Gonadal hormones masculinize and defeminize reproductive behaviors during puberty in the male Syrian hamster

Kalynn M. Schulz, Heather N. Richardson, Julia L. Zehr, Andrew J. Osetek, Tami A. Menard, and Cheryl L. Sisk*

Department of Psychology and Neuroscience Program, Michigan State University, East Lansing, MI 48824, USA

Received 14 August 2003; revised 5 December 2003; accepted 8 December 2003

Abstract

Three experiments were conducted to test whether testicular hormones secreted during puberty masculinize and defeminize the expression of adult reproductive behavior. Experiment 1 tested the hypothesis that gonadal hormones during puberty masculinize behavioral responses to testosterone (T) in adulthood. Male hamsters were castrated either before puberty (noTduringP) or after puberty (TduringP). All males were implanted with a 2.5-mg T pellet 6 weeks following castration and tested once for masculine reproductive behavior 7 days after the onset of T replacement. TduringP males displayed significantly more mounts, intromissions, and ejaculations than noTduringP males. Experiment 2 tested the hypothesis that gonadal hormones during puberty defeminize behavioral responses to estrogen (EB) and progesterone (P). Eight weeks following castration, noTduringP and TduringP males were primed with EB and P and tested for lordosis behavior with a stud male. Behavioral responses of males were compared to that of ovariectomized (OVX) and hormone primed females. NoTduringP males and OVX females displayed significantly shorter lordosis latencies than TduringP males. Experiment 3 investigated whether prolonged T treatment or sexual experience could reverse the deficits in masculine behavior caused by the absence of T during puberty. Extending the T treatment from 7 to 17 days did not ameliorate the deficits in masculine behavior caused by absence of T during puberty. Similarly, when the level of sexual experience was increased from one to three tests, the deficits in masculine behavior persisted. These studies demonstrate that gonadal hormones during puberty further masculinize and defeminize neural circuits and behavioral responsiveness to steroid hormones in adulthood.

D 2004 Elsevier Inc. All rights reserved.

Keywords: Puberty; Testosterone; Organizational effects; Reproductive behavior; Masculinize; Defeminize; Adolescence; Sexual differentiation; Sensitive period

Introduction

The organizational–activational hypothesis (Phoenix et al., 1959) proposes that exposure to steroid hormones early in development masculinizes and defeminizes neural circuits, programming behavioral responses to hormones in adulthood. Since the organizational–activational hypothesis was first proposed, many studies have demonstrated that disruption of perinatal testicular hormone secretion by manipulations such as prenatal stress or neonatal castration reduces the capacity for masculine behavior and increases the capacity for feminine reproductive behavior in adulthood (Eaton, 1970; Gerall et al., 1967; Grady et al., 1965; Ward and Weisz, 1980; Whalen and Edwards, 1967; for review see Ward and Ward, 1985). Thus, the perinatal period is important for the sexual differentiation of behavior by gonadal steroid hormones.

Neonatal castration followed by assessment of behavioral responses to steroid hormones in adulthood has been a commonly used approach for analyzing the contribution of neonatal hormones to the process of behavioral masculinization and defeminization. However, because neonatal castration also prevents exposure of the nervous system to hormone secretions during puberty, this approach confounds the contribution of neonatal hormones to the process of sexual differentiation of behavior with that of pubertal hormones. Furthermore, while many studies have employed prepubertal castration as part of their experimental methods, the purpose of these investigations was not necessarily to assess the role of pubertal hormones in the masculinization...
and defeminization of reproductive behavior. Thus, while the results of some studies employing prepubertal castration suggest that the absence of testosterone (T) during puberty alters adult reproductive behavior (Adkins-Regan et al., 1989; Ford, 1990; Gotz and Dorner, 1976; Larsson, 1967; Sodersten, 1973), the results of other studies do not (Dixon, 1993; D’Occhio and Brooks, 1980; Epple et al., 1990; Larsson et al., 1976; Shrenker et al., 1985), and various methodological considerations make it difficult to draw a firm conclusion. For example, in previous studies, the ability of steroid hormones to activate behavior in males castrated prepubertally was not always directly compared to males castrated as adults (D’Occhio and Brooks, 1980; Epple et al., 1990; Larsson et al., 1976), steroid hormones were not readministered in adulthood before behavior testing (Dixon, 1993), only one measure of reproductive behavior was reported (Larsson, 1967), and sexual behavior may have been influenced by other social experiences such as aggressive encounters (Shrenker et al., 1985).

One indication that further organization and masculinization of behavior occur during puberty is that hormonal treatments that fully activate masculine reproductive behavior in adult males are less effective in activating behavior in prepubertal males (Baum, 1972; Sisk et al., 1992; Sodersten et al., 1977). For example, 1 week of testosterone propionate, dihydrotestosterone, or estradiol benzoate treatment increases mounts, intromissions, and ejaculations in adult but not prepubertal male Syrian hamsters (Meek et al., 1997; Romeo et al., 2001, 2002). Even up to 2 weeks of T treatment fails to activate reproductive behavior in a 28-day-old male hamster (unpublished data). These data suggest that the prepubertal male brain is not fully organized to mediate masculine reproductive responses to steroid hormones.

We, therefore, hypothesize that puberty is a second stage of sexual differentiation during which gonadal hormones fine-tune neural circuits to allow full maturation of sex-typical responses to hormones in adulthood. The current study addresses this possibility by testing whether the presence or absence of gonadal hormones during puberty alters masculine responses to T and feminine responses to estradiol benzoate (EB) and progesterone (P) in adulthood. This hypothesis predicts that males gonadectomized (GDX) before puberty will display lower levels of masculine reproductive behavior than males GDX after puberty when both groups are treated with T in adulthood (Experiment 1). Furthermore, the hypothesis predicts that males GDX before puberty will display higher levels of feminine reproductive behavior than males GDX after puberty when both groups are treated with EB and P in adulthood (Experiment 2). We report here that the absence of gonadal hormones during puberty reduces masculine responsiveness to T in adulthood (Experiment 1), increases feminine responsiveness to EB and P in adulthood (Experiment 2), and that the deficits in masculine behavior are not reversed by prolonged T treatment or sexual experience (Experiment 3).

Methods

Animals

Eighteen-day-old male Syrian hamsters (Mesocricetus auratus) were obtained from Harlan Sprague–Dawley laboratories (Madison, WI) and arrived with their mothers. Males were housed with mothers and littermates until weaning at 21 days of age. All animals were housed in clear polycarbonate cages (12 × 4 × 8 in.) with ad libitum access to food (Telkad Rodent Diet No. 8640, Harlan) and water. Animals were exposed to a 14 h light/10 h dark schedule (lights off at 1200 h EST), and the temperature was maintained at 21 ± 2°C. All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Michigan State University All-University Committee for Animal Use and Care.

Experimental design

Experiment 1A

One group of males (n = 6) was GDX before puberty at 21 days of age (noT during P), and one group of males (n = 7) was GDX after puberty at 63 days of age (T during P). Six weeks following GDX, all males were implanted with a 2.5-mg 3-week time-release T pellet (Innovative Research, Sarasota, FL) to clamp circulating T at adult physiological levels. We have previously verified that 7-day treatment with these pellets results in plasma concentrations of T between 2 and 5 ng/ml in both prepubertal and adult males (Meek et al., 1997; Romeo et al., 2003). Behavior tests with a receptive female were conducted 7 days after the onset of T replacement.

Experiment 1B

Because the noT during P and T during P males of Experiment 1A were tested at 70 and 112 days of age, respectively, a parallel experiment was conducted to assess whether any behavioral differences between these groups were associated with chronological age. One group of males (n = 7) was GDX and implanted with a 2.5-mg T pellet at 63 days of age (same age as noT during P group at beginning of T replacement), and one group of males (n = 8) was GDX and implanted with a 2.5-mg T pellet at 105 days of age (same age as T during P group at beginning of T replacement). One week following T implantation, males were tested for reproductive behavior with a stimulus female (70 and 112 days of age).

Experiment 2

Eighteen males were GDX before puberty at 22 days of age (noT during P), and 18 males were GDX after puberty at 63 days of age (T during P). Seven weeks after GDX, half of the males in each group were injected with EB and P, and half were injected with oil and given one 2-min lordosis test in which behavior was manually induced by the experi-
menters. Manually stimulated lordosis tests were conducted with the intention of comparing the levels of lordosis behavior induced by manual stimulation with levels induced during interactions with a stud male. However, the manually stimulated lordosis data are not reported here due to difficulties encountered in reliably inducing lordosis by manual stimulation of the hind flank area in both males and control females. One week after manual stimulation testing, males received the same hormone or oil treatment and were tested for lordosis behavior with a stud male. Hormone-primed males received two EB (10 μg in 0.05 ml of sesame oil) injections 72 and 48 h before behavior testing, and one P (500 μg in 0.1 ml of sesame oil) injection 6 h before behavior testing. Oil-injected males received the same volume of injection as the hormone-injected males and at the same time relative to testing.

Twenty-four females were ovariectomized (OVX) after puberty between 60 and 79 days of age. Females had been hormone-primed and tested for sexual behavior once in a previous experiment but had not been exposed to steroid hormones or a copulatory test in 7 weeks before this study. All females received the same 2-week hormonal priming and behavioral testing schedule as the hormone-primed males. Half of the females were hormone-primed and tested for behavior at the same time as the noTduringP males, and half were hormone-primed and tested at the same time as the TduringP males.

**Experiment 3**

Fig. 1 shows a schematic of the experimental design. Eighteen males were GDX before puberty at 21 days of age (noTduringP), and 16 males were castrated as adults at 63 days of age (TduringP). Six weeks following GDX, all males were implanted with a 2.5-mg 3-week time-release T pellet (Innovative Research). The noTduringP and TduringP males were further divided into two groups to evaluate the effect of sexual experience on responsiveness to T. One group was tested for behavior three times, at 7, 12, and 17 days after the onset of T treatment. The other group was tested only once at 17 days after the onset of T treatment. Although the T capsules were described by the manufacturer as 3-week time release, we discovered in a previous experiment that plasma T concentrations are undetectable 14 days after implantation of Innovative Research T pellets. Therefore, a second pellet was implanted 10 days after the first pellet. This guaranteed that T was present during the last behavior test, which occurred 7 days after the second pellet was implanted.

**Testosterone radioimmunoassay**

Immediately following the last behavior test in Experiment 3, animals were weighed, administered an overdose of sodium pentobarbital (130 mg/kg i.p.), and blood-sampled via cardiac puncture. All males had experienced 17 days of T treatment at the time of blood sampling. Plasma concentrations of T were measured in duplicate 50-μl samples within a single assay using the Coat-A-Count Total T Kit (Diagnostic Products, Los Angeles, CA). This assay has been previously validated in our laboratory (Parfitt et al., 1999). The intraassay CV was 9.2%, and the lower limit of detectability was 0.1 ng/ml.

**Tests for reproductive behavior**

**Masculine reproductive behavior**

All tests for masculine behavior were conducted 1.5–4 h after lights were out (Experiments 1 and 3). The male was placed in a 10-gal glass aquarium (51 × 26 × 31.5 cm) and allowed to acclimate 5 min before the introduction of a receptive stimulus female; the behavior tests were 15 min. Ovariectomized stimulus females were brought into behavioral estrus with an injection of 10 μg of EB (0.2 mg/ml) in sesame oil 48 h before testing and an injection of 250 μg of progesterone (5 mg/ml) in sesame oil 3 h before testing.

The behavioral tests were videotaped under dim red light illumination. Videotapes were scored to assess the number of vaginally oriented mounts, intromissions, ejaculations, and the latencies to ejaculate. The criteria for these behaviors have been described previously (Meek et al., 1997). Videotapes were scored blind to experimental condition by a single observer.

**Feminine reproductive behavior**

All tests for feminine reproductive behavior occurred between 3 and 6 h after lights were out (Experiment 2). After the experimental male or female experienced a 5-min acclimation period in a 10-gal glass aquarium (51 × 26 × 31.5 cm), a sexually experienced stud male was placed in the
aquarium for a 10-min period. To ensure high levels of behavior during testing, stud males were allowed to interact with a sexually receptive female for 1 min immediately before being placed in the test aquarium with the experimental male or female. The behavior tests were videotaped under dim red light illumination. One observer blind to experimental condition later scored the lordosis latencies and the lordosis durations.

Statistical analysis

Experiments 1A and B
In Experiment 1A, two-tailed t tests compared the mounts, intromissions, ejaculations, and ejaculation latencies of noT during P and T during P males. In Experiment 1B, two-tailed t tests compared the behavior of males that differed only by chronological age. These two groups were age-matched to the noT during P and T during P males from Experiment 1A (70 and 112 days old, respectively).

Experiment 2
Two one-way ANOVAs were conducted to compare the lordosis latencies and lordosis durations of three EB- and P-treated groups: T during P males, noT during P males, and females that were ovariectomized as adults. If a one-way ANOVA was significant, Fishers PLSD post hoc tests were conducted to determine specific statistical differences between T during P males, noT during P males, and OVX females. Differences were considered significant if \( P < 0.05 \). The T during P and noT during P males treated with oil were not included in statistical analyses, because these groups did not display any lordosis behavior.

Experiment 3
Two two-way between subject ANOVAs were conducted on the behavioral data. The first two-way ANOVA examined time of GDX (noT during P, T during P) and duration of T treatment (7 or 17 days) in males that were all sexually naive. The second two-way ANOVA examined time of GDX (noT during P, T during P) and level of experience (one or three tests) in males that were all exposed to T for 17 days. Because some of the same behavioral data were included in both of these analyses, the Bonferroni correction for multiple comparisons was employed, and the significance level was set at \( P < 0.025 \). Plasma concentrations of testosterone from terminal blood samples were also analyzed by a two-way ANOVA (time of GDX × level of experience). Differences in this analysis were considered significant when \( P < 0.05 \).

Results

Experiment 1A
T during P males displayed significantly more mounts \([t(11) = 11.78, P = 0.0056]\), intromissions \([t(11) = 22.13, P = 0.0006]\), and ejaculations \([t(11) = 5.38, P = 0.04]\) than noT during P males (Fig. 2). In addition, T during P males displayed significantly shorter latencies to ejaculate than noT during P males \([t(11) = 5.26, P = 0.04; \text{Fig. 2}]\).
Experiment 1B

No differences in the number of mounts \( t(1,13) = 1.24, P = 0.28 \), intromissions \( t(1,13) = 2.37, P = 0.15 \), or ejaculations \( t(1,13) = 0.25, P = 0.62 \) were observed between two groups of males that differed only by chronological age (70 vs. 112 days; data not presented here).

Experiment 2

No behavioral differences were found between the two groups of OVX females tested on different days, and so their data were combined. The one-way ANOVA comparing the lordosis latencies of the EB- and P-treated TduringP males, noTduringP males, and OVX females was significant \( F(2,38) = 10.77, P = 0.0002 \; \text{[Fig. 3]}. \) Fisher’s PLSD post hoc tests revealed that TduringP males displayed significantly longer lordosis latencies than both noTduringP males and OVX females. In contrast, no significant difference in lordosis latency was found between noTduringP males and OVX females.

The overall ANOVA was also significant for lordosis duration \( F(2,38) = 22.34, P < 0.0001 \; \text{[Fig. 3]}. \) Females displayed significantly longer lordosis durations than both TduringP \( (P < 0.0001) \) and noTduringP males \( (P < 0.0004) \). TduringP males displayed shorter lordosis durations than noTduringP males, but this trend was not statistically significant \( (P = 0.08) \).

Experiment 3

After 17 days of T treatment, mean plasma T concentrations were within adult physiological range in all groups (3.8–5.6 ng/ml). A two-way ANOVA did not reveal any effects of time of GDX or level of experience on plasma...
concentrations of T or any interaction between these variables.

The second two-way ANOVA compared sexually naive TduringP and noTduringP groups that differed only in the duration of T treatment (7 and 17 days; Fig. 4). This ANOVA (time of GDX × duration of T) revealed main effects of time of GDX for mounts \( F(1,30) = 23.09, P < 0.0001 \), intromissions \( F(1,30) = 25.87, P < 0.0001 \), and ejaculations \( F(1,30) = 11.19, P = 0.0022 \). TduringP males displayed significantly higher numbers of mounts, intromissions, and ejaculations than noTduringP males. TduringP males also displayed significantly shorter latencies to ejaculate than noTduringP males \( F(1,30) = 5.60, P = 0.0246 \). This ANOVA also revealed main effects of duration of T treatment (7 or 17 days) for ejaculations \( F(1,30) = 10.11, P = 0.0034 \) and a trend toward significance for intromissions \( F(1,30) = 5.04, P = 0.0323 \), such that males exposed to T for 17 days displayed significantly higher numbers of intromissions and ejaculations than males exposed to T for 7 days. Males exposed to T for 17 days also displayed significantly shorter ejaculation latencies than males exposed to T for only 7 days \( F(1,30) = 11.02, P = 0.0023 \). Duration of T treatment did not affect the number of mounts. No interactions were observed between time of GDX and duration of T treatment for any of the behaviors measured.

The third two-way ANOVA compared groups of TduringP and noTduringP males that received the same duration of T treatment (17 days) but differed in their level of sexual experience (one or three tests; Fig. 5). The \( 2 \times 2 \) ANOVA (time of GDX × level of sexual experience) detected main effects of time of GDX for mounts \( F(1,30) = 22.68, P < 0.0001 \), intromissions \( F(1,30) = 16.77, P = 0.0003 \), and ejaculation latency \( F(1,30) = 6.22, P = 0.0183 \). TduringP males displayed significantly more mounts, intromissions, and shorter ejaculation latencies than noTduringP males. TduringP males also displayed more ejaculations, but this difference was not significant after the Bonferroni adjustment for multiple comparisons was applied \( F(1,30) = 4.67, P = 0.0388 \). The \( 2 \times 2 \) ANOVA also revealed a main effect of level of sexual experience for mounts \( F(1,30) = 6.17, P = 0.0188 \), but not intromissions, ejaculations, or ejaculation latencies. Males that experienced three sexual behavior tests displayed significantly more mounts than males that experienced only one. No interaction between time of GDX and level of sexual experience was found for any of the behaviors quantified.

Discussion

The current study demonstrates that the absence of testicular hormones during puberty results in reduced masculine behavioral responses to T (Experiments 1 and 3) and increased feminine behavioral responses to EB and P (Experiment 2) in adulthood. Males that were GDX before puberty displayed fewer mounts, intromissions, and ejaculations than males that were GDX after puberty, as well as longer latencies to ejaculate. Males that were GDX before puberty also displayed shorter lordosis latencies than males GDX after puberty, and importantly, males GDX before puberty lordosed as quickly as females. Furthermore, increased duration of T treatment and repeated experience with a female did not reverse the deficits in masculine behavior caused by the absence of gonadal hormones during puberty (Experiment 3). The differences in masculine behavior between males GDX before or after puberty are not attributable to chronological age, because control males that were age-matched to the TduringP and noTduringP males did not.

![Fig. 5. Number of mounts, intromissions, and ejaculations displayed by sexually experienced or naïve males that were GDX before or after puberty. The shaded b’s in the schematic indicate the behavior tests being compared in the statistical analysis. All males were treated with testosterone for 17 days before testing. All values are expressed as means ± SEM.](image-url)
differ in their levels of masculine reproductive behavior (Experiment 1B). Taken together, these results suggest that gonadal hormones secreted during puberty cause long-lasting organizational change in the neural circuitry underlying reproductive behavior, thereby altering behavioral responsiveness to gonadal steroid hormones in adulthood.

These findings are the first clear demonstration that the presence of testicular hormones during puberty both masculinizes and defeminizes the capacity for reproductive behavior in males. Neonatal castration is a common method of assessing the contribution of neonatal hormones to the process of sexual differentiation. Because neonatal castration prevents exposure of the nervous system to gonadal hormones during both the neonatal and pubertal periods of development, previous experimental designs have confounded the contribution of neonatal hormones to the process of sexual differentiation with the contribution of pubertal hormones. The results of this study suggest that puberty is a second period of nervous system and behavioral organization by gonadal hormones. We propose a two-stage model for the full maturation of adult male social behaviors: perinatal sexual differentiation of neural circuits, followed by peripubertal finishing of this process, which results in the sex-specific activation of behavior by steroid hormones in adulthood (Fig. 6). Thus, the contribution of pubertal hormones to the expression of adult reproductive behavior includes not only an activational component, but also an organizational component, which permits the activation of behavior by steroid hormones.

The two-stage developmental model may also extend to other male social behaviors that appear to be organized by T during puberty. For instance, adult male rats reduce social interactions when in unfamiliar environments, and this sexually dimorphic response to novel environments develops during puberty (Primus and Kellogg, 1990). Although the masculine response to novel environments is not regulated by T in adulthood, depriving males of T during puberty via prepubertal GDX prevents its development altogether (Primus and Kellogg, 1990). Furthermore, administering prepubertal castrates with T throughout puberty maintains the normal development of this social response (Primus and Kellogg, 1990). T during puberty may also organize scent-marking behavior. In male hamsters, flank marking is a T-dependent behavior that communicates dominance status to male conspecifics (Albers et al., 1988; Johnston, 1981). Males that are GDX before puberty, however, do not respond to T in adulthood with increased flank marking during social interactions (Schulz-Wilson et al., 2002). Similarly, male-typical territorial scent-marking in tree shrews requires the presence of T during puberty (Eichmann and Holst, 1999). Thus, the proposed two-stage model of behavioral development may apply to many sexually differentiated social behaviors.

While increased gonadal secretions of steroid hormones are a hallmark of pubertal development (“gonadal puberty”), substantial alterations in nervous system structure and function occur independently of gonadal hormones during puberty (“brain puberty”). For example, the overproduction and subsequent pruning of striatal dopamine receptors, which occur during puberty in male rats, do not depend on gonadal hormones (Andersen et al., 2002). Thus, just as the onset of gonadal hormone secretions is developmentally timed to occur during puberty, so is steroid-independent brain maturation (for review, see Andersen, 2003; Spear, 2000). While it is clear that these two developmental processes interact during puberty to organize many adult male social behaviors, an important question for developmental neurobiology and psychobiology is one of timing: does the full maturation of adult male social behaviors depend on the temporal coordination of gonadal puberty and brain puberty? If gonadal puberty and brain puberty must be synchronized, then puberty may be a sensitive period for steroid-dependent organization of behavior. Alternatively, the second wave of organization by gonadal hormones may be possible at any time after the perinatal critical period but occurs during puberty, simply because T secretions normally increase during this time. Distinguishing between these possibilities and others is necessary for a complete understanding of the relationships between brain and gonadal puberty and the maturation of social behaviors.

While the current study did not directly test whether puberty fits the criteria for a sensitive period, the results are certainly suggestive. For instance, the behavioral deficits resulting from the absence of testes during puberty persisted even after 17 days of T replacement in adulthood. Since 17 days of adult T treatment are almost as long as the normal time course of the pubertal increase in gonadal hormone secretion and emergence of reproductive behavior, it appears that the effect of T on neural circuits and behavior is different during puberty than after puberty. Furthermore, T treatment before puberty does not activate reproductive behavior in 28-day-old males (Meck et al., 1997), even after 2 weeks of T treatment (unpublished study), indicating that the effects of T on neural circuits and behavior are different before puberty than after puberty. Taken together, these data suggest that a second window of maximal sensitivity to steroid-dependent organization may open at the onset and close at the offset of puberty.

Fig. 6. Two-stage model for the complete maturation of male social behavior. Perinatal steroid hormone secretions sexually differentiate neural circuits underlying behavior, whereas pubertal steroid hormone secretions fine-tune or finish the process of behavioral masculinization and defeminization.
Determining whether puberty is a sensitive period for nervous system development is important to our understanding of life situations that cause shifts in the normal timing of exposure to hormones in humans. For instance, eating disorders and extreme exercise can delay gonadal maturation and consequently deprive the nervous system of hormones during puberty. Similarly, the effects of anabolic steroid use in teenagers may be profound and long-lasting if puberty is a sensitive period for the organization of the nervous system and behavior. Future work in our laboratory will be aimed at determining whether puberty is a sensitive period for hormone-dependent organization of the brain. These investigations will increase our understanding of the consequences for behavior when the timing of exposure of the adolescent brain to gonadal hormones is disrupted.

Acknowledgments

We would like to thank Jane Venier for her exceptional technical assistance. This work was supported by a grant from the National Science Foundation IBN 99-85876.

References


