis. During lordosis the female deflects her tail laterally and arches her back so that both her head and perineum are elevated. After a brief mount the male dismounts, the behavior of which depends on whether or not he has ejaculated (which usually occurs only after a series of intromissions, and is followed by a brief refractory period). High levels of sexual receptivity can be induced in intact or ovariectomized females by estrogen-progesterone priming, and some female sex behavior may be elicited from the castrated male by the same procedure (60). In many studies quantification of female sex behavior (and thus, as in this study, quantification of degree of defeminization) is accomplished

by the lordosis quotient, which is the number of lordosis responses divided by the number of mounts, multiplied by 100 (60).

Possible Androgen Receptor Involvement in Defeminization/Masculinization

Despite the previously mentioned massive accumulation of evidence indicating that defeminization is dependent upon the estrogen receptor, some recent research suggests that the androgen receptor may also play a role in sexual differentiation of the CNS. Gladue and Clemens (10) report that long-term (days 10-22 post-conception) prenatal exposure to the non-steroidal anti-androgen flutamide can attenuate the defeminization process. Olson (61) has shown that female sex behavior can be attenuated by postnatal treatment with the synthetic anti-androgen R-1881, which is believed to bind to androgen receptor and is not metabolized to other androgens or estrogens.

Additionally, Meany et al. (62) have shown, with studies utilizing flutamide and androgen-insensitive testicular feminization mutation (Tfm) males, that the sexual differentiation of social play (a masculinization event) is very probably mediated by the androgen receptor system.

Flutamide

In the initial biological profile, Neri states that flutamide is a highly potent anti-androgen with no androgenic, estrogenic, anti-estrogenic, corticoid, progestational, anti-progestational, or anti-gonadotrophic properties (63). However, it has been shown that prenatal exposure to flutamide can attenuate defeminization in the rat (10). On the basis of

research by Gladue et al. comparing the effects of flutamide and an anti-aromatase on hormonally-mediated behavior (64), McEwen has suggested that the effects of flutamide on defeminization are due to anti-aromatase activity (4). As this drug is becoming more commonly used in sexual differentiation research, clarification of this point is needed to avoid data misinterpretation.

Clomiphene

The effects of clomiphene, historically described as an anti-estrogen (65), are enormously complex and poorly understood. In humans, low doses of clomiphene are commonly used to induce ovulation by antagonistic blockade of estrogen-mediated negative feedback mechansims. However, at high doses clomiphene exerts an estrogenic effect upon this system, and a review of the literature provides evidence that clomiphene may act as an estrogen agonist, an antagonist, or both depending on the species of animal, the tissue, and the dosages administered (66).

A recent study by Bowman et al. (67) on the effects of clomiphene upon estrogen-mediated events in the uterus, pituitary and brain of the adult female Sprague-Dawley rat indicated the following. Pure agonism was demonstrated for food intake regulation and body weight, while pure antagonism was demonstrated for female sexual behaviour, prolactin secretion and possibly the production of uterine luminal fluid. Partial agonism was observed upon FSH and LH secretion and uterine weight. Also, an attempt was made to correlate these findings with 'cytoplasmic' receptor data previously obtained by Kurl and Morris (68). Even though there is a tremendous amount of evidence that clomiphene exerts its effects through the

estrogen receptor system by high affinity binding to the receptor with resulting depletion of the 'cytoplasmic' form of receptor (68,69,70,71,72,73,74), no consistent relationship between the receptor distributions observed by Kurl and Morris and the final effects observed by Bowman et al. were found.

Clark and Guthrie (66), in studies performed upon rat uterus, suggest that clomiphene exerted both agonistic and antagonistic effects over the same doses. This conclusion was arrived at by noting that clomiphene alone had an estrogenic effect upon uterine weight, but, at the same dosage, attenuated the effects of additionally injected estrogen benzoate. On the basis of this and other data, Clarke and Guthrie state that generalized statements regarding the estrogenicity or anti-estrogenicity of clomiphene are invalid.

Research by Adashi et al. (69) indicates that, while nuclear processing in the rat uterus is routinely observed between one and five hours after a single injection of estradiol, a 10.0 mg dose of clomiphene results in persistent, dose related accumulation of receptor in the nucleus for at least 14 days. It was determined that this phenomenon was the result of prolonged retention of 'nuclear' receptor rather than a slow release from the injection site. It was also determined that clomiphene induces the synthesis of estrogen receptor in uterine tissue. Similar receptor results were found in the pituitary, but only very short-lived alterations in receptor distribution could be discerned in the hypothalamus. While experiments using 0.5 and 5.0 mg/kg dosages of clomiphene in adult rats indicated that such CNS mediated parameters as food intake and body weight were significantly affected (67), brain cytoplasmic receptor studies indicated that clomiphene does not enter the brain of the adult rat at these same dosages (68).

Only one published study in the rat examines the behavioral effects of clomiphene following neonatal administration (75). No clomiphene treated animals in this study were ever observed to lordose over multiple trials, while controls exhibited a mean lordosis quotient of 66.1. Poor design, however, weakens these findings. First, no animals were gonadectomized, a procedure that allows for some degree of standardization of the hormonal milieu and aids in relating data to changes imposed upon the brain rather than changes imposed upon the gonads. Second, this study did not make use of estrogen-progesterone priming, which is normally used in sex differentiation studies. Third, the article states that "The most probable way in which the drug acts in this study is for it to have gone to estrogen receptors and there either modified the receptor shape or permanently bonded to it," without any mention of the aromatase theory or the probable role of the estrogen receptor in sexual differentiation of the CNS.

Research Objectives

In this study, neonate rats were subjected to a variety of injections in order to further elucidate the effects of progesterone, clomiphene and flutamide, and to clarify the role of the androgen receptor in differentiation of the CNS. Testing of the following hypotheses was accomplished by examination of the adult behavior and hypothalamic morphology of these animals.

Progesterone. While alpha-fetoprotein is certainly the primary mechanism responsible for protecting the hypothalamus in the rat, it does not seem to be in other animals (52,53). Progesterone has been suggested as a possible mechanism in these other species (1,2,4,9). Additionally, research performed by Kinci and Maqueo suggests that progesterone plays a

protective role in the rat (22).

It was hypothesized that progesterone could exert an attenuatory effect upon defeminization in the rat.

Clomiphene. Only one study in the rat on the behavioral effects of neonatal treatment with clomiphene has been published (75). and the results of this study are weakened by poor experimental design.

It was hypothesized that clomiphene acted as a powerful estrogen agonist upon neonatal hypothalamic tissue.

Flutamide. There is evidence from which it has been inferred that flutamide has anti-aromatase activity (10,64). As this drug is coming into more common use in the investigation of the role of the androgen receptor in differentiation of the CNS. it is important that this possibility be investigated.

It was hypothesized that flutamide has anti-aromatase activity.

The Androgen Receptor. There is a growing body of evidence that the androgen receptor system may play a role in differentiation of the CNS (10.61.62).

It was hypothesized that the androgen receptor could affect the defeminization and/or masculinization of the hypothalamus.

MATERIALS AND METHODS

Animal Treatment

Neonate rats of the Sprague-Dawley strain were obtained from the Lappin Hall colony of Morehead State University. On the day of birth, animals were sexed and assigned to one of 14 experimental groups or a control group. Animals were then subjected to subcutaneous injections according to the schedule shown in Table 3. Testosterone propionate, estrogen benzoate, progesterone and clomiphene citrate were obtained from Sigma Chemical Company. Flutamide (4'-nitro-3'-trifluoromethylisobutyranilide) was graciously provided by the Schering Corporation. Animals were then housed at 22° C under a 14:10 day-night cycle (on at 0400 hours, off at 1800 hours) and supplied with water and Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.) ad libitum.

At 60 ± 5 days all animals were anesthetized with ether or pentabarbital (The Butler Co., Columbus, Ohio) and subjected to bilateral gonadectomy. Surgery was performed on females using a dorsal approach, and a medial, trans-scrotal approach was used on males. Blood vessels and muscle layers were tied with 4-0 chromic. Skin closure on females was done with wound clips; males were closed with 4-0 silk, using an interrupted stitch. Immediately following surgery, all animals were given a single IP injection of penicillin (3.0 mg in 0.5 ml sterile saline). The animals were then allowed a two-week recovery period before behavioral testing.

Behavioral Testing

All animals were given a 10.0 µg IM injection of estrogen benzoate in 0.1 ml peanut oil

Table 3. Schedule of Treatments Administered to Animals During Critical Period.

Group Number	Sex	N	Treatment	Day(s) Post-Partum Injections Given
1	male female	3 3	1.0 mg testosterone propionate	1 & 6
2	male female	4 4	0.25 mg estrogen benzoate	1 & 6
3	male female	5 5	0.25 mg clomiphene	1
4	male female	5 5	1.0 mg progesterone	1, 3, 5, 7, & 9
5	female	5	0.1 ml propylene glycol followed by 1.0 mg testosterone propionate	1
6	female	5	0.5 mg flutamide followed by 1.0 mg testosterone propionate	1
7	male female	6 5	0.5 mg flutamide	6
8	male female	5 5	0.5 mg flutamide	1 & 6
9	male female	4 7	1.0 mg progesterone	1
10	male female	8 3	1.0 mg progesterone	6
11	male female	5 6	0.5 mg flutamide followed by 0.25 mg estrogen benzoate	1 & 6

Table 1. (Continued From Previous Page).

Group Number	Sex	N	Treatment	Day(s) Post-Partum Injections Given
12	male female	3 6	0.1 ml peanut oil only	1
13	male female	4 0 (a)	2.0 mg flutamide followed by 0.25 mg estrogen benzoate	1 & 6
14	male	11	0.25 mg clomiphene	1 & 6
15	male	3	0.25 mg clomiphene & 0.5 mg flutamide	1 & 6

Note. Total N = 125.

Note. Flutamide was administered in 0.1 ml of propylene glycol. Clomiphene citrate was administered in 0.1 ml sterile saline. All other treatments were administered in 0.1 ml peanut oil.

(a) While it had been planned to include females in this group, it proved impossible to do so because of their extremely high death rate.

at -72, -48 and -24 hours. Four hours prior to testing, a 500.0 µg IM injection of progesterone was administered. Actual testing commenced at 1830 hours, 30 minutes after the beginning of the animals nocturnal cycle, and normally continued (three animals at a time) until 0300 hours, 60 minutes prior to the end of the nocturnal cycle. Testing took place in clear plexiglass cages under dim red lighting, which has been shown to provide minimum interference with the animals' normal nocturnal behavior (60).

Lordosis behavior (which is indicative of the degree of defeminization) was quantified for each animal using the lordosis quotient: LQ = frequency of lordosis in response to mounts

÷ number of mounts x 100. Animals were placed in the home cage of a sexually vigorous male. Testing was begun after a 10 minute adaptation period, or upon the first mount that stimulated lordosis, whichever came first. Testing continued for ten mounts or for 45 minutes. If no mounts and no signs of sexual receptivity occurred within the first 20 minutes, testing was discontinued and a lordosis quotient of 0 was recorded.

Evaluation of the Sexually Dimorphic Nucleus of the Preoptic Area

Within 24 hours of the completion of behavioral testing and while under light ether anesthesia, animals were first intracardially perfused with 0.9% saline and then with 10.0% formalin. The animals were then decapitated and the heads stored in 10.0% formalin for 24 hours. Utilizing a ventral aproach, the brains were then removed and placed in numbered culture tubes containing 10% formalin for at least two weeks.

Brains were embedded in paraffin, sectioned at 40.0 µm, and nissl stained with cresyl violet acetate (Sigma Chemical Company) according to the method of Powers and Clark (76). Two sections were selected on the basis of the surrounding anatomy as being the aproximate center of the SDN-POA (see Figure 4). These sections were projected onto a sheet of white paper, and the SDN-POA, along with any pertinent surrounding structures, were traced. A planimeter that had been callibrated with a similarly projected and traced 1.0 mm grid was used to obtain an average square mm measurement of the SDN-POA from those tracings.

Statistical Analysis

All data were first screened with SAS means and univariate procedures. 2-way ANOVA

and Duncan's Multiple Range Test were then performed on all data using SAS General Linear Models (GLM) procedures. Results of $p \leq .05$ were considered to be significant.

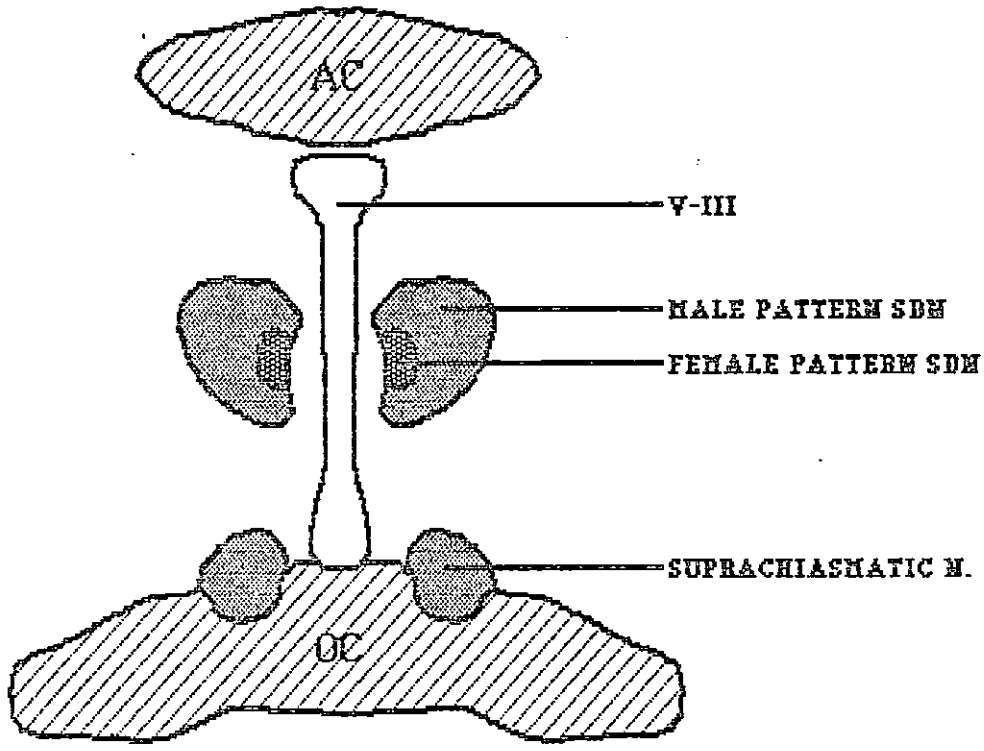

Figure 4. Illustration of Coronal Section Through the Approximate Center of the SDN-POA. AC, anterior commisure; OC, optic chiasma. V-III, third ventricle. Adapted from Gorski et al. (59).

RESULTS

Analysis of Defeminization

The LQ data were initially screened with SAS means and univariate procedures (see Table 4 and Figure 5 for partial output). Since this screening revealed no violations of the mathematical requirements for analysis of variance, a two-way ANOVA was performed on the LQ data. This test indicated that the effect of treatment was significant, $F = 5.9$, $p = .0001$. The sex of the animal also had a significant effect, $F = 177.222$, $p = 0.0001$. However, the treatment group by sex interaction was also significant, $F = 11.75$, $p = 0.0001$, thus precluding interpretation of these effects (see Table 6). The interaction was analyzed by means of a Duncan's post hoc test, the full results of which are shown in Table 8. A simplified summary of those results is given in Table 9.

Progesterone. The group 4, female results indicate that 1.0 mg doses of progesterone given on days 1,3,5,7 and 9 post-partum can have a significant defeminization effect (Table 8, summary Table 9). However, since neither group 9 or 10 (1.0 mg of progesterone on day 1 or 6, respectively) achieved statistical significance (Table 8), group 4 results may be a pharmacological effect.

Clomiphene. The group 3, female results indicate that a single, 0.25 mg injection of clomiphene given on day 1 post-partum can have a significant defeminization effect (Table 8, summary Table 9). Considering the role of the estrogen receptor in differentiation of the CNS, this data indicates that clomiphene acted as an extremely potent estrogen agonist.

Flutamide and the Androgen Receptor System. In order to determine if flutamide

exerted anti-aromatase effects, one group of females were treated with 0.5 mg flutamide followed by 1.0 mg testosterone propionate (group 6), and another group was treated with testosterone propionate only (group 5). Group 6 was found to be significantly less defeminized than group 5, indicating that flutamide significantly attenuated testosterone propionate-induced defeminization (Table 8, summary Table 9).

Additionally, comparison of group 11 and group 2 animals (respectively, 0.5 mg flutamide followed by 0.25 mg estrogen benzoate on days 1 & 6 post partum, and 0.25 mg estrogen benzoate only on days 1 & 6 post-partum) revealed no significant difference between males or females (Table 8). This suggests that flutamide does not affect estrogen benzoate-induced defeminization. Group 13, male (which was treated with four times the dose of flutamide used in group 11, followed by 0.25 mg estrogen benzoate) was also not significantly different from group 2, male (Table 8), again suggesting that flutamide is not capable of attenuating defeminization when a supply of estrogen is available to the animal for this process.

Group 7, male (0.5 mg flutamide on day 6 post-partum) evidenced no significant attenuation of defeminization in comparison to control males (Table 8), suggesting that this effect of flutamide may be limited to early critical period. Group 8, male (0.5 mg flutamide on days 1 & 6 post-partum), while not significantly more feminine in their behavior than controls, did show a higher (22 as compared to 15.8) LQ mean (Table 8).

No significant differences were found between testosterone propionate- and estrogen benzoate-defeminized animals (Table 8, groups 1 and 2).

Evaluation of the SDN-POA

The SDN-POA data were initially screened with SAS means and univariate procedures (see Table 5 and Figure 6 for partial output). Although this screening revealed several violations of the mathematical requirements for analysis of variance, a two-way ANOVA was nonetheless performed on the SDN-POA data. This test indicated that the effect of treatment groups was not significant, $F = 1.25$, $p = 0.33$. The effect of sex was also not significant, $F = 3.88$, $p = 0.065$ (see Table 7). However, it should be noted that the total N for this data was only 38, which strongly increases the probability of type II error.

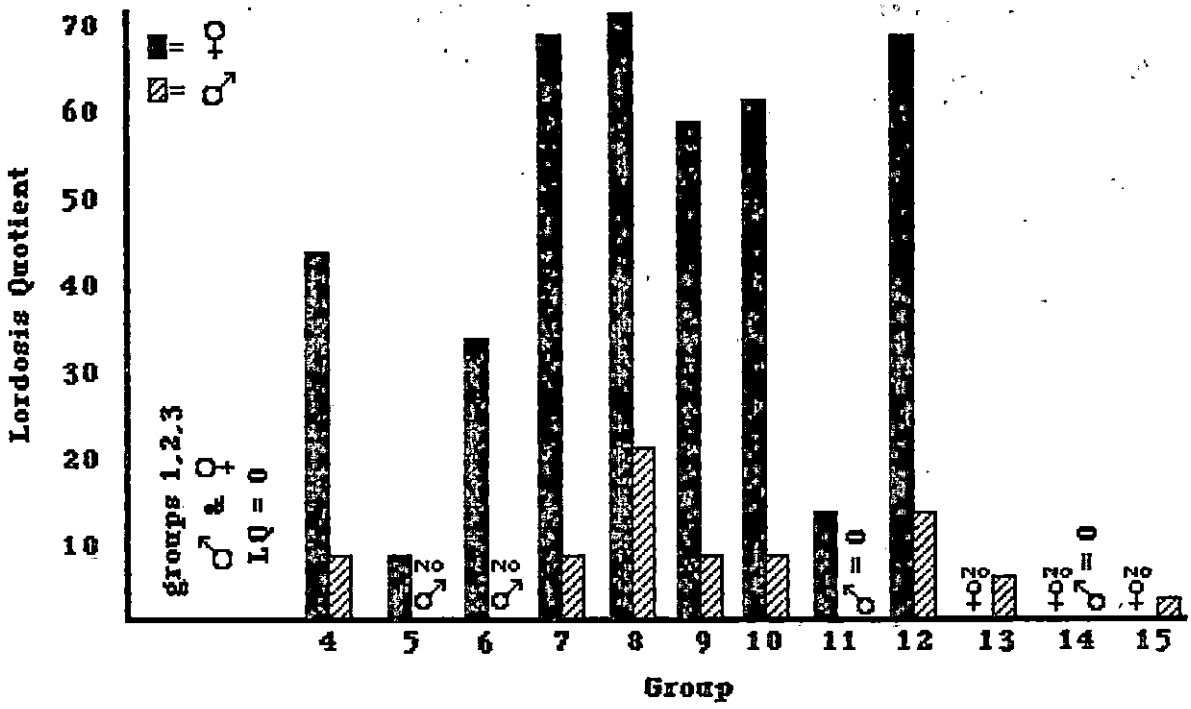

Figure 5. Bar Graph of Group LQ Means.

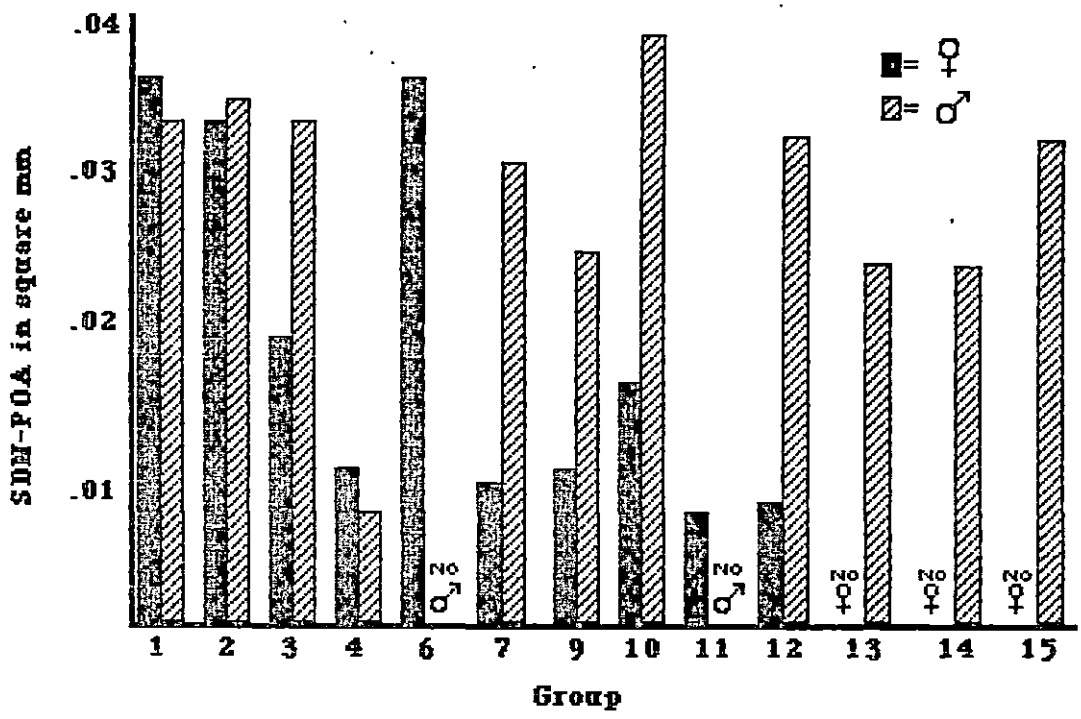

Figure 6. Bar graph of Group SDN-POA Means.

Table 4. SAS Statistics Output on the Lordosis Quotient Data. SEM, standard error of the mean; CV, coefficient of variation. The CV is the ratio between the standard deviation of the sample and the mean of the sample expressed as a percent.

Group	Sex	N	Mean	Std. Deviation	SEM	CV
1	F	3	0	0	0	
1	M	3	0	0	0	
2	F	4	0	0	0	
2	M	4	0	0	0	
3	F	5	0	0	0	
3	M	5	0	0	0	
4	F	5	41.22	6.03	2.70	14.62
4	M	5	5.32	8.69	3.89	163.34
5	F	5	10	17.32	7.75	173.21
6	F	5	33.32	21.71	9.71	65.14
7	F	5	68	8.37	3.74	12.30
7	M	6	7.92	10.05	4.10	126.97
8	F	5	70	24.49	10.95	34.99
8	M	5	22	13.04	5.83	59.27
9	F	7	55.71	12.72	4.81	22.83
9	M	4	6.25	7.22	3.61	115.41
10	F	3	60	10	5.77	16.67
10	M	8	5.54	8.10	2.86	146.26
11	F	6	11.10	11.87	4.85	106.92
11	M	5	0	0	0	
12	F	4	67.50	17.08	8.54	25.30
12	M	3	15.83	16.71	9.65	105.54
13	M	4	5	10	5	200
14	M	10	0	0	0	
15	M	3	2.20	3.81	2.20	173.21

Table 5. SAS Statistics Output on the SDN-POA Data. SEM, standard error of the mean; CV, coefficient of variation. The CV is the ratio between the standard deviation of the sample and the mean of the sample expressed as a percent. SDN in square mm.

Group	Sex	N	Mean	Std. Deviation	SEM	CV
1	F	1	0.036			
1	M	1	0.032			
2	F	1	0.032			
2	M	2	0.036	0.0057	0.0040	15.713
3	F	2	0.018	0.0078	0.0055	44.447
3	M	1	0.032			
4	F	1	0.012			
4	M	2	0.009	0.0042	0.0030	47.140
5	F	0				
6	F	2	0.036	0.0057	0.0040	15.713
7	F	1	0.012			
7	M	4	0.030	0.0185	0.0093	61.704
8	F	0				
8	M	0				
9	F	2	0.011	0.0014	0.0010	12.856
9	M	1	0.024			
10	F	1	0.016			
10	M	3	0.043	0.0197	0.0114	45.750
11	F	2	0.009	0.0042	0.0030	47.140
11	M	0				
12	F	2	0.011	0.0014	0.0010	12.856
12	M	1	0.032			
13	M	1	0.024			
14	M	6	0.024	0.0142	0.0058	58.157
15	M	1	0.032			

Table 6. ANOVA Summary: Dependent Variable - LQ. Total N - 122.

Source	DF	Type IV SS	F Value	PR > F
Group	14	9918.91	5.90	0.0001
Sex	1	21285.60	177.22	0.0001
Group by Sex	9	12703.64	11.75	0.0001

Table 7. ANOVA Summary: Dependent Variable - SDN-POA. Total N - 38

Source	DF	Type IV SS	F Value	PR > F
Group	12	0.0026	1.25	0.3305
Sex	1	0.0007	3.88	0.0653
Group by Sex	7	0.0006	0.47	0.8416

Table 8. Full Results of SAS Duncan's Multiple Range Test for LQ. Alpha = 0.05. DF = 97. MSE = 120.11. This chart is interpreted by comparing the letters listed for any two means. If the two means being compared have one or more letters in common, they are not significantly different.

Grouping		Mean	N	Group	Sex
	A	70	5	8	F
	A	68	5	7	F
	A	67.50	4	12.(CONTROL)	F
	A	60	3	10	F
B	A	55.71	7	9	F
B	C	41.22	5	4	F
D	C	33.32	5	6	F
D	E	22	5	8	M
F	E	15.83	3	12.(CONTROL)	M
F	E	11.10	6	11	F
F	E	10	5	5	F
F	E	7.92	6	7	M
F	E	6.25	4	9	M
F	E	5.54	8	10	M
F	E	5.32	5	4	M
F	E	5	4	13	M
F		2.20	3	15	M
F		0	3	1	F
F		0	4	2	F
F		0	5	3	M
F		0	5	3	F
F		0	10	14	M
F		0	3	1	M
F		0	5	11	M
F		0	4	2	M

Table 9. SAS Duncan's Multiple Range Test for LQ Summary. The groups listed below consist of females only; no male groups achieved statistical significance. Groups with the symbol ' are significantly defeminized in comparison to controls. Groups with the symbol * are significantly different from each other. C, clomiphene; EB, estrogen benzoate; F, flutamide; P, progesterone; PG, propylene glycol; TP, testosterone propionate.

Group Number	N	Treatment	Day(s) Post-Partum Injections Given
'1	3	1.0 mg TP	1 & 6
'2	4	0.25 mg EB	1 & 6
'3	5	0.25 mg C	1
'4	5	1.0 mg P	1,3,5,7 & 9
*'5	5	0.1 ml PG followed by 1.0 mg TP	1
*'6	5	0.5 mg F followed by 1.0 mg TP	1
'11	6	0.5 mg F followed by 0.25 mg EB	1 & 6

Note. In some cases, groups which did not achieve statistical significance were of importance in the interpretation of the results of this study. A discussion of these cases is included in the text of this chapter.

DISCUSSION

Progesterone

The hypothesis that progesterone could attenuate defeminization in the rat was rejected. This conclusion contradicts the work of Kincl and Maqueo (22), whose results (obtained from neonatal treatment of female rats) suggest that progesterone can protect the neonatal rat hypothalamus from the effects of testosterone. Differences in the results obtained by Kincl and Maqueo and those obtained in this study may be explained by the considerable differences in the experimental protocol used to examine the effects of progesterone. Kincl and Maqueo administered much smaller doses of testosterone propionate (50.0 and 100.0 µg) and much greater doses of progesterone (3000.0 µg) than those used in this study. Additionally, the rats were not subjected to these treatments until day 5 post-partum, and the effects were interpreted purely on the basis of whether or not the animals' ovaries appeared to be luteal on day 45 post-partum.

Although this study does not provide any evidence that progesterone can protect the rat hypothalamus against the effects of testosterone, it does not eliminate the possibility that progesterone could be of importance in other animals, or could have effects in the rat different from those found in this study at different dosages. Progesterone is transformed to 5-alpha-dihydroprogesterone by 5-alpha-reductase, the enzyme which transforms testosterone to DHT. This fact, combined with possible sex differences in serum progesterone in some species, has led several researchers to suggest that progesterone may play a role in differentiation of the CNS (1,2,4,9). The presence of ovaries has been shown to inhibit the

defeminizing effects of testosterone in the rat (21). A future study should attempt to determine if this effect is due to progesterone by providing a careful examination of the effects in the rat of progesterone in physiological, rather than pharmacological, quantities. Additionally, an examination of the effects of progesterone in the guinea pig would be particularly commendable as a future study, considering this animal's apparent lack of alpha-fetoprotein (53).

Clomiphene

The estrogenic/anti-estrogenic effects of clomiphene are enormously complex and poorly understood. There is evidence that clomiphene may act as an estrogen agonist, an antagonist, or both depending on the species of animal, the tissue, and dosages administered (66,67). It is believed that clomiphene exerts its effects through the estrogen receptor system by long-term, high affinity binding to the receptor (69), with resulting depletion of the 'cytosolic' form of the receptor (68,69,70,71,72,73,74).

Animals treated with clomiphene in this study were highly defeminized, as evidenced by their failure to lordose (Table 8, summary Table 9). This suggests that, at a dosage of 0.25 mg, this drug acted as an agonist upon neonatal rat hypothalamic tissue. It should, however, be noted that Clarke and Guthrie (66) suggest that clomiphene may act as both an agonist and an antagonist over the same doses in uterine tissue. They found that, while clomiphene alone had an estrogenic effect upon uterine weight, the same dosage would attenuate the agonism of injected estrogen. A similar situation could exist in the neonatal rat hypothalamus.

Some qualitative data of interest was obtained on clomiphene-treated animals. two of five males and three of five females in group 3 actively attacked the non-experimental male. A

similar behavioral observation was made in clomiphene-treated males of group 14, five of ten males actively attacking the non-experimental male. This level of aggression was not noted in testosterone or estrogen treated animals, and was surprising considering that these animals did not have any gonadally-produced testosterone available to them at the time of testing. While it is possible this was a behavioral result of the neonatal administration of clomiphene, another explanation may be derived from the fact that clomiphene seemed to cause considerable genital malformation and resulting infection of the genital region in these animals, and thus the aggressive behavior observed may have been the result of pain.

Despite the considerable differences in experimental protocol, these results are in basic agreement with those obtained by Fitzpatrick et al., who also found that a complete repression of lordosis behavior followed the neonatal administration of clomiphene (75).

Flutamide and the Androgen Receptor System

This study shows that neonatal administration of flutamide can significantly attenuate testosterone-induced defeminization, and that this effect seems to be more pronounced during the first few days post-partum (Table 8, summary Table 9). Furthermore, flutamide does not seem to be capable of affecting estrogen-induced defeminization (Table 8). On the basis of research by Gladue et al. comparing the effects of flutamide and an anti-aromatase on hormonally-mediated behavior (64), McEwen has suggested that the effects of flutamide on defeminization are due to anti-aromatase activity (4). The results obtained in this study indicate that flutamide does indeed affect defeminization by exerting an inhibitory influence upon aromatase activity. However, on the basis of recent research by Roselli and Resko (77), I

suggest that this is not a direct inhibition of the enzyme, but rather a consequence of blocking the androgen receptor.

Roselli and Resko (77) have provided extremely convincing evidence that aromatase activity in the hypothalamic-preoptic area is controlled by testosterone through a receptor mechanism. Aromatase activity in this area of the adult, male rat brain fell by 60 percent following castration, and Silastic implantation of testosterone or DHT reversed the effect of castration. Neither progesterone or estradiol implantation affected the post-castration levels of aromatase activity. Furthermore, flutamide was found to block the testosterone or DHT induced reversal of post-castration aromatase levels, while having no effect when given alone to castrated rats. Research following the methodology of Roselli and Resko needs to be performed on neonatal tissue to determine if this mechanism of androgen receptor control of aromatase activity exists during critical period. If this is indeed the case, it would suggest an important role for the androgen receptor system in the regulation of defeminization, possibly acting as a local modulator of aromatase activity and thus defeminization. A model such as this could provide an explanation for the anti-defeminization effect of flutamide observed in this study, and in the research of Gladue and Clemens (10). This model could also explain the results obtained by Olson, who has shown that the anti-androgen R-1881 can attenuate defeminization (61).

No other evidence of androgen receptor influence upon defeminization was found in the lordosis studies, though it should be remembered that there is strong evidence of androgen receptor involvement in the development of masculine behavior. Meany et al. (62) have shown that the sexual differentiation of social play (a masculinization event) is dependent on the androgen receptor. It is unfortunate that the amount of data on the SDN-POA was so limited that

an a priori prediction of non-significance could be made. However, it is worth noting (bearing in mind that statistical significance was not achieved) that the SDN-POA seems to be masculinized by both testosterone and estradiol, indicating that this particular masculinization event involves aromatase.

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