

Sexual Differentiation of Aromatase Activity in the Rat Brain: Effects of Perinatal Steroid Exposure*

CHARLES E. ROSELLI AND SCOTT A. KLOSTERMAN

Department of Physiology and Pharmacology, Oregon Health Sciences University, Portland, Oregon 97201

ABSTRACT

Androgens regulate aromatase activity in the medial preoptic area and other components of the brain circuit that mediates male sexual behavior. The levels of aromatase activity within these brain regions are greater in males than in females. As the activation of copulation requires aromatization of testosterone to estradiol, this quantitative enzymatic difference between sexes could contribute to the greater behavioral response displayed by males. The present study was designed to test the hypothesis that gender differences in brain aromatase activity of adult rats are dependent on the sexual differentiation of the brain that occurs during perinatal exposure to gonadal hormones. Aromatase activity was measured *in vitro* in microdissected brain samples using a sensitive radiometric assay. We examined the effect of pre- and postnatal treatment with testosterone propionate or diethylstilbestrol on basal levels and androgen responsiveness of aromatase in adults. In addition, we examined what effect

prepubertal gonadectomy exerts on enzyme regulation. Our results demonstrate that perinatal treatments with gonadal hormones that are known to differentiate sexual behavior can completely masculinize the capacity for aromatization in the adult female. The process that differentiates aromatase expression appears to depend on androgen exposure and, in part, local estrogen synthesis, as diethylstilbestrol was able to substitute for testosterone propionate. We also observed that prepubertal gonadectomy reduced the levels of aromatase activity measured in adult brain, suggesting that gonadal hormones that are secreted during puberty may enhance the expression of aromatase activity in adulthood. From this study, we conclude that testosterone and/or its estrogenic metabolites act on the developing brain to determine the gender-specific capacity for aromatization and to regulate androgen responsiveness within components of the neural circuitry that mediates male sexual behavior. (*Endocrinology* **139**: 3193–3201, 1998)

ONE MANIFESTATION of a male-differentiated brain is an enhanced responsiveness to hormonal activation of male-typical sexual behaviors by testosterone (T) (1–4). It is well established that this behavioral sexual dimorphism is determined at least in part by exposure to androgen during the critical period for sexual differentiation of neural tissue (5). The critical period in rats begins in late gestation and continues into the first week to 10 days of postnatal life (5). Male rats that are deprived of androgen exposure during the critical period by gonadectomy (Gdx) before day 10 of age exhibit an impaired behavioral response to T as adults (6). On the other hand, female rats that are exposed to T before day 10 of age and injected again with T as adults display levels of copulatory behaviors comparable to those of gonad-intact genetic males (7–9). However, it is not androgen *per se* that is responsible for masculinizing the brain. According to the aromatization hypothesis, the effects of T on behavior depend to a large extent upon its cellular conversion to estrogen by cytochrome P450 aromatase in the rat brain (5).

Behavioral gender differences in androgen responsiveness are presumably related to specific morphological and/or biochemical brain characteristics that differ between males and females. Several brain regions involved in the regulation of sexual behavior are known to be sexually dimorphic (10).

There is evidence that perinatal androgen exposure masculinizes the brain by altering the number of neurons in specific brain areas, as well as their connectivity and neurotransmitter content (10–13). Moreover, region-specific sex differences in steroid receptor binding [protein and messenger RNA (mRNA)] have been demonstrated in the rat brain. Males have greater androgen receptor concentrations and/or greater numbers of androgen receptor-containing neurons within brain areas mediating the hormonal activation of sexual behavior by androgen (2, 14–16). As with brain differentiation, the conversion of T to estradiol by aromatase is an important part of the pathway mediating copulatory behaviors in adult rats (17). Thus, it is also possible that the capacity for conversion of T to active estrogenic metabolites in the adult mammalian brain is influenced by perinatal exposure to gonadal steroids.

Aromatase activity (AA) is highest within the medial amygdala (MA), bed nucleus of the stria terminalis (BNST), medial preoptic nucleus (MPN), periventricular preoptic area (PVPOA), anterior hypothalamus (AH), and ventromedial nucleus of the hypothalamus (VMN) of adult rats (18, 19). These nuclei in large part comprise the brain circuitry that regulates masculine sexual behavior (20). AA in these nuclei is stimulated by androgens and has been referred to as androgen dependent (19). The induction of aromatase by T is mediated through an androgen receptor mechanism that regulates aromatase mRNA levels (21, 22). Expression of androgen-dependent AA in the adult brain is greater in males than in females because of normal sex differences in circulating androgen levels (19). However, the mechanism of enzyme induction is also sexually dimorphic because equiv-

Received January 26, 1998.

Address all correspondence and requests for reprints to: Dr. Charles E. Roselli, Department of Physiology and Pharmacology L334, Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Road, Portland, Oregon 97201-3098. E-mail: roselliec@OHSU.edu.

* This work was supported by NSF Grant IBN-9421759 (to C.E.R.) and NIH Grant P30-HD-18185.

alent physiological doses of T stimulate aromatase to a greater extent in males than in females (2). Dose-response studies indicate that the sex difference is apparent over a range of circulating T concentrations and constitutes a gender difference in the efficacy of T stimulation (23). Measurements of aromatase mRNA in androgen-treated gonadectomized (Gdx) rats demonstrate that the sex difference in regulation is exerted pretranslationally (24). Taken together, these results suggest a sexually dimorphic mechanism that could potentially limit the action of T in females and may in part account for the enhanced expression of T-stimulated sexual behaviors in males.

A major issue that needs to be resolved is whether the adult gender difference in the expression and regulation of brain aromatase is developmentally determined. Therefore, the present study tested the hypothesis that exposure to T during the critical perinatal period for sexual differentiation accounts for the sex difference in the neural expression of androgen-regulated aromatase in adults. In addition, as the aromatase hypothesis predicts that androgen-derived estrogens are responsible for sexual differentiation in rats (17), we examined whether perinatal estrogen exposure is also capable of altering the phenotypic expression of aromatase in the adult. Finally, because it has been suggested that puberty is associated with an increase in neural responsiveness to T (25–27), we examined what effect, if any, prepubertal Gdx exerts on enzyme regulation in adults. The present study has addressed these issues by using a micropunch technique to measure basal and androgen-stimulated AA in a group of limbic and basal forebrain nuclei that are known to be involved in the modulation of androgen-dependent behaviors and neuroendocrine functions. Comparisons are made among control males, control females, and females treated perinatally with androgen or estrogen.

Materials and Methods

Animals

Pregnant Sprague-Dawley rats were obtained from Simonsen Laboratories (Gilroy, CA) and timed to arrive on gestational day 14 or 15. The determination of the day of conception was designated gestational day 0 (GD0) was carried out by the supplier and was defined by the presence of a copulatory plug. The rats were housed individually and maintained on a 12-h light, 12-h dark schedule. Food and water were available *ad libitum*. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the NIH and were approved by the institutional animal care and use committee of the Oregon Health Sciences University.

Experimental protocol

The experimental protocol is diagrammed in Fig. 1. Rat fetuses were treated prenatally with daily sc injections of testosterone propionate (TP; 2 mg/100 μ l oil), diethylstilbestrol (DES; 10 μ g/100 μ l oil), or 100 μ l sesame oil vehicle to pregnant dams from GD16 to GD19. At birth, all pups were weighed, and their anogenital distances were measured with a micrometer. When possible, litters were adjusted to no more than eight pups. After birth, the pups were given sc injections of TP (0.1 mg/50 μ l oil), DES (1 μ g/50 μ l oil), or 50 μ l sesame oil vehicle each day beginning on the day of delivery and continuing through the fifth postnatal day (PD). All pups in a litter were given the same treatment and were kept separate from all other litters. The five perinatal treatment groups were: 1) control δ , males treated pre- and postnatally with the sesame oil vehicle; 2) control ϕ , females treated pre- and postnatally with the sesame oil vehicle; 3) perinatal TP ϕ , females treated pre- and postna-

Experimental Design

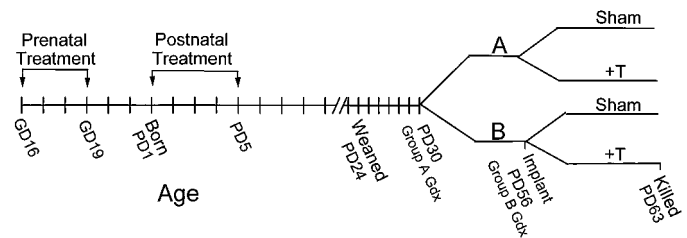


FIG. 1. Experimental design and treatment time line. Rat fetuses were treated prenatally with daily sc injections of TP (2 mg/100 μ l oil), DES (10 μ g/100 μ l oil), or 100 μ l sesame oil vehicle to pregnant dams from GD16 to GD19. After birth, the pups were given sc injections of TP (0.1 mg/50 μ l oil), DES (1 μ g/50 μ l oil), or 50 μ l sesame oil each day beginning on the day of delivery and continuing through the fifth postnatal day (PD). All pups in a litter were given the same treatment and were kept separate from all other litters. The five perinatal treatment groups were: control δ , males treated pre- and postnatally with the sesame oil vehicle; control ϕ , females treated pre- and postnatally with the sesame oil vehicle; perinatal TP ϕ , females treated pre- and postnatally with TP; postnatal TP ϕ , females treated prenatally with vehicle and postnatally with TP; and perinatal DES ϕ , females treated pre- and postnatally with DES. Pups were weaned and weighed on PD 24 and housed according to sex. Group A rats were Gdx on PD 30 before the onset of puberty. Group B rats were Gdx on PD 56 as adults. On PD 56, half of the rats in both groups received 3-cm SILASTIC brand capsules filled with crystalline T, whereas the other half were sham implanted. The rats were killed 7 days later, and basal (Gdx) and androgen-stimulated (Gdx+T) levels of AA were measured in microdissected brain nuclei.

tally with TP; 4) postnatal TP ϕ , females treated prenatally with vehicle and postnatally with TP; and 5) perinatal DES ϕ , females treated pre- and postnatally with DES. These treatments were chosen because they were shown previously to masculinize brain anatomy and adult sexual behaviors (7, 8, 28).

Pups were weaned and weighed on PD 24, and housed according to sex. To determine the effect of prepubertal gonadectomy on the capacity for aromatization in brain, half of the juvenile rats in each treatment group were bilaterally Gdx under ketamine-xylazine anesthesia on PD 30 before the onset of puberty. On PD 56, the remaining rats in each treatment group were Gdx. At this time half of the rats in both the juvenile Gdx and adult Gdx groups received 3-cm SILASTIC brand capsules (Dow Corning, Midland, MI) filled with crystalline T, and the remaining rats were sham implanted. This dose of T produces hormone levels typical of an adult male rat (29) and was used in the present experiment to compare T-stimulated AA in males and females. Seven days later, all of the rats were decapitated, and their brains were rapidly removed and frozen on dry ice. The brains were stored at -80°C until they were microdissected and assayed for AA. Trunk blood was collected, and serum was harvested for determination of circulating T concentrations. Generally, brains from Gdx and Gdx plus T-treated rats from all perinatal treatment groups ($n = 20$) were processed together to measure AA. A total of 124 rats were used to complete the study; each group contained 4–7 animals.

Tissue dissections

Frozen brains were sectioned coronally at 300- μ m intervals beginning where the anterior commissure crosses the midline and extending caudally 3.6 mm as depicted in the brain map of Palkovits and Brownstein (30). The tissue sections were thaw mounted onto glass microscope slides and stored overnight at -80°C . Selected brain nuclei and regions were dissected bilaterally using 500- and 1000- μ m calibrated stainless steel cannulas, as described previously (19). The tissue punches were expelled into 500- μ l propylene microtubes and chilled on ice until homogenized.

AA assay

Tissue punch dissections collected from individual animals were homogenized using a tissue sonicator (E/MC Corp., Hauppauge, NY) in 125 μ l phosphate buffer (10 mM KH_2PO_4 , 100 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.4). Aliquots of these homogenates (100 μ l) were then incubated for 1 h at 37 C with [1β - ^3H]androstenedione (New England Nuclear-Dupont, Boston, MA; SA, 24.1 Ci/mmol). AA was estimated by quantifying the amount of $^3\text{H}_2\text{O}$ generated by the stereospecific loss of the C-1 β tritium, which is proportional to the amount of estrogen formed (31). This assay has been validated in our laboratory, and the procedural details have been described previously (31). Protein concentrations were determined using the Lowry method (32). AA was expressed as femtomoles of $^3\text{H}_2\text{O}$ produced per h/mg protein.

Testosterone assay

Trunk blood was collected after decapitation and allowed to clot at 4 C overnight. They were then centrifuged ($1500 \times g$ for 30 min), and sera were harvested. T levels were measured in individual samples by RIA after extraction and chromatography on Sephadex LH-20 using previously described methodologies (33). All samples were assayed in duplicate in a single RIA. The percent recovery, water blanks, and intraassay coefficient of variation were 79%, 0.7 pg/tube, and 7.3%, respectively.

Statistical analysis

The AA data for each brain nucleus were analyzed by parametric 3×3 ANOVA (perinatal treatment \times age at castration \times adult treatment). When a significant main effect of perinatal treatment was found in a tissue ($P < 0.05$), multiple one-way ANOVAs were performed across treatment groups. *Post-hoc* comparisons were then made between perinatal treatments with *post-hoc* Newman-Keuls tests and were considered significant at $P < 0.05$ (34).

Results

Effects of perinatal treatments on T-stimulated AA in brain punches

Three-way ANOVA revealed significant main effects of perinatal treatment, age at castration, and adult treatment on AA in the BNST, MPN, PVPOA, and VMN. A significant interaction between perinatal treatment and adult treatment was also found in PVPOA and VMN. No other significant interactions were revealed. The results in these four tissues are summarized in Figs. 2-5, respectively. In each figure, A shows the results from rats Gdx on PD30, and B shows the results for rats Gdx on PD56. To simplify the data presentation, only comparisons of perinatal treatment groups with control males and control females are noted in the figures. As expected, adult T treatments significantly stimulated AA in these tissues regardless of prior perinatal treatment (compare gray vs. black bars) or age at castration (compare A, Gdx on PD 30, with B, Gdx on PD 56). The induction of AA by T was significantly greater in control males than in control females. In the prepubertal Gdx group, the percentages by which AA levels in males was greater than those in females were 174% (BNST), 154% (MPN), 212% (PVPOA), and VMN (122%). In the adult Gdx group, the percentages by which AA levels in males were greater than those in females were 134% (BNST), 212% (MPN), 184% (PVPOA), and 143% (VMN). In BNST, MPN, and PVPOA, females that were treated perinatally with TP or postnatally only with TP, exhibited significantly greater levels of T-stimulated AA than control females, whereas T-stimulated AA levels in these groups were not significantly different from levels in control males. This effect

Bed n. of the Stria Terminalis

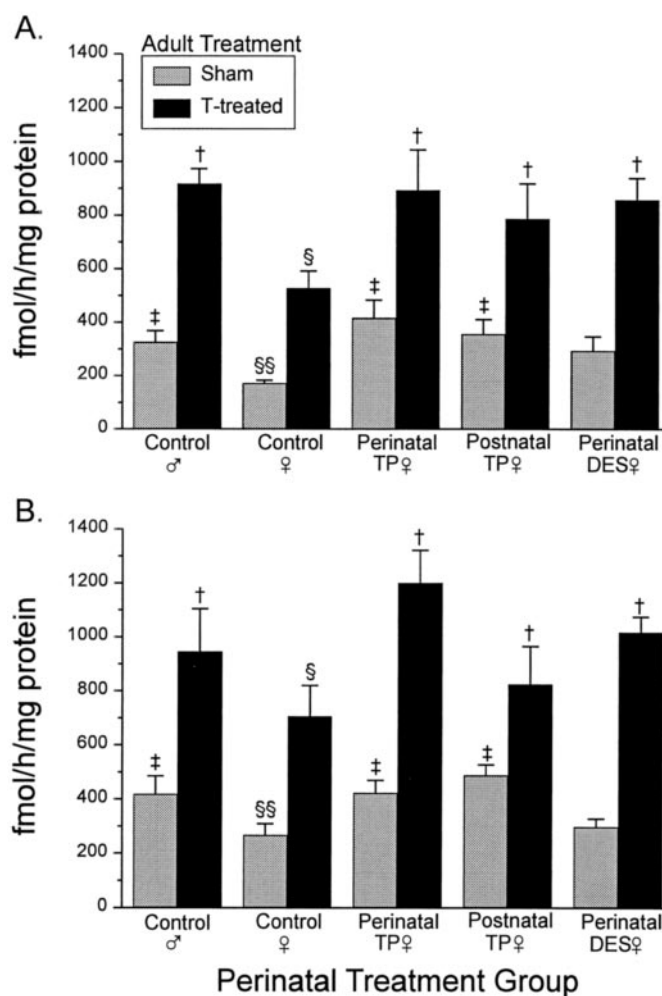


FIG. 2. Effects of perinatal treatments on T-stimulated AA in the BNST. A, Rats Gdx on PD30. B, Rats Gdx on PD56. All rats received sc T-filled SILASTIC implants (30 mm) on PD56 and were killed 1 week later on PD 63. See *Materials and Methods* for descriptions of the perinatal treatment groups. Data are presented as the mean (bars) \pm SEM (lines; $n = 4-7$ /group). Three-way ANOVA revealed significant main effects of perinatal treatment ($F_{4,104} = 7.68$; $P < 0.0001$), age at castration ($F_{1,104} = 7.12$; $P < 0.01$), and adult T treatment ($F_{1,104} = 183$; $P < 0.0001$) on AA. Newman-Keuls *post-hoc* comparisons: §§, $P < 0.05$ vs. sham control δ ; ‡, $P < 0.05$ vs. sham control δ ; §, $P < 0.05$ vs. T-treated control δ ; †, $P < 0.05$ vs. T-treated control δ .

was observed regardless of whether the rats were Gdx on PD 30 or as adults. In VMN, a significant effect of perinatal and postnatal TP treatments was observed only in rats that were Gdx as adults. Perinatal DES treatment masculinized the T-stimulated induction of AA in BNST, MPN, and PVPOA, but not in VMN. Gonadectomized (*i.e.* unstimulated) levels of AA did not differ between perinatal treatment groups in the MPN and VMN. However, in the PVPOA and BNST, AA levels in control males, perinatal TP-treated females, and postnatal TP-treated females were generally higher than the

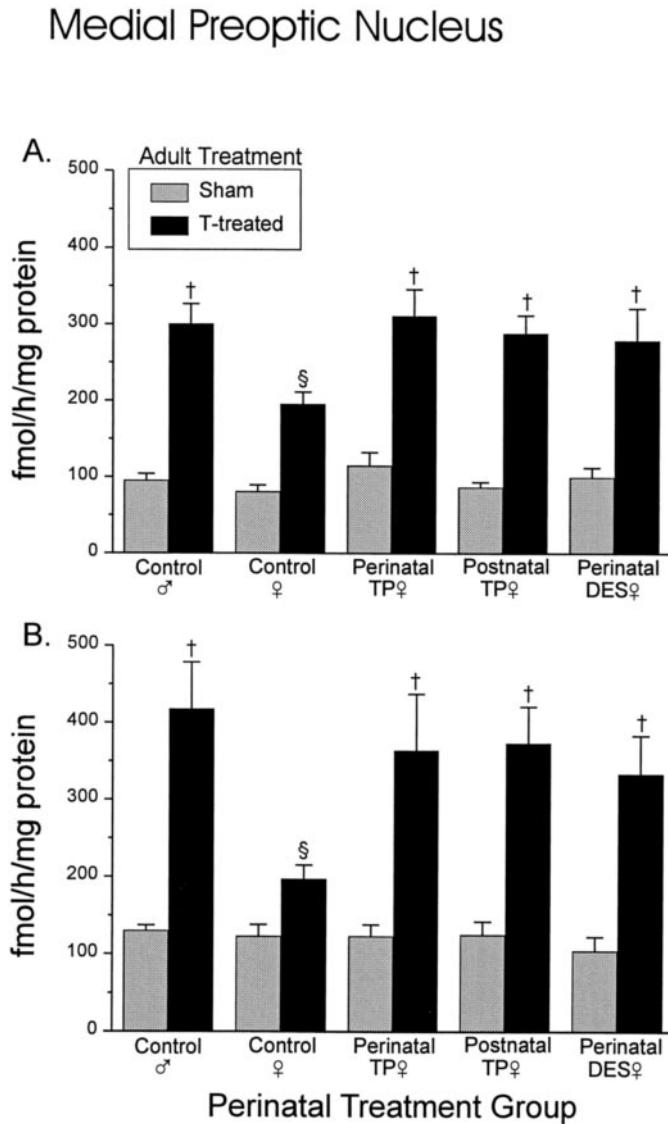


FIG. 3. Effects of perinatal treatments on T-stimulated AA in the MPN. A, Rats Gdx on PD30. B, Rats Gdx on PD56. Data are presented as the mean (bars) \pm SEM (lines; $n = 4-7$ /group). Three-way ANOVA revealed significant main effects of perinatal treatment ($F_{4,104} = 4.20$; $P < 0.005$), age at castration ($F_{1,104} = 9.67$; $P < 0.005$), and adult T treatment ($F_{1,104} = 276$; $P < 0.0001$) on AA. Newman-Keuls *post-hoc* comparisons were made between perinatal treatment groups. See Fig. 2 for symbol designations.

levels in control females and perinatal DES-treated females. In general, both Gdx and T-stimulated levels of AA in all four tissues were higher in rats Gdx at 56 days of age (Figs. 2B–5B) than in rats Gdx at 30 days of age (Figs. 2A–5A), which accounts for the significant main effect due to age at Gdx.

The results for the anterior hypothalamus and medial amygdala are summarized in Figs. 6 and 7, respectively. Three-way ANOVA revealed a significant main effect of adult T treatment, but no effect of perinatal treatment or age of castration in these tissues. In general, T treatment stimulated AA in adults regardless of any prior perinatal treatment (compare gray vs. black bars) or pubertal treatments (compare A, Gdx on PD 30, with B, Gdx on PD 56).

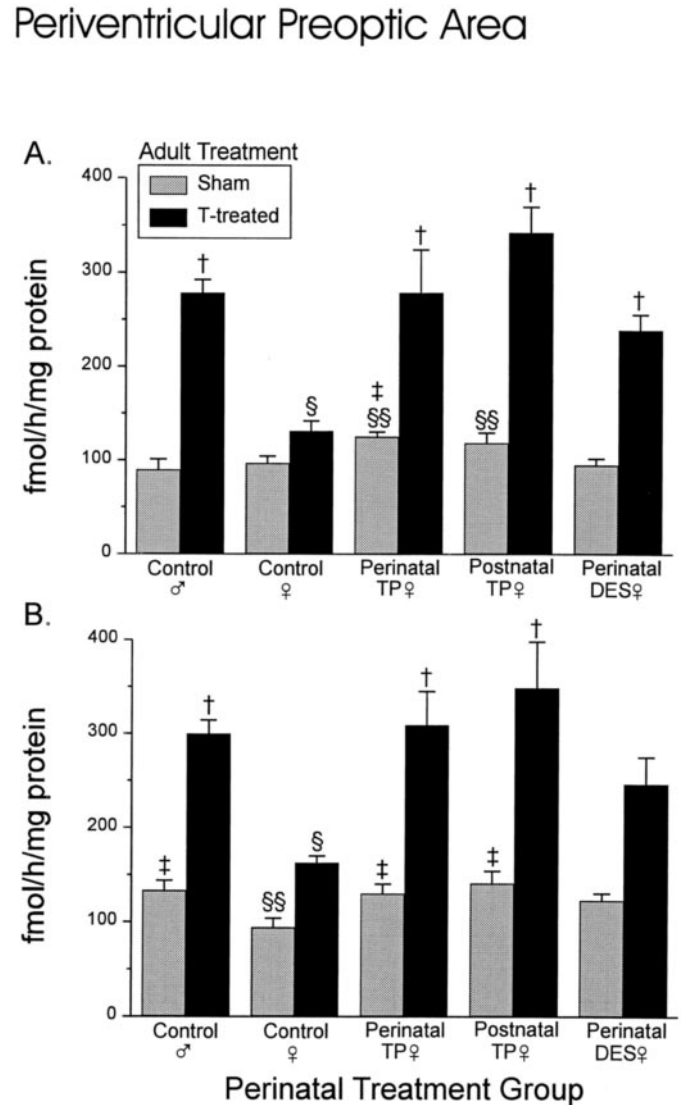


FIG. 4. Effects of perinatal treatments on T-stimulated AA in the PVPOA. A, Rats Gdx on PD30. B, rats Gdx on PD56. Data are presented as the mean (bars) \pm SEM (lines; $n = 4-7$ /group). Three-way ANOVA revealed significant main effects of perinatal treatment ($F_{4,103} = 18.74$; $P < 0.0001$), age at castration ($F_{1,103} = 8.26$; $P < 0.005$), and adult T treatment ($F_{1,103} = 312$; $P < 0.0001$), and a significant interaction between perinatal treatment and adult treatment ($F_{4,103} = 4.88$; $P < 0.005$). Newman-Keuls *post-hoc* comparisons were made between perinatal treatment groups. See Fig. 2 for symbol designations.

Effects of perinatal treatments on anogenital distances, body weights, and genitalia

The average anogenital distances and body weights of the experimental groups on PD 63 are shown in Fig. 8. There was a significant effect of perinatal treatment on anogenital distances, but no effect of age at castration or adult treatment. In general, anogenital distance was greatest in control males, intermediate in females treated peri- or postnatally with TP, and lowest in control females and females treated perinatally with DES. There was a significant effect of perinatal treatment group, age at castration, and adult treatment on body weight, with a significant interaction between perinatal treat-

Ventromedial n. of the Hypothalamus

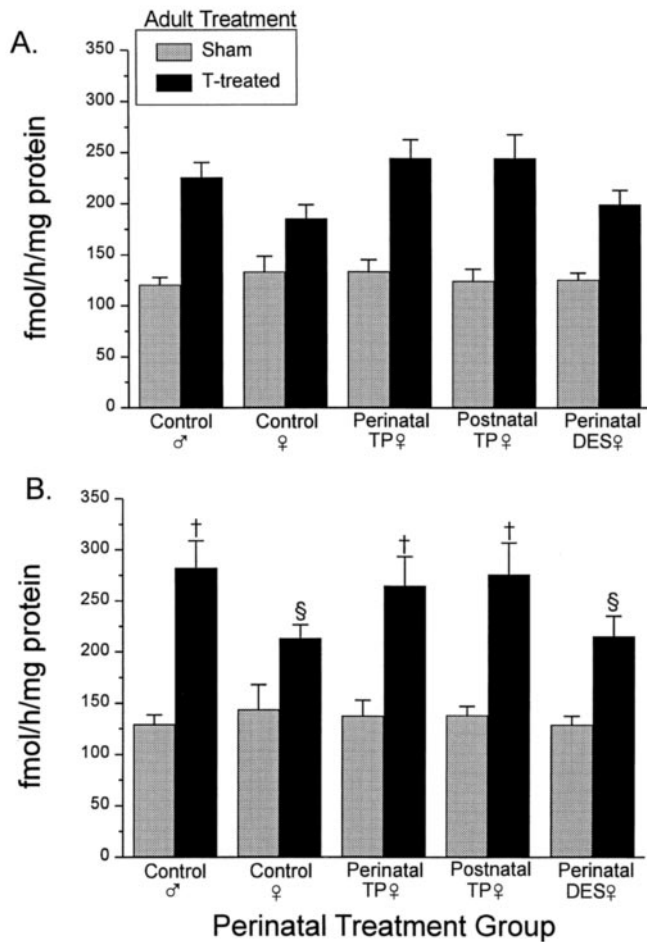


FIG. 5. Effects of perinatal treatments on T-stimulated AA in the VMN. A, Rats Gdx on PD30. B, Rats Gdx on PD56. Data are presented as the mean (bars) \pm SEM (lines; $n = 4-7$ /group). Three-way ANOVA revealed significant main effects of perinatal treatment ($F_{4,101} = 3.30$; $P < 0.05$), age at castration ($F_{1,101} = 8.53$; $P < 0.005$), and adult T treatment ($F_{1,101} = 206$; $P < 0.0001$) and a significant interaction between perinatal treatment and adult treatment ($F_{4,101} = 4.25$; $P < 0.005$). Newman-Keuls *post-hoc* comparisons were made between perinatal treatment groups. See Fig. 2 for symbol designations.

ment and age at castration (Fig. 9). In general, control males weighed more than any other treatment group. However, females that were treated postnatally with TP or perinatally with DES and castrated as adults were significantly heavier than control females, but were not as heavy as control males (Fig. 9B). Females that were gonadectomized prepubertally weighed more than their counterparts that were gonadectomized as adults.

Females that received perinatal TP were phenotypical masculinized. They exhibited an enlarged clitoris that was indistinguishable visually from the penis of the normal male. They also possessed prostates and seminal vesicles in addition to a uterus and ovaries. Adult androgen treatment stimulated the growth of the male-typical glands. The clitori of females that received postnatal TP were slightly enlarged

Anterior Hypothalamus

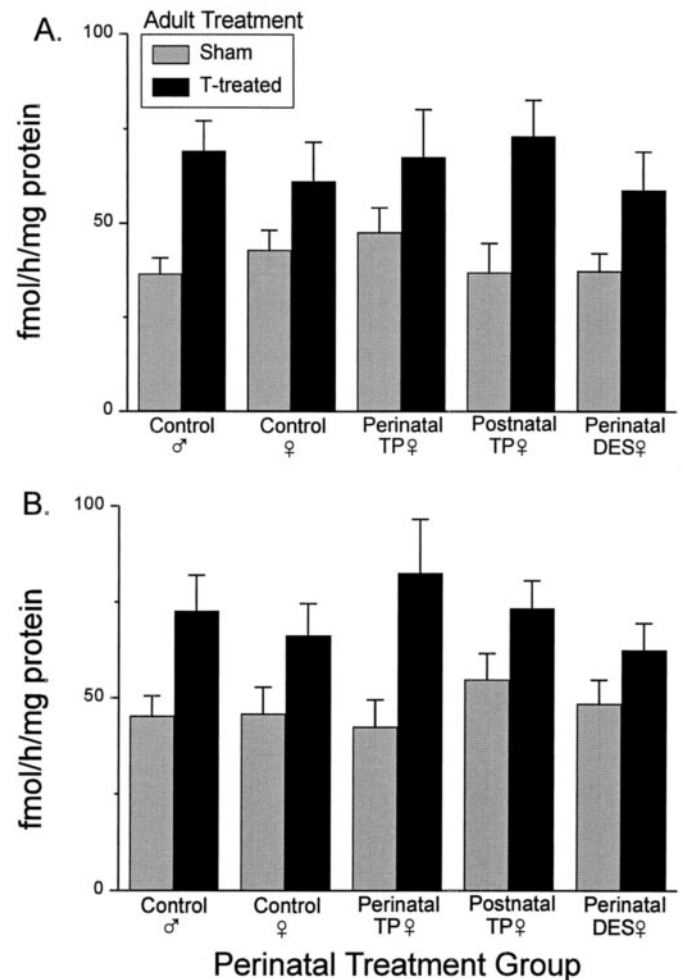


FIG. 6. Effects of perinatal treatments on T-stimulated AA in the AH. A, Rats Gdx on PD30. B, Rats Gdx on PD56. Data are presented as the mean (bars) \pm SEM (lines; $n = 4-7$ /group). Three-way ANOVA revealed significant main effects of adult T treatment ($F_{1,102} = 44.96$; $P < 0.0001$) only.

compared to those of normal females. Postnatal TP females did not possess male-typical internal genitalia. The clitoris and uterus of females that were treated with DES perinatally were not visually different from those of normal females.

Serum T levels

The levels of serum T are summarized in Fig. 10. Equivalent levels of T were achieved in all groups 1 week after Gdx and androgen treatment. However, females treated with TP perinatally or postnatally exhibited a slight, but significant, elevation in serum T levels in the sham group Gdx at 30 days of age.

Discussion

The present experiment demonstrates that manipulation of androgen levels during perinatal development can per-

Medial n. of the Amygdala

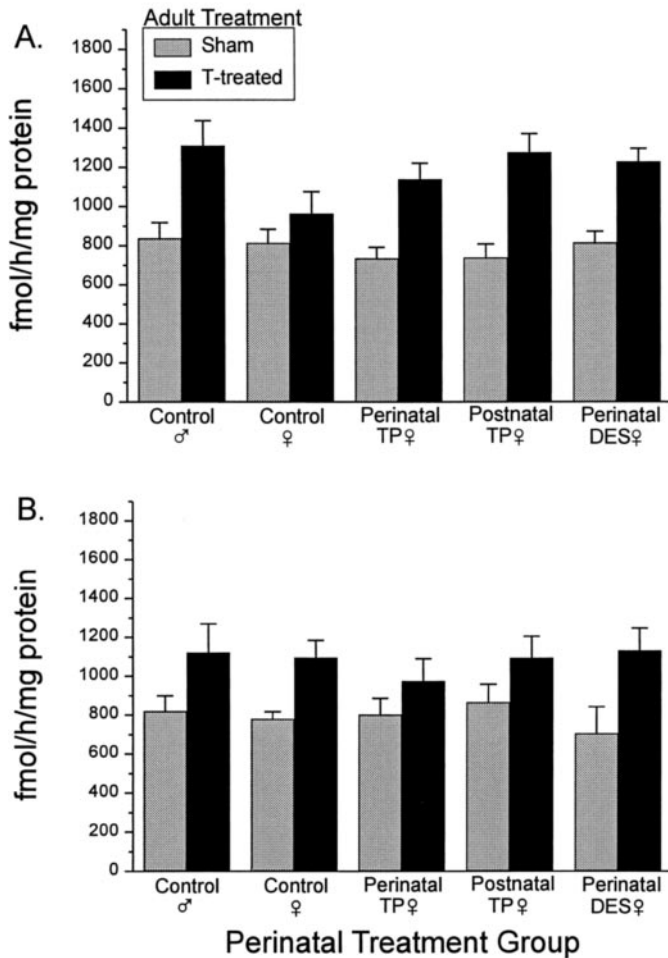


FIG. 7. Effects of perinatal treatments on T-stimulated AA in the MA. A, Rats Gdx on PD30. B, Rats Gdx on PD56. Data are presented as the mean (bars) \pm SEM (lines; $n = 4-7$ /group). Three-way ANOVA revealed significant main effects of adult T treatment ($F_{1,103} = 63.34$; $P < 0.0001$) only.

manently alter the gender-specific capacity for aromatization within components of the neural circuitry that mediates male sexual behavior. Extended exposure of genetic females to TP during gestational days 16–19 and PD 1–5 increased the subsequent expression of AA in adulthood to levels characteristic of genetic males. However, treatment of genetic females with TP from PD1–PD5 also completely masculinized the capacity for aromatization, suggesting that the critical period for this effect encompasses the first week of life. These effects on aromatase expression are temporally correlated with the critical developmental period during which sexual behaviors and neural structures are sexually differentiated in rats (5) and lend support to the hypothesis that maturation of aromatase-containing neurons is a differentiated characteristic of the male brain. Our results further indicate that although TP was able to substitute fully for testes and its

Ano-genital Distance

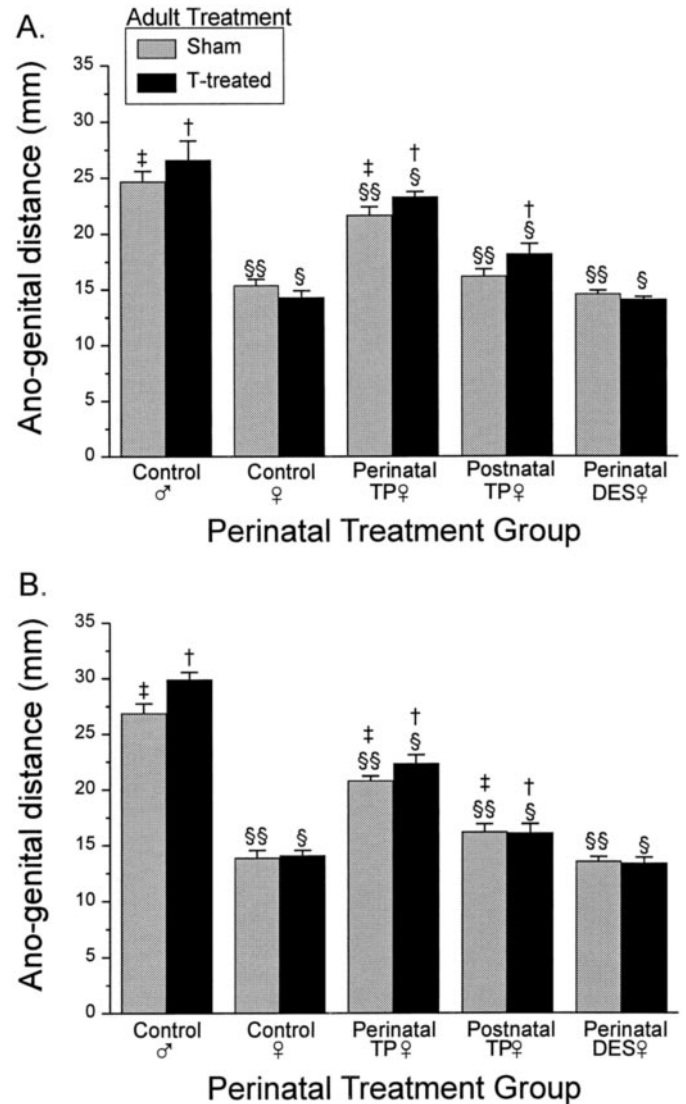


FIG. 8. Effects of perinatal treatments on anogenital distances at the time of death (63 days old). Data are presented as the mean (bars) \pm SEM (lines; $n = 4-7$ /group). Three-way ANOVA revealed significant main effects of perinatal treatment ($F_{4,99} = 215$, $P < 0.0001$). Newman-Keuls *post-hoc* comparisons were made between perinatal treatment groups. See Fig. 2 for symbol designations.

secretions in determining the capacity for neural aromatization, aspects of its effect may well be mediated by locally produced estrogen. This conclusion is based on our observation that perinatal treatment with the synthetic estrogen, DES, in large part mimicked the effect of perinatal TP treatment. Perinatal DES exposure of genetic females increased the androgen-induced expression of AA in adulthood to the levels observed in genetic males. Thus, these results indicate that the conversion of androgens into estrogens probably plays a major role in the differentiation of aromatase neurons.

We demonstrated that the organizational effects of andro-

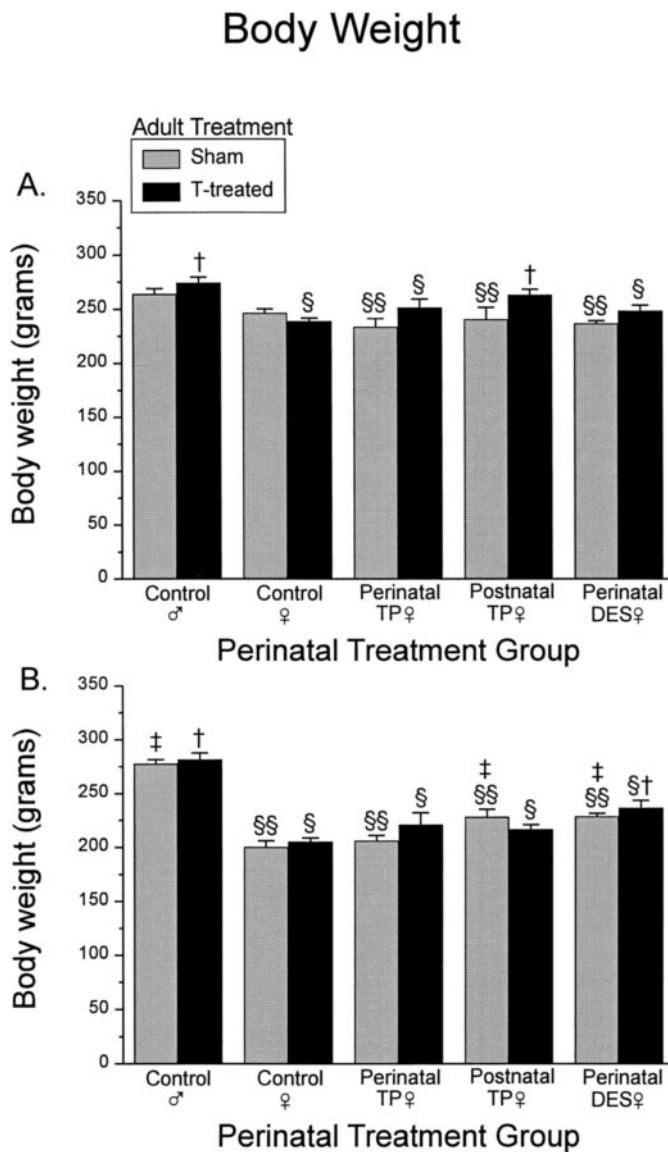


FIG. 9. Effects of perinatal treatments on body weights at the time of death (63 days old). Data are presented as the mean (bars) \pm SEM (lines; $n = 4-7$ /group). Three-way ANOVA revealed significant main effects of perinatal treatment ($F_{4,104} = 45, P < 0.0001$), age of castration ($F_{1,104} = 52, P < 0.0001$, and adult T treatment ($F_{1,104} = 7.6, P < 0.01$), and a significant interaction between perinatal treatment and age of castration ($F_{4,104} = 10.8, P < 0.0001$). Newman-Keuls *post-hoc* comparisons were made between perinatal treatment groups. See Fig. 2 for symbol designations.

gen and estrogen are brain region specific. An effect on the expression of androgen-stimulated AA was evident in the BNST, MPN, PVPOA, and VMN, but not in the AH and MA. These results generally agree with our previous studies that identified gender differences in the induction of brain aromatase by T within these same nuclei (2, 23). Our results confirm and extend the work of Steimer and Hutchison (35), who demonstrated that the sex difference in the induction of aromatase in the preoptic area of rats is abolished by neonatal Gdx and exposure to TP from PD 2 to 70. However, in this early study, neonatal Gdx and extended treatment with TP appeared to substantially decrease the responses of both

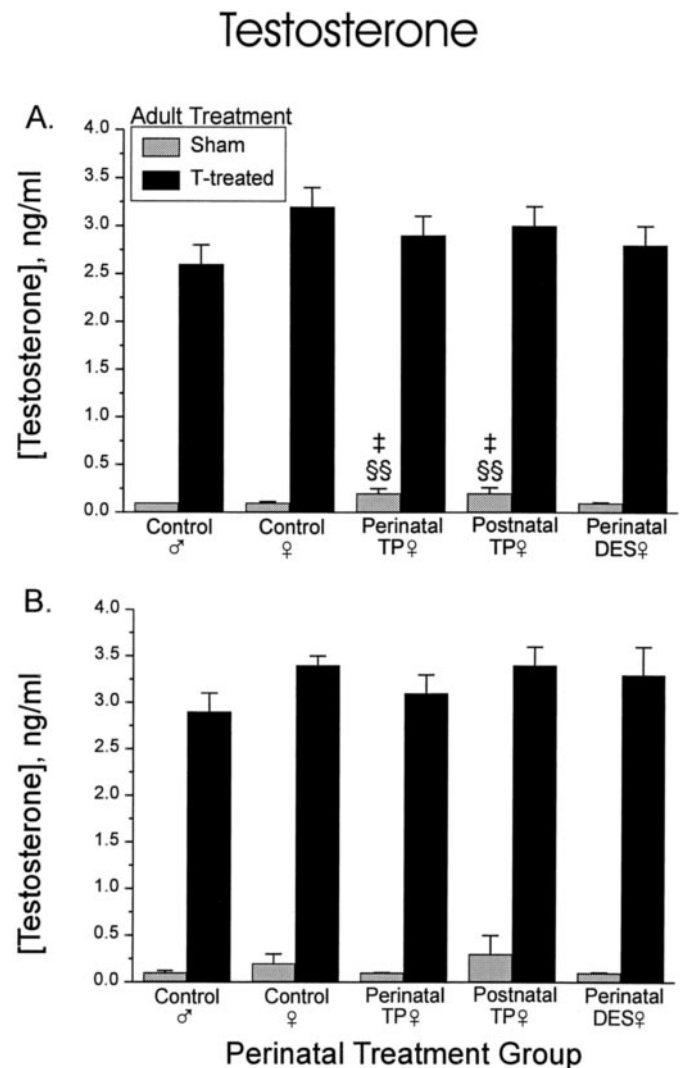


FIG. 10. Serum testosterone concentrations (mean \pm SEM) in Gdx rats that underwent sham surgery or received sc SILASTIC implants (30 mm) filled with crystalline T. Two-way ANOVA revealed significant effect of adult treatment ($F_{9,104} = 1701, P < 0.0001$) but not perinatal treatment or age of castration.

sexes to TP compared with that of normal adult males. In contrast, the perinatal treatments used in the present experiment were sufficient to completely masculinize the expression of AA, so that the enzyme activity in hormone-treated genetic females was equivalent to that in control males.

The induction of AA by T is transcriptionally mediated through a specific androgen receptor mechanism (36). Therefore, one important inference that can be drawn from the current study is that the perinatal exposure to androgen permanently alters the adult responsiveness to T. Moreover, the cellular basis for the effect of early androgen and estrogen exposure on androgen-dependent aromatase expression may relate in part to the differentiation of androgen receptor-positive neurons or their connections. Consistent with this idea, the distribution of androgen receptors and androgen-dependent aromatase exhibit an extensive regional overlap in the rat brain (37-40), and sex differences in androgen

receptor concentrations are found largely within the same brain regions as sex differences in aromatase (2).

In the current study, significant sex differences in the expression of basal levels of AA were found in the BNST and PVPOA of Gdx adults. Basal levels of aromatase were significantly higher in control males than in control females, but were not significantly different from those in females treated perinatally with TP. Sex differences in AA that are present after Gdx could be due to gender differences in androgens produced by the adrenals, but no sex-related differences in plasma T were detected in the Gdx sera in this study. Moreover, in a preliminary unpublished study, we did not detect differences between Gdx and Gdx-adrenalectomized rats in the levels of AA measured in brain punch samples. Thus, the sex differences in the Gdx levels of AA most likely reflect actual differences in the number of aromatase neurons independent of their capacity for hormonal induction and suggest that greater numbers of aromatase-containing cells are present within certain regions of the masculinized adult brain. Consistent with this hypothesis, Wagner and Morell (38) reported that adult male rats tended to have higher numbers of aromatase mRNA-expressing cells than females, but not more aromatase mRNA per cell. Likewise, a sex difference in the number of aromatase-immunoreactive cells has been found in the preoptic area of adult Japanese quail (41) and shrew (42). Finally, Beyer and Hutchison (43) have shown that numbers of aromatase-immunoreactive neurons are higher in hypothalamic cultures originating from male compared with female mice.

Interestingly, perinatal exposure to TP, but not DES, masculinized the basal levels of AA in the BNST and PVPOA. This observation contrasts with our finding that both TP and DES exposure masculinized the capacity for androgen induction of AA in these nuclei as well as in PVPOA and MPN. The fact that these two end points (*i.e.* basal and androgen-induced aromatase) can be differentially affected by perinatal steroid exposure suggests that they may represent independent processes that have different hormonal requirements for differentiation. Both end points appear to be sexually differentiated by androgen, but aromatization may be required to determine the capacity for induction of aromatase by androgen, *i.e.* androgen responsiveness, whereas androgen *per se* may determine the absolute number of aromatase neurons that develop or survive. There are precedents for considering that both local estrogen synthesis and unmetabolized androgens contribute to male-specific developmental processes in the perinatal brain. Locally synthesized estrogens are thought to irreversibly masculinize the size and connectivity of several brain nuclei, including the sexually dimorphic nucleus of the preoptic area (44). On the other hand, T, not its aromatized metabolites, affects the survival of neurons in the spinal nucleus of the bulbocavernosus (10). Moreover, it was recently demonstrated that T treatment increases the absolute number of aromatase-immunoreactive neurons in hypothalamic cultures and that, unlike estrogen, T stimulated their morphological differentiation (45). Taken together, these studies suggest that androgen and estrogen exert independent, perhaps complementary, effects on the developing brain that, in turn, affect

the basal levels and androgen responsiveness of aromatase in the adult.

Rats that were Gdx on PD 56 exhibited significantly higher levels of basal and T-stimulated aromatase in BNST, MPN, and PVPOA than rats that were Gdx on PD30. This effect was observed regardless of sex or perinatal treatment. In VMN, the effects of perinatal T treatments reached significance only in the group that was Gdx on PD 56. These data suggest that peripubertal exposure to gonadal secretions can enhance the expression of AA in certain areas of the adult brain, which may, in turn, enhance adult responsiveness to T. There was no effect of prepubertal Gdx on the expression of aromatase in AH or MA, indicating that this response was exerted in a regionally specific manner. The nature of the effect we observed was subtle and appeared to be secondary to the effect exerted by perinatal androgen exposure. However, if confirmed, this observation may help explain the results of earlier behavioral studies which demonstrated that pubertal exposure to testosterone sensitizes the neural circuits mediating male sexual behavior to the activational effects of T in adulthood (25–27, 46).

In conclusion, the present experiment has demonstrated that AA in the neural centers mediating masculine sexual behavior is sexually differentiated in the rat. We have shown that manipulations of the early hormonal milieu, which are known to differentiate sexual behavior, also irreversibly determine the capacity for aromatization in the adult. The process that differentiates aromatase appears to depend on androgen exposure and in part local estrogen synthesis and is brain region specific. As the activation of copulation by androgen requires aromatization of T to estradiol (47–49), this quantitative enzymatic difference between sexes could contribute in part to the greater behavioral responsiveness displayed by males. Finally, our data suggest that although exposure of the brain to steroid hormones during perinatal life appears to be necessary for sexual differentiation of aromatase expression to occur, gonadal hormones also appear to exert additional effects during puberty.

Acknowledgments

The authors gratefully acknowledge the valuable assistance of Joan West, recipient of an NSF Research Experience for Undergraduate award, and Jobin Nash, recipient of an American Heart Summer Fellowship award.

References

1. Mode A, Gustafsson J-A, Sodersten P, Eneroth P 1984 Sex differences in behavioral androgen sensitivity: possible role of androgen metabolism. *J Endocrinol* 100:245–248
2. Roselli CE 1991 Sex differences in androgen receptors and aromatase activity in microdissected regions of the rat brain. *Endocrinology* 128:1310–1316
3. Debold JF, Clemens LG 1978 Aromatization and the induction of male sexual behavior in male, female, and androgenized female hamsters. *Horm Behav* 11:401–413
4. Czaja JA 1984 Sex differences in the activational effects of gonadal hormones on food intake and body weight. *Physiol Behav* 33:553–558
5. MacLusky NJ, Naftolin F 1981 Sexual differentiation of the central nervous system. *Science* 211:1294–1302
6. Grady KL, Phoenix CH, Young WC 1965 Role of the developing rat testis in differentiation of the neural tissues mediating mating behavior. *J Comp Physiol Psychol* 59:176–182
7. Sachs BD, Pollak EI, Krieger MS, Barfield RJ 1973 Sexual behavior: normal male patterning in androgenized female rats. *Science* 181:770–771
8. Ward IL 1969 Differential effect of pre- and postnatal androgen on the sexual behavior of intact and spayed female rats. *Horm Behav* 1:25–36

9. Gray GD, Davis HN, Dewsbury DA 1976 Masculine sexual behavior in male and female rats following perinatal manipulation of androgen: effects of genital anesthetization and sexual experience. *Horm Behav* 7:317-329
10. Breedlove SM 1992 Sexual dimorphism in the vertebrate nervous system. *J Neurosci* 12:4133-4142
11. Goy RW, McEwen BS 1980 *Sexual Differentiation of the Brain*. MIT Press, Cambridge
12. De Vries GJ 1990 Sex differences in neurotransmitter systems. *J Neuroendocrinol* 2:1-13
13. Reisert I, Pilgrim C 1991 Sexual differentiation of monoaminergic neurons-genetic or epigenetic? *Trends Neurosci* 14:468-473
14. McGinnis MY, Katz SE 1996 Sex differences in cytosolic androgen receptors in gonadectomized male and female rats. *J Neuroendocrinol* 8:193-197
15. Herbison AE 1995 Sexually dimorphic expression of androgen receptor immunoreactivity by somatostatin neurons in rat hypothalamic periventricular nucleus and bed nucleus of the stria terminalis. *J Neuroendocrinol* 7:543-553
16. Lisciotto CA, Morrell JI 1994 Sex differences in the distribution and projections of testosterone target neurons in the medial preoptic area and the bed nucleus of the stria terminalis of rats. *Horm Behav* 28:492-502
17. Naftolin F, Ryan KJ, Davies IJ, Reddy VV, Flores F, Petro Z, Kuhn M, White RJ, Takaoka Y, Wolin L 1975 The formation of estrogens by central neuroendocrine tissues. *Recent Prog Horm Res* 31:295-319
18. Selmanoff MK, Brodtkin LD, Weiner RI, Siiteri PK 1977 Aromatization and 5 α -reduction of androgens in discrete hypothalamic and limbic regions of the male and female rat. *Endocrinology* 101:841-848
19. Roselli CE, Horton LE, Resko JA 1985 Distribution and regulation of aromatase activity in the rat hypothalamus and limbic system. *Endocrinology* 117:2471-2477
20. Sachs BD, Meisel RL 1994 The physiology of male sexual behavior. In: Knobil E, Neil J (eds) *The Physiology of Reproduction*. Raven Press, New York, pp 3-105
21. Roselli CE, Salisbury RL, Resko JA 1987 Genetic evidence for androgen-dependent and independent control of aromatase activity in the rat brain. *Endocrinology* 121:2205-2210
22. Abdelgadir SE, Resko JA, Ojeda SR, Lephart ED, McPhaul MJ, Roselli CE 1994 Androgens regulate aromatase cytochrome P450 messenger ribonucleic acid in rat brain. *Endocrinology* 135:395-401
23. Roselli CE, Klosterman SA, Fasasi TA 1996 Sex differences in androgen responsiveness in the rat brain: regional differences in the induction of aromatase activity. *Neuroendocrinology* 64:139-145
24. Roselli CE, Abdelgadir SE, Jorgensen E, Resko JA 1996 Sex differences in androgen regulated cytochrome P450 aromatase mRNA in the rat brain. *Endocrine* 5:59-65
25. Sisk CL, Berglund LA, Tang YA, Venier JE 1992 Photoperiod modulates pubertal shifts in behavioral responsiveness to testosterone. *J Biol Rhythms* 7:329-339
26. Sodersten P, Damassa DA, Smith ER 1977 Sexual behavior in developing male rats. *Horm Behav* 8:320-341
27. Baum MJ 1972 Precocious mating in male rats following treatment with androgen or estrogen. *J Comp Physiol Psychol* 78:356-367
28. Dohler KD, Coquelin A, Davis F, Hines M, Shryne JE, Gorski RA 1984 Pre- and postnatal influence of testosterone propionate and diethylstilbestrol on differentiation of the sexually dimorphic nucleus of the preoptic area in male and female rats. *Brain Res* 302:291-295
29. Roselli CE, Ellinwood WE, Resko JA 1984 Regulation of brain aromatase activity in rats. *Endocrinology* 114:192-200
30. Palkovits M, Brownstein MJ 1988 *Maps and Guide to Microdissection of the Rat Brain*. Elsevier, New York
31. Roselli CE, Resko JA 1991 *In vitro* assay of aromatase activity in the central nervous system. In: Greenstein B (ed) *Neuroendocrine Research Methods*. Harwood, Chur, Switzerland, vol 2:937-951
32. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ 1951 Protein determination with the Folin phenol reagent. *J Biol Chem* 193:265-267
33. Resko JA, Malley A, Begley D, Hess DL 1973 Radioimmunoassay of testosterone during fetal development of the rhesus monkey. *Endocrinology* 93:156-161
34. Winer BJ 1962 *Statistical Principles in Experimental Design*. McGraw-Hill, New York
35. Steimer T, Hutchison JB 1990 Is androgen-dependent aromatase activity sexually differentiated in the rat and dove preoptic area. *J Neurobiol* 21:787-795
36. Roselli CE, Abdelgadir SE, Resko JA 1997 Regulation of aromatase gene expression in the adult rat brain. *Brain Res Bull* 44:351-357
37. Sar M, Stumpf WE 1975 Distribution of androgen-concentrating neurons in rat brain. In: Stumpf WE, Grant LD (eds) *Anatomical Neuroendocrinology*. Karger, Basel, pp 120-133
38. Wagner CK, Morrell JI 1996 Distribution and steroid hormone regulation of aromatase mRNA expression in the forebrain of adult male and female rats: a cellular-level analysis using *in situ* hybridization. *J Comp Neurol* 370:71-84
39. Simerly RB 1993 Distribution and regulation of steroid hormone receptor gene expression in the central nervous system. *Adv Neurol* 59:207-226
40. Tsuruo Y, Ishimura K, Fujita H, Osawa Y 1994 Immunocytochemical localization of aromatase-containing neurons in the rat brain during pre- and postnatal development. *Cell Tissue Res* 278:29-39
41. Foidart A, De Clerck A, Harada N, Balthazard J 1994 Aromatase-immunoreactive cells in the quail brain: effects of testosterone and sex dimorphism. *Physiol Behav* 55:453-464
42. Rissman EF, Harada N, Roselli CE 1996 Effect of vorozole, an aromatase enzyme inhibitor, on sexual behavior, aromatase activity and neural immunoreactivity. *J Neuroendocrinol* 9:199-210
43. Beyer C, Green SJ, Hutchison JB 1994 Androgens influence sexual differentiation of embryonic mouse hypothalamic aromatase neurons *in vitro*. *Endocrinology* 135:1220-1226
44. Gorski RA 1985 Sexual differentiation of the brain: possible mechanisms and implications. *Can J Physiol Pharmacol* 63:577-594
45. Beyer C, Hutchison JB 1997 Androgens stimulate the morphological maturation of embryonic hypothalamic aromatase-immunoreactive neurons in the mouse. *Dev Brain Res* 98:74-81
46. Larsson K 1967 Testicular hormone and developmental changes in mating behavior of the male rat. *J Comp Physiol Psychol* 63:223-230
47. Lephart ED, Simpson ER, McPhaul MJ 1992 Ovarian aromatase cytochrome P-450 mRNA levels correlate with enzyme activity and serum estradiol levels in anestrus, pregnant and lactating rats. *Mol Cell Endocrinol* 85:205-214
48. Clancy AN, Zumppe D, Michael RP 1995 Intracerebral infusion of an aromatase inhibitor, sexual behavior and brain estrogen receptor-like immunoreactivity in intact male rats. *Neuroendocrinology* 61:98-111
49. Vagell ME, McGinnis MY 1997 The role of aromatization in the restoration of male rat reproductive behavior. *J Neuroendocrinol* 9:415-421