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Hormonal influences on sexually differentiated behavior in nonhuman primates

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Abstract

Sexually dimorphic behavior in nonhuman primates results from behavioral predispositions organized by prenatal androgens. The rhesus monkey has been the primary primate model for understanding the hormonal organization of sexually dimorphic behavior. Historically, female fetuses have received high prenatal androgen doses to investigate the masculinizing and defeminizing effects of androgens. Such treatments masculinized juvenile and adult copulatory behavior and defeminized female-typical sexual initiation to adult estrogen treatment. Testosterone and the nonaromatizable androgen, 5α-dihydrotestosterone, produced similar effects suggesting that estrogenic metabolites of androgens are not critical for masculinization and defeminization in rhesus monkeys. Long duration androgen treatments masculinized both behavior and genitalia suggesting that socializing responses to the females' male-like appearance may have produced the behavioral changes. Treatments limited to 35 days early or late in gestation differentially affected behavioral and genital masculinization demonstrating direct organizing actions of prenatal androgens. Recent studies exposed fetal females to smaller doses of androgens and interfered with endogenous androgens using the anti-androgen flutamide. Low dose androgen treatment only significantly masculinized infant vocalizations and produced no behavioral defeminization. Females receiving late gestation flutamide showed masculinized infant vocalizations and defeminized interest in infants. Both late androgen and flutamide treatment hypermasculinized some male juvenile behaviors. Early flutamide treatment blocked full male genital masculinization, but did not alter their juvenile or adult behavior. The role of neuroendocrine feedback mechanisms in the flutamide effects is discussed. Sexually differentiated behavior ultimately reflects both hormonally organized behavioral predispositions and the social experience that converts these predispositions into behavior.

Keywords: Sexual differentiation; Primate; Rhesus monkey; Anti-androgen; Flutamide; Behavior; Aromatization; Genitalia; Copulatory behavior; Vocalization; Social behavior

1. Introduction

Nonhuman primates, like humans have a long developmental life span, live in complex social groups and exhibit striking sexually differentiated behavioral patterns both during development and in adulthood. Additionally, nonhuman primates have important biological similarities to humans, including a prenatal period of sexual differentiation, making them ideal for investigating basic mechanisms of sexual differentiation. Sexual differentiation of behavior has been investigated in few of the many nonhuman primate species, with the vast majority of studies using rhesus monkeys. While these studies have elucidated a great deal about sexual differentiation in a nonhuman primate, we know little if anything about the extent or the mechanisms of sexual differentiation of behavior in apes, new world primates, or nonmacaque species. However, the range of treatments investigated in rhesus monkeys and the diverse social conditions employed have revealed a number of important relationships that help frame research in other primate species, including humans. Thus this review primarily focuses on hormonal influences on sexual

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differentiation of behavior in rhesus monkeys, with other nonhuman primate species discussed where appropriate. Hormonal mechanisms of sexual differentiation in rhesus monkeys have been investigated in the context of a long history of studies on the role that hormones play in sexual differentiation.

The organizational hypothesis, the notion that androgens or their metabolites alter the developing nervous system during specific developmental periods permanently inducing behavioral characteristics of male and females, has become a central tenet of behavioral neuroendocrinology since the pioneering study of Phoenix et al. [78]. While specific details of hormonally induced organization continue to be debated [5,21], there is little doubt that exposure to steroid hormones during some period of developmental sensitivity permanently alters the responsivity of individuals to their environment. Most studies of the organizational effects of steroids on the sexual differentiation of behavior come from studies of altricial species who are born prior to complete neural differentiation, [101]; guinea pigs and nonhuman primates being the only precocial mammalian species whose sexual differentiation has been extensively studied. Whether this developmental distinction explains differences in sexual differentiation remains unresolved. There is little doubt, however, that these precocial species differ from the more typically studied altricial species in the timing of sexual differentiation and in the role of estrogenic metabolites of androgens in sexual differentiation [101]. This is of particular importance for considerations of human sexual differentiation, as the dominant rat and mouse models of sexual differentiation seem unlikely to apply to human sexual differentiation. In contrast, studies of nonhuman primates are likely to be more directly applicable to humans.

2. Basic processes of behavioral sexual differentiation

A cascade of physiological and cellular events leads to anatomical and psychological sexual differentiation of male and female mammals. Sex determination is the process by which the male or female pathway is selected and then sexual differentiation elaborates the chosen pathway [26]. Sexual differentiation is achieved through the processes of masculinization and defeminization that sculpt the developing fetus in a male or female direction. The sexual differentiation cascade is well understood, even if many molecular details remain to be elucidated. Similarly the evidence that sexual differentiation results from the processes of masculinization and defeminization is convincing.

Genes on the Y chromosome in males trigger a cascade of sexually differentiating events resulting from the expression of these genes, as well as autosomal genes [5]. A critical step in this process is the differentiation of the indifferent primordial gonad into testes as a result of products of the Sry gene on the Y chromosome interacting with products of the X chromosome genes, Sox9, and autosomal genes [54]. Without this system of genes, the gonad differentiates into an ovary. Testicular differentiation, and the activation of the hypothalamic–pituitary–gonadal axis (HPG), results in the production of testicular hormones which direct the differentiation of masculine and suppress feminine characteristics. This developmental cascade from gene expression to morphological and behavioral differentiation is the principle pathway for sexual differentiation, but nonhormonal pathways may also influence sexual differentiation [4]. Evidence from mice suggests that the Sry gene is transcribed in the developing male, but not female brain, raising the possibility of a direct effect of Sry transcripts on neural organization [55,67]. While these findings are intriguing and demonstrate that a full description of sexual differentiation will likely contain surprises, it is apparent that the actions of testicular hormones play a large and critical role. The role of steroid hormones on behavioral differentiation in primates is the focus of this chapter. This is preceded by a brief description of the cascade of differentiating events that testicular hormones influence.

Mammalian sexual differentiation is biased in a female direction [52], meaning that morphogenic processes produce female endpoints in sexual differentiation more easily than they produce male endpoints. This bias towards female endpoints in sexual differentiation has resulted in referring to the production of females as the "default" path, meaning this pattern occurs most easily. Unfortunately, others have equated default with passive or inactive and the term has become politicized and lost its original sense that masculine characteristics are imposed on an essentially female life-plan [52]. This concept is valuable as it implies that the failure of a process necessary to produce a male trait will lead to the creation of a female phenotypic trait instead. The converse is not true; that when a process necessary for full female sexual differentiation is blocked, a male characteristic arises. Thus, while there can be no doubt that female differentiation requires a suite of active morphogenic processes [21], it is also the case that male differentiation requires two specific processes that allow the male phenotype to emerge from what is essentially female-biased differentiation.

The nomenclature used to describe the processes through which sexual differentiation is achieved has been historically quite confusing. With little precision, terms like feminization and demasculinization have been used in descriptions of sexual differentiation even though there is little evidence to support their existence in mammals. Demasculinization, for example, would require the preexistence of a masculine characteristic that is eliminated or suppressed during sexual differentiation. While this term is appropriate in birds and other nonmamma-

lian species [53], such pre-existing masculine traits have not been demonstrated in mammalian sexual differentiation. Instead, investigators use demasculinization when they are actually describing the prevention of the masculinization of an underlying feminine trait [22]. The case for feminization is a bit better, when one is addressing sex differences in cognitive function [21], but is not yet applicable to differentiation of sexual behavior. Instead, it now seems likely that there are only two fundamental differentiating processes necessary to create a male: masculinization and defeminization. Masculinization imposes male-like characteristics on the developing organism, whereas defeminization suppresses female-like characteristics that would otherwise arise. These processes are involved whether the endpoints are anatomical or behavioral and operate in concert or independently.

This review refers to these two processes as underlying sexual differentiation:

A. Masculinization. The production of male-typical anatomical and behavioral characteristics. Anatomically, these would be the presence of penis, scrotum, testes, and internal Wolffian duct derivatives. Behaviorally, this refers to male-typical copulatory behavior, partner-preference, and male-typical patterns of infant vocalization, juvenile mounting and high energy expenditure play.

B. Defeminization. The suppression of female-typical anatomical and behavioral characteristics that would have differentiated without hormonal intervention. Anatomically this results in suppression of the development of uterus, fallopian tubes, portions of the vagina, and vaginal opening. Behaviorally, this refers to the suppression of infant vocalization patterns, and juvenile patterns of social affiliation and interest in infants. In adulthood, defeminization suppresses sexual receptivity (receptive defeminization) and female sexual initiation (proceptive defeminization).

The original notion for these separate processes came from anatomical investigations of sexual differentiation and is modeled after the differentiation of the Müllerian and Wolffian duct systems into the male or female internal reproductive organs. Since these internal nongonadal reproductive structures arise from separate male and female primordial structures, normal sexual differentiation needs to result in full development of only one duct system. Thus, errors in sexual differentiation can result in derivatives of either, both, or neither duct system. It remains unclear whether this anatomical model fully applies to behavioral differentiation. It is apparent from the widespread occurrence of bisexual characteristics in mammals [35] that it is possible for both masculine and feminine endpoints to co-exist suggesting that masculinization and defeminization may be operating on separate neural primordia underlying masculine and feminine behavioral traits.

2.1. Developmental stage at birth and sexual differentiation

Species vary widely in the extent of their development at birth. Following a distinction first developed in ornithology, species that are born with their eyes closed and lacking the capacity to function relatively independently at birth are termed altricial while species with more completely developed offspring are termed precocial.

Many of the most often studied species are those where sexual differentiation is only partially completed at birth. In these species a significant portion of the differentiating process occurs when the offspring is no longer attached to the maternal circulation. Typically, many neural systems in these species are not fully developed at birth and they are born with their eyes closed, poor motor coordination, and without the capacity for thermoregulation. Although the gonad and duct systems have differentiated in utero, the external genitalia have only begun to differentiate and distinguishing males from females typically requires measuring the distance from the penile/clitoral glans to the anus,(ano-genital distance), which is longer in males than females.

Whether a species has altricial young follows no clear phylogeny. Species as diverse as rats and ferrets produce altricial young. While altricial offspring are characteristic of many laboratory rodents, not all rodents are altricial. The guinea pig is the principle exception. It appears in some orders, such as the carnivores, that altricial offspring are the rule.

Altricial and precocial species probably reflect two different reproductive strategies that are expressed in different patterns of neural development [23]. In general precocial species are characterized by a greater brain to body-weight ratio than are altricial species [73]. In addition, a substantially greater portion of brain development occurs in utero in precocial than in altricial species. This difference in development may reflect a life-history difference in that altricial species produce young rapidly with short maturation times and rapid brain growth [57]. In contrast, precocial species develop more slowly, even though both species may reach maturity at comparable times [92].

3. Sexually differentiated behavior in rhesus monkeys

Rhesus monkeys are born with their genitals and internal reproductive anatomy completely differentiated. Unlike altricial species, like rats, hamsters, and mice, morphological sexual differentiation occurs prenatally in rhesus monkeys and likely apes and old world primates in general. Monkeys have an approximately 168 day gestation with the testes differentiating between gestation day 38 and 40 [85]. Fetal testes become steroidogenically active around gestation day 40 and secrete androgens

throughout gestation with peak levels at gestation days 40–75 then declining for the rest of gestation with another apparent increase around gestation day 140 [85]. Throughout the prenatal period males experience significantly higher levels of testosterone (T), though there are no apparent differences between the sexes in either 5α-dihydrotestosterone (DHT) or androstenedione [84,85]. The fetal ovaries are apparently quiescent at this time since females show significantly elevated LH levels in comparison to males and luteinizing hormone (LH) levels are suppressed by exogenous T in fetally ovariectomized females [18]. Thus fetal males are exposed to elevated levels of T from their own testes and females are exposed to lower, but quantifiable T levels, presumably of maternal origin since the fetal ovary is inactive during this time.

As is the case in humans, rhesus monkeys have a period of infant and juvenile dependency and development, where behavioral predispositions fully develop. Rhesus monkeys develop about four times more quickly than do humans (e.g., developmentally, a rhesus monkey year equals four human years). Thus, while sharing many important features with humans; rhesus monkeys make a practical and valuable model system for investigating behavioral sexual differentiation.

Rhesus monkeys display several sexually differentiated patterns of juvenile and adult behavior [59,98]. The primary sexually differentiated behavioral patterns are higher levels of juvenile mounting and high energy expenditure play (rough play) in males and higher interest in infants [47] and greater association with adult females in juvenile females [59,98]. In addition, infant vocalizations have been found to be sexually differentiated [94]. In adult rhesus monkeys, play rarely occurs, but adult males display greater levels of mounting, now accompanied by intromissions and ejaculations, whereas females show increased interest in infants [60] and higher levels of sexual initiation [60,106]. Vocalizations continue to be sexually differentiated with the primary sex difference being volubility with adult males rarely vocalizing and females displaying routine vocalization [94].

4. Social influences on behavioral sexual differentiation

Social context and rearing conditions affect the expression of infant and juvenile sex differences. A previous review of social influences on sexually differentiated behavior concluded that some patterns of sexually differentiated behavior differ between males and females almost completely as a result of social context and rearing history. In contrast, other behaviors appear to differ between the sexes across a range of a social and rearing conditions [98]. This review focuses on those behavioral patterns where sex differences either occur in more than one social environment, or, like infant vocalizations, that have not yet been studied under multiple social contexts and thus the effect of socialization processes is unknown. Other behavioral patterns such as juvenile aggression that have been suggested to be sexually differentiated [43,44], now appear to be very situationally variable, occurring in some environments and not in others [98].

Understanding the social conditions of rearing is important for assessing whether hormones affect sexual differentiation of behavior. Three principle systems for rearing laboratory rhesus monkeys have been employed with markedly different effects on sexually differentiated behavior. Harlow developed a minimal socialization procedure that he claimed produced fully socialized monkeys [44]. This procedure minimized social input from any other monkeys except peers (peer-rearing), and even peer access was severely limited to 60 min or less per day. Although males reared using the Harlow rearing paradigm showed consistent levels of male-typical play, males reared in this manner were not sexually functional as adults [36]. In response to the limitations created by the Harlow rearing system, a system of continuous housing of 4–6 mother–infant pairs was developed (mother-peer), which resulted in the expression of rhesus typical juvenile behavior and sexually competent adult males [36]. More recently, sex differences in behavior have been studied in large age-graded socially stable groups of outdoor housed rhesus monkeys (social-group rearing) [47,48,59,94,98]. These groups, some of which have been studied for more than 30 years, duplicate significant aspects of rhesus social organization, absent from other rearing conditions. These groups have an enduring matrilineal structure, with the presence of offspring at almost every developmental stage. Thus infants are reared under conditions where interactions can occur with mothers, peers, siblings, and related adult females. This complexity allows the investigation of sex differences in behavior, such as interest in infants, not possible in the more limited social contexts historically employed in laboratory studies. Where relevant the specific rearing condition, peer-rearing, mother-peer rearing, or social group rearing, will be referred to in assessing how hormones affect the development of sexually differentiated behavior.

5. Neonatal hormonal secretions and behavioral sexual differentiation

In male rhesus monkeys testicular activity falls on the day of birth and then increases, remaining at post-pubertal levels for the first 2–3 months of life [65]. In contrast there does not appear to be a similar activation in female rhesus monkeys, although there may be a gonadal restraint on female gonadotropin secretion during the neonatal period [79]. Suppression of male neonatal T secretion appears to influence the timing of puberty

[63,64], but has no striking effects on either juvenile [105] or adult male behavior [17]. Neither neonatal castration [37,81] nor suppression of neonatal T secretion using gonadotropin hormone releasing hormone (GnRH) agonists or antagonists in males have produced substantial evidence that neonatal hormonal secretions are generally involved in masculinization or defeminization in males or in normal female sexual differentiation [10,71,98,105]. Males who were exposed to supraphysiological levels of T neonatally initiated proximity with their mothers less than did either females or males whose neonatal T had been suppressed [105]. However, even though supraphysiological T levels altered this social behavior, males with suppressed T were neither significantly different from normal males or females. While this finding suggests that some aspects of juvenile social behavior may be sensitive to neonatal hormonal influences, the effects are not striking. It seems more likely that hormonal influences during the neonatal period elaborate predispositions that are organized prenatally. In this regard, the effects of neonatal androgen may more closely parallel their effects on genitalia. Penile differentiation is determined prenatally, but the penis is sensitive to androgens neonatally, such that suppression of the neonatal T secretion produces a smaller penis [11,105] and supraphysiological T results in a larger penis [11,105], or clitoris in the case of T treated females [11]. The effects of neonatal T on penile growth disappeared soon after the T treatment ended [11,105]. However, T treatment produced a longer lasting increase in clitoral size, but did not alter the basic anatomy of this clitoris suggesting that some aspects of genital anatomy, at least in females, can still be organized postnatally [11].

In contrast to the limited effects of neonatal T in macaques, there appears to be a period of neonatal sexual differentiation in new world monkeys during which T significantly affects the adult sexual behavior potential of males [14,19]. In both tamarins and marmosets neonatal castration reduces adult masculine sexual behavior, but does not completely eliminate responsiveness to the ability of T to activate male sexual behavior [14,19]. Similarly, neonatal T treatment of genetic females partially masculinizes genitalia and also partially masculinizes, but does not defeminize their behavior [1,2]. While further studies are needed, it appears that within the primate order there may be significantly different patterns of the effects of neonatal T on sexual differentiation.

6. Sex differences in maternal treatment of infants

Sex differences in juvenile and adult behavior could result from differential maternal responses to male and females infants resulting in differential patterns of development. While the notion that sex differences in social behavior stem from differential maternal socialization is attractive there are no data to support this notion. Rhesus monkey mothers do not appear to react differently to males and females in regard to time spent grooming, restraining or interacting with infants of each sex [59,98]. There are two patterns of maternal behavior that may be differentially expressed to male and female infants. Goy et al. [40] reported that mothers inspect the genitals of their male offspring more frequently than they do those of female offspring. The original report was obtained from 4 to 6 member mother–infant groups in relatively sparse surroundings, thus the behavior might have reflected limited options in the social environment. However, this maternal difference was also seen in larger more socially complex groups housed in outdoor compounds offering many behavioral opportunities [105]. In this latter case, the extent to which mothers inspected their male offspring's genitals was proportional to penis size. The only other maternal behavior expressed differentially to male and female infants is maternal responsiveness to infant distress vocalizations with mothers responding more reliably to male than female infant vocalizations [94]. This difference is described more fully in the following section on prenatal influences on behavioral sex differences. Taken together there is limited evidence that mothers treat infants differently on the basis of sex independently of sex differences in the infant's behavior. Inspection of infant genitals seems to be the only reliable maternal sex difference in infant treatment. Thus, it seems very unlikely that the behavioral sex differences described in the following sections stem from differential maternal socialization. It is more likely that they result from hormonal modulation of nervous system development producing behavioral predispositions that differ between males and females.

7. Prenatal hormonal influences on behavioral sexual differentiation

Prenatal hormonal influences on behavioral differentiation has been investigated primarily by exposing female fetuses to supraphysiological levels of androgens, by injecting their mothers with either esterified testosterone (testosterone enanthate, propionate, or cypionate) or dihydrotestosterone (dihydrotestosterone propionate) in amounts ranging from 5 to 25 mg/day [37]. One study of Japanese macaques, a species closely related to rhesus monkeys, employed abdominal implants of silastic packets containing crystalline testosterone, which produced maternal T levels (~75 ng/ml) comparable to those produced by injections [16]. Treatments have typically resulted in very high maternal androgen levels, which produces fetal levels one-eighth to one-tenth of the maternal levels [86]. Thus a treatment that produces 75–125 ng/ml in the mother will produce levels within the normal fetal range for males, but levels 10- to 20-fold higher than normal in fetal females [86,87]. These

amounts are sufficient to completely masculinize female genitalia when the treatment is started early enough in gestation. The behavioral effects of such treatments are thought to reveal the processes involved in normal masculine sexual differentiation on the assumption that masculine characteristics are imposed upon an essentially female anlagen. By varying the timing of the prenatal treatment the effects of androgens on genital anatomy can be separated from some of their effects on sexually differentiated behavior [40]. In general androgen treatments starting around 35 days of gestation and continuing through gestation day 75 affect both the development of reproductive anatomy and some behavioral patterns. Treatments starting after gestation day 100 have no detectable effect on female reproductive anatomy, but have been reported to alter aspects of juvenile behavior [40]. The role of timing of androgen exposure is addressed more fully under the specific behaviors in the sections that follow. These androgen treatments have not been reported to have any effect on male offspring, possibly because the serum androgen levels in male fetuses of treated mothers do not differ from the endogenous levels produced by fetal males [86].

Recently we have used lower androgen doses and the anti-androgen flutamide administered prenatally to investigate sexual differentiation in my laboratory [48]. Treatments were done on pregnant time-mated females [110] living as members of 65–125 member social groups containing multiple adult males and females and their offspring. Pregnant females received either testosterone enanthate (20 mg/week, IM in oil vehicle), or flutamide (30 mg/kg twice daily in dimethlyl sulfoxide (DMSO) vehicle), or vehicle (twice daily DMSO). The timing of treatments was varied such that about half of the subjects' mothers received hormonal treatments starting on gestation day 35 or 40 through gestation day 70 (early treatments) or gestation day 110 or 115 through gestation day 145 (late treatments). All treatments were administered within the pregnant female's social group and infants were delivered within the social group and mothers and offspring remained in the group for the duration of the longitudinal study.

Testosterone treatment produced substantially lower maternal T levels than reported in previous studies with amounts ranging from 2.4 to 21.7 ng/ml at the treatment nadir [48]. Females exposed to these levels of testosterone early in gestation (early androgen females or EAF) were not genitally masculinized, although their neonatal gonadotropin secretion was altered, suggesting that significant androgen had reached the fetus, but at levels below those necessary to masculinize genitalia [48]. Females exposed to testosterone late in gestation (LAF) and females exposed to flutamide early (EFF) or late (LFF) in gestation showed no clear effects of treatment on genital anatomy or neonatal neuroendocrine function [48]. Males exposed to flutamide early in gestation (EFM) had significantly less masculinized penises and in two cases, a urethral meatus that was not integral to the penile shaft as is typical of males. Thus EFM penises were both smaller and less typically masculine than those of control males. Males exposed to flutamide late in gestation (LFM) had male typical genitals, but their penises were significantly smaller than those of control males. Androgen treatment either early (EAM) or late (LAM) in gestation had no measurable effect on male genital anatomy. These findings demonstrate that the penis remains sensitive to androgen levels after an early period when it differentiates from the genital tubercle. Prenatal treatment did not affect neonatal gonadotropin secretion, but did increase T secretion during the first two postnatal weeks in LFM subjects [48].

The range of prenatal treatments and the differing social conditions under which rhesus monkeys have been studied allow some generalizations about the role that prenatal androgens play in sexual differentiation of behavior. The sections that follow are organized around specific behavioral endpoints. The specific nature of the behavioral sex difference is first described and then the effects of alterations in the prenatal hormonal environment are discussed. Although much work remains to be done to fully articulate the role that prenatal androgens play in behavioral differentiation, it is apparent that their effects are pronounced and that androgens are critical for masculinization of behavior, whether the endpoint is one requiring concurrent hormonal activation, like adult copulatory behavior, or a pattern not needing hormonal activation, like juvenile rough play.

7.1. Sex differences in infant vocalizations

Rhesus monkeys use vocalizations for a variety of social functions, including threats and aggression, warning calls, and communicating distress and fear [28–30]. These vocalizations are quite specific referential calls requiring a period of development to be used in the correct social context. Development of this vocal proficiency is sexually differentiated, at least in pigtail macaques, with females developing vocal fluency earlier than do males [27]. Vocalizations are also used to indicate emotional distress and infants, in particular vocalize intensely when separated from, or are rejected by their mothers [94]. Both male and female infants vocalize, but with different frequencies and intensities. When vocalizations are categorized into discrete call types (Table 1), females utilized more call types and had longer separation-rejection vocalization bouts than did males. In addition, males used more high energy call types (noisy screams and geckers), whereas females used more low energy, "pleasant" calls (coos and arched screams) when separated [94]. In addition to sex differences in the nature of the calls, males and females differed in the efficacy with which their calls elicited retrieval by their mother.

Table 1
Vocal features of separation-rejection vocalizations in infant rhesus macaques

Call type	Acoustic properties	General contextual usage
Coo[a,b]	Tonal, some harmonics, start frequency < 3 kHz	To solicit aid, indicate need for "friendly contact"
Girney[a,c]	Similar to coo, but with variable frequency	Nursing call
Gecker[c]	Rapid pulses of energy	Distress call accompanied by convulsive jerking of the body
Noisy scream[d]	Broadband, high frequency	In adults, physical aggression from higher-ranking individual
Tonal scream[d]	Tonal, narrow frequency range	In adults, aggression from kin or higher-ranking individual without physical contact
Arched scream[d]	Tonal call with inverted "U" shape	In adults, aggression from a lower-ranking individual without physical contact
Undulated scream[d]	Nasal sounding, with many narrow-band harmonics, and an atonal quality	In adults, aggression without physical contact by higher-ranking individual
Pulsed Scream[d]	Short, rapid pulses of energy	In adults, aggression from kin member

[a] Ref. [72].
[b] Ref. [88].
[c] Ref. [58].
[d] Ref. [30] (adapted from [94]).

Mothers were significantly more likely to retrieve their male infants than their female infants following a separation call [94]. This difference may reflect the differences in call types that males and female's employ in separation-rejection vocalizations. Possibly, the broad spectrum, high energy nature of the male infant's calls makes it difficult for its mother to ignore, unlike the less intense calls of female infants.

Sex differences in infant vocalizations have not been extensively studied and only a single study has investigated whether prenatal androgen exposure affects their expression. Subjects were treated with testosterone enanthate, flutamide, or vehicle as previously described. Table 2 summarizes the effects of prenatal hormonal manipulations on infant vocalizations for sexually differentiated call parameters. In females, exposure, early in gestation, to androgen levels that did not masculinize their genitalia completely masculinized their infant separation-rejections vocalizations (EAF, Table 2). Similar androgen exposure late in gestation produced less pronounced masculinization of vocalizations, but still resulted in masculinization of three of six vocal characteristics (LAF, Table 2). Surprisingly, prenatal flutamide treatment also had a masculinizing effect on female vocalizations with early flutamide treated females having three of six parameters masculinized and late flutamide treated females have five of six parameters masculinized (EFF and LFF in Table 2, respectively). Prenatal androgen treatment had no clear effect on male vocalizations as both early androgen treated (EAM) and late androgen treated (LAM) males had four or five masculine vocal characteristics of the six measured. Early flutamide treated males (EFM, Table 2) had the fewest masculine vocal characteristics with three of six. Late flutamide males (LFM, Table 2) were indistinguishable from the androgen treated males Table 3.

These results show that infant vocalizations are sensitive to prenatal hormonal manipulations, but the results are somewhat contradictory. In females, exposure to either androgen or flutamide prenatally masculinized female infant vocalizations either strongly, as in the case of early androgen or late flutamide exposure, or partially, as in late androgen treatment or early flutamide treatment. In contrast to its effects in females, where it appeared to act as an androgen, flutamide treatment partially blocked masculinization of vocalizations

Table 2
Summary of prenatal treatment effects on female-typical and male-typical separation-rejection vocalizations in infant rhesus monkeys

Vocal feature	Prenatal treatment group[a]							
	EAF	LAF	EFF	LFF	EFM	LFM	EAM	LAM
Percent coo usage	M	F	M	M	F	—	M	F
Percent gecker usage	M	M	M	M	F	—	M	M
Percent arched scream usage	M	—	—	—	—	F	F	M
Total number of calls	M	—	F	M	M	M	M	—
Types of vocalizations	M	M	F	M	M	M	M	M
Vocalization duration (seconds)	M	M	M	M	M	M	M	M

M, male-typical (e.g., differs significantly from control females, but not from control males).
F, female-typical (e.g., differs significantly from control males, but not from control females).
—, does not differ from either control males or control females.
[a] EAF, early androgen female; EAM, early androgen male; EFF, early flutamide female; EFM, early flutamide male; LAF, late androgen female; LAM, late androgen male; LFF, late flutamide female; and LFM, late flutamide male (adapted from [94]).

Table 3
Summary of effects of prenatal hormonal manipulations in relation to dosage and gestational timing on anatomical and behavioral endpoints in male and female rhesus monkeys

Prenatal treatment (references)	Sex	Genital anatomy	Infant vocalization	Rough play	Mounts	Infant interest
Early flutamide 35 or 40 days [47,48,94]	♀	↑female-like	↔	↔	↔	↔
	♂	↓masculinization	↔	Not different from control ♀ or ♂	Not different from control ♀ or ♂	↔
Early testosterone 35 or 40 days (Low) [47,48,94]	♀	↔	↑♂	Not different from control ♀ or ♂	↔	↔
	♂	↔	↔	Not different from control ♀ or ♂	↔	↔
Late flutamide 35 or 40 days [47,48,94]	♀	↔	↑♂	Not different from control ♀ or ♂	↔	↓interest
	♂	↓penis length	↔	↔	↑mounts	↔
Late testosterone 35 or 40 days (Low) [47,48,94]	♀	↔	↔	↔	↔	↔
	♂	↑penis length (not significant)	↔	↑rough play	↔	↔
Early testosterone 25 days (High) [40]	♀	masculinized	NS	↔	↑mounts	NS
Late testosterone 25 days (High) [40]	♀	↔	NS	↑rough play	↑mounts	NS
>50 days TP or DHTP (High) [31,32,34]	♀	masculinized	NS	↑rough play	↑mounts	NS
DESDP > 100 days [38]	♀	↔	NS	↑rough play	>control ♀ <control ♂	NS
	♂	↔	NS	↔	↔	NS
DESDP 25 days late gestation [38]	♀	↔	NS	↔	↔	NS

Key: ♀, female; ♂, male; ↔, no effect; ↑, increased; ↓, decreased; NS, not studied; DHTP, 5α-dihydrotestosterone propionate; TP, testosterone propionate; DESDP, diethylstilbestrol dipropionate.

whether given early or late in gestation. Why the different effects of flutamide? One possibility is that flutamide does not cross into the brain in very high concentrations and thus, while it works excellently as an anti-androgen peripherally, it is less effective centrally. Prenatal flutamide treatment did result in increased maternal T levels [48], which would have been high for females, but lower than male endogenous T levels. If flutamide does not enter the brain in very high concentrations, then the elevated maternal T could actually expose developing females to higher levels of androgen than would occur naturally. In males, as a result of their higher androgen levels some reduction by flutamide could result in partial blockade of masculinization. This, however, seems unlikely to be the whole story, as will be seen when other behavioral end points are considered. Prenatal flutamide treatment turns out to be particularly problematic and does not appear to have actions completely consistent with its capacity to weakly occupy androgen receptors.

7.2. Sex differences in juvenile social behavior

The findings of Phoenix et al. [78] that prenatal hormones altered adult responsiveness to the effects of gonadal hormones on adult sexual behavior led some to suggest that steroid hormones primarily organized sensitivity to hormonal activation [108]. While this likely applies to many aspects of adult sexual behavior, the existence of sexually differentiated behavior that does not rely upon hormonal activation for its expression provides the opportunity to distinguish the organization of behavioral patterns from the organization of sensitivity to hormonal activation. The demonstration by Harlow [44] of sexually differentiated infant and juvenile behavior in monkeys provided an excellent behavioral system for investigating the effects of prenatal hormones on the organization of behavior. While several of the sexually differentiated patterns described by Harlow, rigidity, threatening, and withdrawal, are only sexually differentiated in very specific and socially impoverished environments [98], two patterns in particular, rough play and juvenile foot-clasp mounting are particularly important behavioral endpoints for demonstrating the effects of prenatal hormone manipulations.

Rough play (also called rough and tumble play, Fig. 1A) is a high energy expenditure whole body play with grasping and tumbling that is exhibited more frequently by males than females in a peer groups [31,43,44], mother peer groups [36,103], and in large social groups [59,105]. Described by Harlow as the primary indicator of proper social adjustment [44,46], it is now apparent that it does not predict adult social competence, but its complete absence indicates severely inappropriate socialization [36].

Juvenile rhesus monkey males display a variety of mounting postures, but of primary importance is the double foot clasp mount (Fig. 1B) that is typical of the mating posture of adult male macaques [3]. This mount is displayed more by males than females [31,44,45,59,105], but is only displayed with any appreciable frequency when males are reared with substantial opportunities for continuous social interaction with

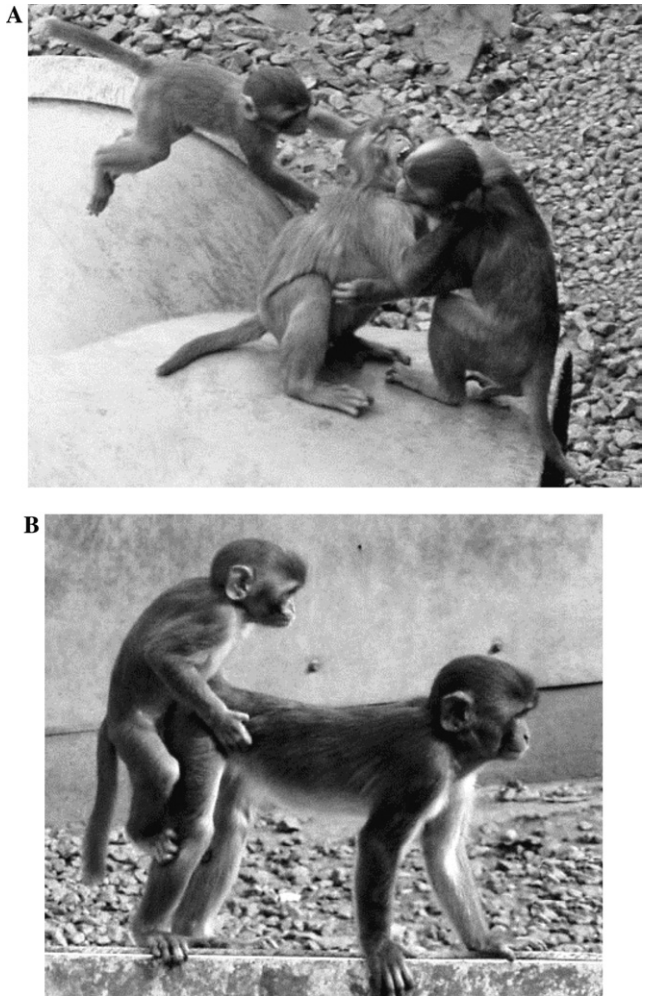

Fig. 1. (A) Rough play in yearling rhesus monkeys. The two male rhesus monkeys at the right engage in friendly wrestling play, characterized by grappling and whole body involvement. Play may involve more than two animals as the infant on the left is about to demonstrate. (B) Foot clasp mounting by an infant male to a yearling rhesus monkey. This mount is a highly cooperative behavior that is of the same form as that used by adult males during copulation (Photos K. Wallen).

peers [36,98,103,104]. In more socially limited contexts foot clasp mounting is almost never displayed [45,46,104]. Unlike rough play, which may be displayed at high frequencies under social rearing conditions not conducive to the development of adult social competency, the routine occurrence of foot-clasp mounting indicates adequate juvenile socialization [36,98].

7.2.1. Studies of exogenous prenatal steroid administration to genetic females

Early studies of the role of hormones on the development and expression of rough play and foot-clasp mounting demonstrated that their juvenile expression was not dependent upon the presence of male gonadal function as neonatally castrated males displayed these behaviors at levels indistinguishable from gonadally intact males [31]. In contrast to the lack of effect of postnatal androgenic influences these behaviors were significantly increased in genetic females whose mothers had been treated with testosterone during much of gestation [31,33,37]. The use of an aromatizable androgen did not appear to be critical as female offspring of mothers treated with 5α-dihydrotestosterone, a nonaromatizable androgen, displayed comparable masculinization of both rough play and juvenile mounting to that produced by prenatal testosterone treatment [32]. Similar effects of prenatal androgen treatment were also found in Japanese macaque females whose mothers received testosterone from approximately gestation day 40 to gestation day 100, of the 168 day gestation, who showed higher levels of mounting, but not rough play than did control females [16]. The androgen levels in this study were lower than those employed by Goy and colleagues in the rhesus monkey as the Japanese macaque offspring exposed to androgen in utero did not have the extensive genital masculinization reported in the rhesus. Thus, if these differences do not reflect species differences in the metabolism and action of androgens, these results suggest that lower levels of androgen are required to masculinize mounting than are required to masculinize rough play. Further evidence that play and mounting have different sensitivities to androgens came from a study in which limited androgen exposure to a 25 day period during gestation, but varied the gestational timing of the 25 day treatment [40].

Exposing genetic females to 10 mg of TP injected daily to their mothers on gestation days 40–64 extensively masculinized their genitalia, producing a fully formed penis, scrotum, and no vaginal opening. Behaviorally, these genitally masculinized females mounted at frequencies not different from control males and higher than control females. However, these females, like Eaton's Japanese macaques exposed to lower levels of T, did not show increased frequencies of rough play [40]. In contrast to the effects of this early androgen treatment on behavior, the same dose of T administered from gestation days 115–139, produced no detectable masculinization of the female's genital anatomy, but both mounting and rough play were significantly elevated over that seen in control females, and in the case of rough play, over the levels displayed by early androgen treated females [40]. Thus, the timing of androgen exposure androgen separated its effects on genital anatomy from its effects on behavior. This study also suggested that late gestation is a period of particular sensitivity of the developing nervous system to prenatal androgens. This might be expected since genital differentiation differs markedly in its time course from that of neural differentiation. While genital differentiation is complete by approximately gestation day 75, cortical neurons have not completely proliferated in some areas of the macaque brain until after gestation day 100 [83]. In addition, synaptogenesis of these neurons occurs and even

continues through the first two postnatal months [9,41]. Evidence that synaptogenesis can be influenced by androgens [66] provides support for the latter part of gestation being a period of particular sensitivity to androgens for behavioral differentiation.

While both aromatizable and nonaromatizable androgens masculinize juvenile behavior, there is some evidence suggesting that estrogens can also masculinize juvenile behavior. Goy and Deputte [38] treated pregnant females with 100 μg per day of diethylstilbestrol dipropionate (DESDP), a dose 33 times that shown to masculinize and defeminize aspects of the behavior of genetic female guinea pigs [50], for more than 100 days of gestation. Females of this long DESDP treatment had normal appearing genitalia, but displayed increased juvenile mounting and rough play. Another group of females received shorter DESDP treatments timed similarly to the late gestation androgen treatments described above. These short-treated DESDP females showed no evidence of behavioral masculinization, in contrast to the significant masculinization produced short TP treatments late in gestation. These DESDP females were only studied for their first year of life while still in the presence of their mothers when the full expression of juvenile sex differences has yet to be realized [36]. Thus, it is hard from these data to determine whether the masculinization produced by long treatments with large amounts of DESDP reflect an involvement of estrogens in masculinization, or a pharmacological effect. Clearly, the DESDP treatment had the capacity to influence sexual differentiation, but the short term nature of this single report prevents reaching any definitive conclusion about the role that estrogens play in the sexual differentiation of juvenile behavior.

Our own studies of prenatal flutamide treatment have not yet provided evidence that the masculinization of juvenile behavior was prevented by either early or late flutamide treatment. In fact, we have evidence, described more fully in the following section, that late flutamide treated males display significantly higher levels of juvenile play and mounting than do control males. We think it unlikely that these data are truly evidence that androgens late in gestation suppress the full masculinization of male juvenile behavior. Instead we think it more likely that these results reflect an increased testicular output during the flutamide block due to the suppression of negative feedback as had previously been demonstrated in rats and humans [24,91,96]. If this is the case, these preliminary findings could be consistent with an estrogen effect on masculinization of juvenile behavior, since AR would be blocked by the flutamide, but ER would not and the increased testicular activity might elevate estrogen levels enough to produce effects similar to those reported for DESDP. Whether or not estrogens or estrogenic metabolites play any role in the normal course of juvenile behavioral differentiation remains an open question.

7.2.2. Alteration of endogenous prenatal androgens

7.2.2.1. Effects on rough play and mounting. The hypothesis that late gestation is a period of increased sensitivity to the organizing actions of androgens on sexually differentiated behavior, was tested using lower doses of exogenous testosterone than previously employed and attempting to block endogenous androgens using the anti-androgen flutamide [48]. We here report the effects of these treatments on rough play and juvenile mounting during the first two years of life [59,105]. Subjects were the offspring of mothers receiving treatments as described for the study of infant vocalizations. All subjects were born into one of two social groups consisting of 75–125 monkeys that had been established at least 20 years prior to the start of the study. The treatments and observation procedures have been previously described [47,48,94]. Early flutamide treatment of males (EFM) prevented full masculinization of the male's penis, whereas late flutamide treatment resulted in a fully formed, but significantly smaller penis. Early flutamide treatment in females (EFF) modified their genitals in a more female-typical direction. None of the androgen treatments significantly affected genital anatomy in either male or female offspring, although males exposed to androgen late in gestation (LAM) had the longest penises of any male group [48].

First year of life. During the first year of life, subjects were distributed between treatments as follows: vehicle control females (VCF) = 7, vehicle control males (VCM) = 7, EAF = 6, LAF = 6, EFF = 7, LFF = 7, EAM = 6, LAM = 4, EFM = 7, LFM = 6. Behavioral data were collected as previously described for the first six months of life [47,48,94]. Overall effects were compared using a one-way analysis of variance with Bonferroni's post hoc test for multiple comparisons between treatment groups.

During the first six months of life, rough play was not sexually differentiated in the presence of the mother, but differed significantly across groups ($F(9,52) = 6.36$, $p < 0.001$) when out of the mother's presence with males displaying much higher rates of rough play that did females ($p = 0.014$, Bonferroni, Fig. 2A). Although there was an overall effect of prenatal treatment on rough play rates, neither prenatal androgen nor anti-androgen treatment significantly increased female rough play compared to control females during the first year (all p's = 1.0, Bonferroni, Fig. 2A). The only suggestion of a prenatal hormonal effect was that the LAF and LFF subjects played at frequencies that were not different from either control males or control females. In contrast in males, prenatal treatments affected rough play. Whereas control males played at higher levels than only control female and EFF subjects, androgen treated males, either early or late had higher rates of rough play than all females groups with the exception of the LFF subjects (Fig. 2A). Contrary to predictions,

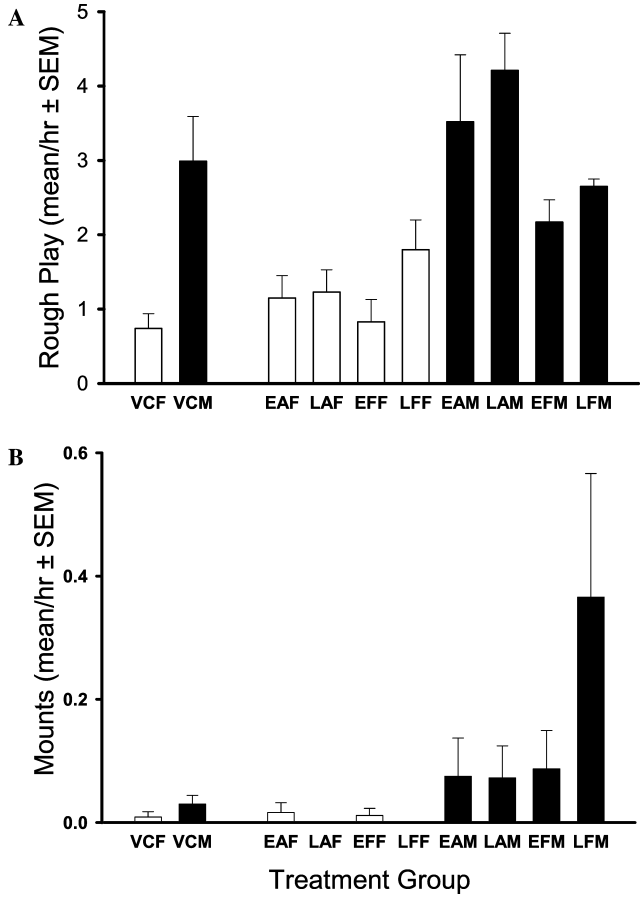

Fig. 2. Rough play (A) and mounting (B) displayed by control and prenatally treated rhesus monkeys during the first year of life. *Abbreviations:* VCF, vehicle treated control female; VCM, vehicle treated control male; EAF, early treated androgen female; LAF, late treated androgen female; EFF, early treated flutamide female; LFF, late treated flutamide female; EAM, early treated androgen male; LAM, late treated androgen male; EFM, early treated flutamide male; and LFM, late treated flutamide male (see text for details of treatments).

administration of flutamide late in gestation did not decrease rates of rough play. Early flutamide treatment produced the lowest rate of male rough play, and EFM subjects did not differ significantly from any other treatment group. Thus they were not significantly more masculinized in their play than females nor significantly less masculinized than the other males.

Mounting is very infrequent during the first 6 months of life, so the measure presented in Fig. 2B includes all properly oriented mounts (penis in correct juxtaposition to the rear of the animal being mounted), not just foot-clasp mounts. Control males mounted more than females, but the frequencies were so low that the difference was not significant. There was an overall treatment effect ($F9,52) = 2.92$, $p = 0.007$, Fig. 2B that stemmed from LFM subjects mounting more than either control males or any of the female control and treatment groups. Thus contrary to our hypothesis, that flutamide late in gestation would block juvenile masculinization, it paradoxically seems to have hypermasculinized these males, an effect not evident in EFM subjects who, as they did in rough play, did not differ significantly from any other group. None of the other apparent differences between treatment groups were significant and there was no evidence that prenatal androgen exposure affected female mounting rates.

Second year of life. During the first year of life, subjects were distributed between treatments as follows: VCF = 6, VCM = 8, EAF = 5, LAF = 6, EFF = 6, LFF = 6, EAM = 6, LAM = 4, EFM = 7, LFM = 4. The difference in subject numbers from year reflect the addition of two unmanipulated male controls to replace animals removed from the study due to illness, and the loss of subjects due to illness where insufficient data could be collected. Behavioral data were collected as previously described for the first six months of life for 13–15 weeks starting on the subject's first birthday and analyzed as previously described [47,48,94]. Since yearlings spend a majority of their time away from their mothers, we collapsed the data across all weeks of observation to provide a total occurrence of the specific behavior.

Rough play varied with prenatal treatment ($F(9,48) = 6.91$, $p < 0.001$). There was a clear sex difference between VCM and VCF subjects ($p = 0.012$, Bonferroni, Fig. 3A). Among the female treatment groups, only EAF subjects displayed significantly less rough play than did control males ($p = 0.009$, Bonferroni) and none of the female treatment groups displayed significantly more rough play than did control females (All p's > 0.17, Fig. 3). Thus, the effect of all prenatal treatments to females, except early androgen administration, was to increase rough play sufficiently such that females were neither completely masculine nor feminine in their pattern of play. As seen with infant vocalizations, late flutamide treatment had the most pronounced masculinizing effect on female rough play, although this was not significantly different from any other female or male group. Although the differences were not significant it is interesting that the increase was greater than either of the testosterone treatments. In addition to control males, males receiving prenatal manipulations late in gestation, regardless of treatment, were the only other male treatment groups to differ from control females (LAM, $p < 0.001$, Bonferroni; LFM, $p = 0.033$, Bonferroni). In addition, LAM subjects differed significantly from all prenatally treated female groups ($p < 0.006$ in all cases, Bonferroni, Fig. 3A). LFM subjects differed significantly from EAF subjects in addition to control females ($p = 0.024$, Bonferroni), making them the most fully masculinized of the male treatment groups after LAM subjects. In contrast the to the LAM and LFM subjects early male treatment groups (EAM and EFM), did not differ significantly from any other group in their rough play. Thus, like the late flutamide females, these groups of males were neither fully masculine nor feminine in

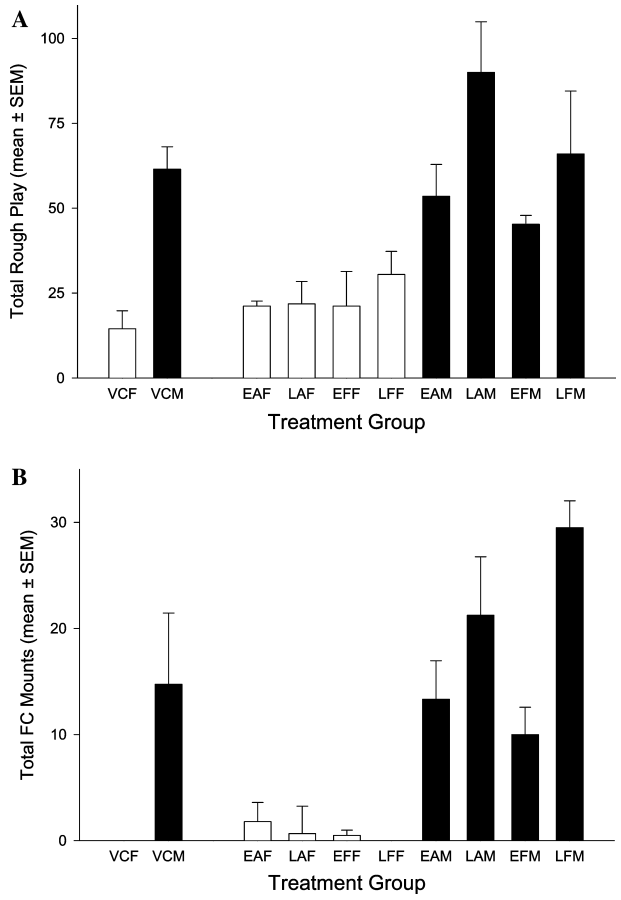

Fig. 3. Rough play (A) and mounting (B) displayed by control and prenatally treated rhesus monkeys during the second year of life. *Abbreviations:* VCF, vehicle treated control female; VCM, vehicle treated control male; EAF, early treated androgen female; LAF, late treated androgen female; EFF, early treated flutamide female; LFF, late treated flutamide female; EAM, early treated androgen male; LAM, late treated androgen male; EFM, early treated flutamide male; and LFM, late treated flutamide male (see text for details of treatments).

their play patterns, suggesting that these early prenatal treatments had partially blocked full masculinization.

The total number of foot clasp mounts displayed during the observation period, with or without pelvic thrusting, varied with treatment. Control males displayed significantly more mounts than did control females ($p = 0.001$, Bonferroni, Fig. 3B) and more than all female treatment groups as well (p's < 0.009 in all cases, Bonferroni, Fig. 3B). None of the female prenatal treatment groups differed significantly from the control females ($p = 1.0$ in all cases, Bonferroni, Fig. 3B), thus unlike the case of rough play, there was no evidence that prenatal androgen or flutamide treatment had any impact on the occurrence of juvenile mounting. Manipulating androgens in males significantly affected their mounting behavior. As was the case with rough play late treatments, either with T or flutamide, produced elevated levels of mounting (Fig. 3B). Males receiving flutamide late in gestation (LFM) showed the highest level of mounting of any group in the study and differed significantly (all p's < 0.004, Bonferroni, Fig. 3B) from all groups except the late androgen treated males (LAM). In contrast, flutamide treatment given early in gestation (EFM) produced males that did not differ significantly from any group, male or female, in their mounting (all p's > 0.11). Late androgen-treated males mounted at higher frequencies than all female groups, but not more frequently than any male treatment group. Males receiving androgens early in gestation (EAM) were similar to late treated males, differing only in that they did not mount more frequently than EAF ($p = 0.063$, Bonferroni, Fig. 3B) subjects and mounted less frequently than LFM subjects ($p = 0.002$, Bonferroni, Fig. 3B).

Taken together these results support several conclusions. First, it appears that late gestation is a period of particular sensitivity of mounting to hormonal manipulations. Second, paradoxically, both flutamide and androgen have similar effects late in gestation. Last, differentiation of mounting is sensitive to hormonal variation early in gestation as well, with early flutamide treatment producing males with the poorest mounting performance. Males who received flutamide early in gestation also had the least masculinized genitals. Thus it is possible that the lower mounting displayed by EFM subjects was in response to their less completely masculinized penile structure. This does not seem the most likely explanation as LFM subjects had penises that were significantly smaller than any other male group, except the EFM males and that were not significantly larger than the penises of EFM subjects and yet they displayed the highest levels of mounting of all groups in the study. While there may be some specific sort of sensory feedback that is provided by a fully formed, but small penis that is not available to a male with a poorly masculinized penis, it seems more likely that the early flutamide treatment altered some motivational characteristic of males so treated.

The effects that we have produced using prenatal flutamide treatments are paradoxical and contradictory if one assumes that flutamide works strictly as an antiandrogen and in the same manner on neural systems and peripheral organ systems. There are several possible explanations for these findings. One possibility is that flutamide simply does not get into the brain in sufficient concentrations to occupy a significant proportion of androgen receptors. No in vivo studies have demonstrated that flutamide enters the brain in levels comparable to that seen in peripheral circulation. Since flutamide is a relatively weak ligand for the androgen receptor [89], any decreased neural concentration could markedly reduce its anti-androgenic effects. Probably more critically, in intact animals flutamide treatment can block negative feedback [42,91,95] and potentially increase endogenous androgens making a central flutamide antiandrogen blockade even less effective. In a study of male

rats, peripherally administered flutamide had no measurable effect when administered to intact male and very effectively inhibited growth of peripheral accessory glands in castrated males to exogenous T, but only reduced, but did not eliminate male copulatory behavior [91]. One study that administered flutamide to pregnant rats found it blocked the masculinization of the corpus callosum [22], there is however, scant additional evidence that peripherally administered flutamide has significant affects on brain structure and function. Thus, one possible explanation for the behavioral masculinization effects that we found with peripherally administered flutamide is that flutamide effectively blocks negative feedback resulting in either increased steroid secretion in pregnant females, such as the increased T secretion we found in our flutamide treated mothers [48], or produces an increased secretion of testicular T in male offspring of flutamide treated mothers. In this regard it may be significant that neonatally, LFM subjects had significantly higher T levels than any other group during the first two weeks postnatally [48]. Whether this reflects some alteration in testicular function (LFM subjects did not have elevated LH at the same time their T was elevated), or some remaining effect of the late flutamide treatment on hypothalamic–pituitary–gonadal function (HPG), remains to be seen. Flutamide's effects on male genitalia in the EFM group demonstrate that sufficient flutamide crossed the placenta to affect peripheral masculinization. However, that behavioral masculinization was not blocked and in fact was enhanced in some cases does not seem compatible with significant levels of flutamide entering the developing brain.

An alternative possibility is that flutamide interferes with negative feedback, but that it is an estrogenic metabolite of the resulting elevated androgens that increases masculinization. Given the capacity of DHT to both masculinize and defeminize adult copulatory behavior and the moderate effects of DESP on juvenile masculinization, this possibility seems unlikely, but it cannot be ruled out at this time. It is apparent from our results that the administration of flutamide to animals with an intact hypothalamic–pituitary–gonadal (HPG) axis does not produce results consistent with a pure anti-androgenic mode of action. In some ways this should be unsurprising given that one of the earliest studies of flutamide in intact male rats reported no anti-androgenic effect on male behavior [91].

In addition to the paradoxical effects of flutamide, there was some suggestion that early androgen exposure also interfered with normal masculinization, whereas when given late in gestation, it appeared to enhance masculinization, as seen in penis size, rough play and mounting. We have no definitive explanation for this difference, which may simply reflect random variation, as the differences are not statistically reliable. However, if they do reflect some more systematic differences the explanation may be similar to that offered for the effects of flutamide involving alterations in negative feedback. From our data on females, it is apparent that the levels of exogenous androgen that we employed produced low levels in the fetus incapable of masculinizing the developing genitalia of females. If the T levels experienced in male fetuses were sufficient to produce negative feedback, but lower then endogenous testicular T levels, the effects would be to reduce male fetal exposure to testosterone, resulting in less complete masculinization. If this mechanism is operating, then our finding that early androgen treatment appeared to reduce masculinization, while late androgen treatment increased masculinization would suggest that endogenous T levels are higher early in gestation than late in gestation. Thus our exogenous androgen treatment produced negative feedback at both gestational times, but resulted in lower total T exposure early in gestation and higher total T exposure late in gestation. Although sampling of endogenous T levels in fetal rhesus monkeys is relatively limited, Resko's data suggest that late gestation T levels are lower than early gestation T levels at the time our fetal males would have been exposed to our exogenous T treatment [87]. This proposed mechanism may also account for a similar finding of reduced masculine behavior in males with congenital adrenal hyperplasia (CAH). Girls with CAH have masculinized genitalia, and show masculinized toy preferences [8], but no increase in rough play [49]. Males with CAH show typical male toy preferences, but significantly reduced rough play compared to unaffected controls [8,49], suggesting that the elevated adrenal androgen they are exposed to is not as high as their endogenous testicular androgen and "clamps" testicular T at a lower level than in control boys and below that level for the full differentiation of play behavior. Comparative studies in rhesus monkeys show that the level of androgen necessary for masculinization of play behavior is higher than that needed for masculinization of mounting. Thus, there is support for the notion that masculinization of different behavioral endpoints may reflect significantly different hormonal thresholds.

7.2.2.2. Interest in infants. Juvenile and prepubertal females exhibit much greater interest in infants than do prepubertal males in several primate species [47], including humans [20,62]. Most of the studies of prenatal hormone manipulations were done in social contexts precluding measurement of interest in infants and thus we know nothing about the effect that long T or DHT treatments may have had on this endpoint. In contrast, subjects in our current studies were reared in complex social groups, where access to infants was a typical aspect of the environment. Subjects were those previously described for vocalizations and juvenile mounting and rough play. A variety of measures of infant interest were collected during approximately 10 h/year of 15 min

focal observations of social behavior for each of the first three years of life on each subject: Behavioral measures included touching, holding, playing with infants, and kidnapping infants [47]. Sex differences in interactions with infants are striking, with effect sizes ranging from 1.3 (frequency of kidnapping in yearling subjects) to 5.1 (frequency of touching infants in yearling subjects). These are among the largest behavioral sex differences reported in rhesus monkeys, or any other species for that matter. Together, 14 measures differed significantly between males and females, and a few of these measures were affected by prenatal treatment to females, but not to males. Females receiving flutamide late in gestation (LFF), showed masculine patterns of interest on five of the 14 measures. To maximize the power of our multiple measures we calculated an index of infant interest that used the deviations from the control males and females across all measure differing significantly between males and females [47]. Fig. 4 illustrates the effect of prenatal treatment on this measure of infant interest. Males differed significantly from all female groups, but the LFF group differed significantly from all other female and male groups, suggesting that they showed less interest in infants than did females, but more interest in infants than did males [47]. This suggests that juvenile interest in infants reflects prenatal hormonal action, but that our treatments were near the threshold for effectiveness in altering interest in infants.

Juvenile interest in infants is of particular interest because of the magnitude of the sex difference and that it occurs at a time when the gonads are quiescent, arguing that it is the expression of an underlying behavioral predisposition requiring no hormonal activation. Particularly intriguing, is that interest in infants in post-pubertal reproductive females appears to be strongly

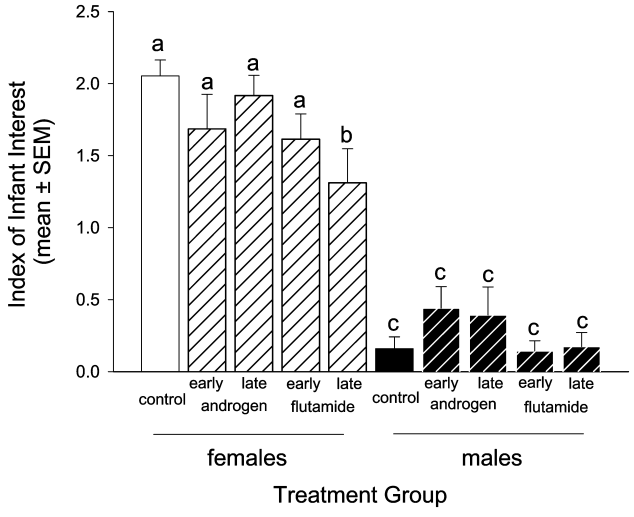

Fig. 4. Index of interest in infants for control male and female rhesus monkeys and males and females receiving testosterone or flutamide treatments early and late in gestation. Bars with differing superscripts differ significantly. (Adapted from [47]).

hormonally activated under our social conditions [60,61]. That hormones do not modulate infant interest before puberty, but do after puberty is also seen in male mounting. Juvenile mounting, like juvenile interest in infants, requires no hormonal activation, whereas adult copulatory mounting does [99]. The mechanism by which a behavior occurs prior to puberty without hormonal activation and then comes under hormonal activational control post pubertally has not been investigated. It is likely, however, that understanding the mechanism of this transition will be important to developing a general understanding of hormonal modulation of behavior.

7.3. Adult sexuality

7.3.1. Masculinization of adult copulatory behavior

Unlike juvenile mounting, adult mounting in rhesus monkey males is steroid-activated and is reduced, but not eliminated by either castration [76] or pharmacologic suppression of testicular function [13,107]. Reinstatement of copulatory behavior in castrated male rhesus monkeys, like castrated male guinea pigs and unlike castrated rats, is achieved by either TP or DHTP treatment [74], but not by estradiol treatment [70,75]. This may not be characteristic of all macaques as the cynomolgus monkey exhibits a more complicated pattern of steroid effects on copulatory behavior. As in rhesus monkeys, DHTP replacement therapy increased ejaculations in castrated male cynomolgus monkeys [68,69]. However, it has been suggested that the DHTP treatment less effectively reinstates behavior than does TP since the same amount of TP as DHTP produces greater copulatory behavior than does DHTP [68,69]. However, this comparison ignored that the TP treatment would have increased both T and DHT from the 5α-reduction of T. In contrast, the DHTP treatment only increased DHT and thus delivers a lower total amount of circulating androgen than does a comparable T dose, which was the case when plasma levels of both T and DHT were compared [69]. Similarly, when brain uptake of these androgens was compared, DHT treatment produced brain levels of androgen about one half that found after T treatment raising the possibility that DHT is behaviorally effective, but must come from the intracellular 5α-reduction of T to reach behaviorally effective brain levels. In addition, the evidence from cynomolgus monkeys that blocking aromatization decreases testosterone activation of copulatory behavior is contradictory. Castrated male cynomolgus monkeys treated as adults with TP and either concurrent fadrozole, an aromatase inhibitor that eliminated more than 98% of the neural aromatization of T, or a control treatment showed a reduction, but not elimination of male copulatory behavior [111]. Concurrent estradiol with the fadrozole had mixed effects, reversing the copulatory

decline in three males, but decreasing it even further in three others [111]. Later studies produced similarly contradictory findings in that the small reduction in male copulatory behavior seen during fadrozole treatment was reduced even farther by the addition of exogenous estradiol [112]. Thus as in the rhesus, aromatization does not appear to be obligatory for the activation of male copulatory behavior. However, it is still possible that aromatization plays some facilitating role in male copulatory behavior.

Prenatal treatments with TP and DHTP have had mixed effects on adult copulatory behavior. However, the interpretation of these results is complicated by the different rearing conditions employed in the studies. The earliest androgenized female rhesus monkeys were reared under restricted social conditions that produce very poor copulatory behavior in the control males [36]. When androgenized females reared under these socially restrictive conditions were tested for male copulatory behavior as adults, exogenous TP treatment did not increase copulation with sexually receptive females [15,75]. Inexplicably, the most extensive test of these androgenized females reared under socially restricted conditions [75] used wild-reared adult males as the comparison group instead of males that had comparable socially restricted rearing experience. It is likely that such males would have shown adult copulatory behavior as deficient as that reported for the socially restricted reared androgenized females [36]. Although these findings have been interpreted as showing that male monkey copulatory behavior is not organized by prenatal androgens [75], a more conservative interpretation would be that the rearing history of these subjects had a greater influence on their adult sexual behavior than did their prenatal hormonal environment [98].

More recent studies of prenatally androgenized females used a mother-peer rearing system that produces robust sex differences in juvenile behavior and sexually competent adult males and females [39,102]. When tested at eight years of age with sexually receptive females, both TP and DHTP prenatally androgenized females displayed increased mounting when treated with exogenous TP [80]. In contrast, ovariectomized control females showed no increases in mounting during adult TP treatment, in fact they were never observed to mount on any test. Although mounting was increased in androgenized females, it was neither at the level typically seen in intact males under these testing conditions nor did those androgenized females with fully masculinized genitalia show intromission or ejaculatory patterns, even after 12 weeks of TP stimulation [80]. Thus, the copulatory behavior of these pseudohermaphrodites is more masculinized than is that of control females, but is less masculinized than that seen in control males.

The moderately masculinized copulatory behavior of these androgenized females may reflect the timing and duration of the prenatal treatments or the fact that they had a markedly different life-history exposure to androgen than do normal males. These androgenized females did not experience the neonatal elevated T typical of normal males [65]. While neonatal T was not found to affect sexually dimorphic patterns of juvenile behavior, its elimination resulted in a later puberty and a lower sex drive in these males as adults [17,63,64]. Thus, the lowered responsiveness of androgenized females to the activating effects of adult TP treatment could reflect a developmental effect of neonatal T. Similarly, these androgenized females were not exposed to any T until 8 years of age, almost 5 years later than a male would typically be exposed to pubertal elevations of androgen. Evidence is accumulating that puberty is a period of significant changes in brain structure and may be a period of increased sensitivity to the organizing effects of androgens [90]. Whether a pubertal period of sensitivity to androgens affects post-pubertal sensitivity to T is not known, but the possibility remains that that full activation of adult male copulatory behavior by T requires not only prenatal organization of neural systems, but also additional neonatal and pubertal T exposure to produce maximal sensitivity. A similar possibility has been reported in human hypogonadal males who were exposed to T for the first time as older adults. Although there were increases in erections and sexual functioning, these changes required months of T therapy, even though T levels increased to within the normal range within 48 h after the start of T therapy [12]. In contrast, normal men reported decreased sexual interest after 2 weeks of testicular suppression with a GnRH antagonist [6]. While the role that androgen during the life-span plays in determining sensitivity and responsiveness for androgen-activation of male copulatory behavior cannot be currently resolved, it is interesting that adult copulatory behavior was masculinized whether the prenatal androgen given to females was aromatizable or not.

Very little is known about the effects of removing endogenous prenatal androgens on the adult copulatory behavior. Our current studies of the effects of prenatal flutamide either early or late in gestation cannot provide a definitive answer, but they do suggest that our prenatal flutamide treatment had little if any effect on central mechanisms regulating male sexual behavior. Insufficient males exposed to exogenous androgen prenatally reached adulthood to analyze their sexual behavior, however we have observed 12 h of mating behavior of our flutamide-treated and control males when they were six or seven years of age and fully adult. All of the males mounted during observations, with the EFM subjects showing the highest frequency of pelvic thrusting mounting ($\mathrm{EFM} = 5.34 \pm 0.75$ mounts/h, $\mathrm{LFM} = 2.85 \pm 0.75$ mounts/h, $\mathrm{VCM} = 3.3 \pm 1.05$ mounts/h, $F(2,17) = 3.76$; $p < 0.05$). However, when social rank was covaried out of the analysis there was no effect of treatment

($F(2,16)=1.99$; $p=0.10$), reflecting that three of the EFM subjects were the highest ranking males in their social groups [56]. Thus, there does not appear to be a treatment effect on this measure of male copulatory behavior. Similarly, males from all three groups were observed to ejaculate, even the EFM subjects who had poorly masculinized genitalia. Only when thrusting mounts without intromissions or mounts without pelvic thrusting were evaluated was an effect of treatment, independent of social rank discovered. Males who received flutamide treatment early in gestation displayed these mount types at greater frequencies than the other two male groups, even when social rank was entered into the analysis (Thrusting mounts without intromissions: EFM = 2.37 ± 0.46 mounts/h, LFM = 1.07 ± 0.43 mou nts/h, VCM = 0.81 ± 0.28 mounts/h, $F(2,16)=4.87$, $p=0.021$; Nonpelvic thrusting mounts: EFM = 1.29 ± 0.32 mounts; h, LFM = 0.40 ± 0.16 mounts/h, VCM = 0.25 ± 0.09 mounts/h; $F(2,16)=7.29$, $p=0.005$,[56]). The increased frequency of these mount types most likely results from the poor genital masculinization of the EFM subjects which reduces penile sensory feedback. Their motivation to mount is clearly unaffected by their prenatal treatment, but the specific form of their sexual behavior reflects their genital anatomy as well as their motivational state. Thus markedly altering male penile anatomy did not appear to have any significant impact on the sexual behavior of these males.

7.3.2. Defeminization of adult sexual behavior

Assessing defeminization in monkeys is problematic in that female monkeys do not display behavior analogous to lordosis in rodents, or the immobility posture in pigs [97]. In fact, it appears that sexual receptivity, in the sense used in studies of rodents, is not hormonally regulated in anthropoid primates [51,100]. In the rhesus monkey, a better measure of female sexual behavior is her propensity to initiate sexual behavior under the activation by ovarian hormones [97,106]. Sexual initiation by rhesus monkey females is overwhelmingly directed towards males and reflects a sexual preference. Thus in the monkey, attraction to males and initiation of sexual behavior [proceptivity 7] is used to assess defeminization and not the willingness of a male or female to accept a male mount as is the case in rodents.

Male monkeys either castrated neonatally or as adults do not attempt to initiate sexual activity with stud males when treated in adulthood with estradiol, unlike ovariectomized, estrogen-treated females who will initiate sex with males [93]. Prenatal treatment with either TP or DHTP defeminizes this behavior as both TP and DHTP pseudohermaphrodites display decreased sexual initiation in comparison to control females [82,93]. Thus, unlike the case for all other laboratory mammals investigated [101] the evidence in the monkey is that androgen itself and not its aromatized metabolites are responsible for proceptive defeminization. This may reflect a difference in behavioral systems, such that receptivity, the primary endpoint for defeminization in rodent studies, utilizes different steroids than does proceptivity. In light of the pronounced effects that estrogens have on partner preference in other mammalian species [101], it seems unlikely that this finding reflects a difference in the hormones differentiating these two behavioral systems. More likely this reflects a characteristic of monkeys, and possibly other primates, where defeminization is androgen regulated and does not require neural aromatization to be effective. The definitive resolution of this issue will require studies that directly manipulate prenatal androgens as well as prenatal aromatization.

The lower doses of androgen that we have employed in our studies have not produced any evidence of defeminization in our females whether or not they received the treatment early or late in gestation. All of our experimental subjects have gone through puberty with some variation in the timing of puberty that reflected late in pregnancy prenatal manipulations, but not early manipulations [109]. However, all females eventually ovulated and all have conceived at least once over their first three breeding seasons. Since in a rhesus monkey group, mating requires female sexual initiation, as males rarely initiate mating [106], the occurrence of pregnancy in these females is presumptive evidence of a lack of proceptive defeminization in these monkeys. Thus whatever hormones produce defeminization in rhesus monkeys; the threshold for defeminization appears to be much higher than doses that alter neonatal neuroendocrine function.

8. Conclusions

Prenatal exposure to nonphysiological levels of exogenous androgen either during the second third or last third of gestation masculinizes the juvenile behavior of genetic females. Only early treatments also masculinize genitalia, thus behavioral masculinization is not the result of the genital masculinization but is an independent effect of prenatal androgen exposure. Similarly blocking endogenous androgen in genetic males significantly reduced genital masculinization, but did not prevent masculinization of behavior, demonstrating the independence of genital and behavioral masculinization. Whether flutamide blockade failed to prevent masculinization of behavior because it did not reach the brain in sufficient quantities or because estrogens, which would not have been blocked by flutamide, are important for male masculinization remains to be resolved.

Across studies, it does not appear, however, that estrogens are critical to male sexual differentiation, although there are still too many gaps in the data to be completely confident of this conclusion. However, it is apparent that the nonaromatizable androgen, DHT

both masculinized and defeminized the behavior of genetic females when administered prenatally. Thus it seems likely that sexual differentiation in the precocial rhesus monkey is more similar to the precocial guinea pig than it is to the other altricial laboratory animals [101]. In both rhesus monkeys and guinea pigs, prenatal DHT masculinizes female behavior, whereas in altricial species like the rat, DHT does not masculinize female behavior unless estrogen is also given [101]. In contrast to the guinea pig, where prenatal DHT does not defeminize genetic females [25], prenatal DHT treatment defeminized female proceptive behavior in rhesus monkeys. Whether this reflects a true species difference or a difference between the hormonal influences on receptivity, which was the measure of female guinea pig sexual behavior, and proceptivity used in the monkey remains to be resolved. Taken together, it seems unlikely that estrogenic metabolites of testosterone are the active agents stimulating behavioral sexual differentiation in rhesus monkeys.

It is apparent from these studies that latter part of gestation is an important period for prenatal hormones to affect brain organization. Consistently across studies using high levels of testosterone, or our studies using lower dosages and anti-androgen treatment, behavioral effects late in gestation were more pronounced than those seen in early gestation. Thus it seems that this period of significant synaptogenesis [9,41] is also an important period for behavioral differentiation. Our finding that late gestation androgen manipulations hypermasculinized the juvenile behavior of males raises the possibility that androgen levels are significantly lower in late gestation than in early gestation, producing incomplete masculinization of males. Future work focusing on behavioral differentiation during late gestation, after reproductive organs are fully differentiated, is necessary to fully describe this important developmental period.

The effects of prenatal hormones on behavioral differentiation are profound and significantly determine developmental trajectories in both males and females. The consistent findings of effects on mounting and rough play across different social contexts, suggests that these behaviors are particularly sensitive to prenatal hormonal influences. However, it is important to remember that social context also significantly affects sexually differentiated behavior. Other patterns of behavior, such as threatening behavior, are sexually differentiated in some social conditions, but not others and prenatal hormones do not consistently affect the development of this behavior [98]. Similarly, prenatal androgens appear to have little effect upon adult copulatory behavior of females reared under restrictive social conditions [77,78], but profoundly alter copulatory behavior when reared under less restrictive conditions [81,82,93]. Thus the effect of prenatal hormonal manipulations reflects an interaction between the specific hormonal manipulation, its timing in gestation, and the social history of the animal. Ultimately, sexually differentiated behavior reflects both the hormonally organized predisposition to engage in a behavior and the social experience and current social context to convert that predisposition into behavioral expression.

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