Effects of Gonadal Steroids during Pubertal Development on Androgen and Estrogen Receptor-α Immunoreactivity in the Hypothalamus and Amygdala

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ABSTRACT: Perinatal development is often viewed as the major window of time for organization of steroid-sensitive neural circuits by steroid hormones. Behavioral and neuroendocrine responses to steroids are dramatically different before and after puberty, suggesting that puberty is another window of time during which gonadal steroids affect neural development. In the present study, we investigated whether the presence of gonadal hormones during pubertal development affects the number of androgen receptor and estrogen receptor α-immunoreactive (AR-ir and ERα-ir, respectively) cells in limbic regions. Male Syrian hamsters were castrated either before or after pubertal development, and 4 weeks later they received a single injection of testosterone or oil vehicle 4 h prior to tissue collection. Immunocytochemistry for AR and ERα was performed on brain sections from testosterone-treated and oil-treated males, respectively. Adult males that had been castrated before puberty had a greater number of AR-ir cells in the medial preoptic nucleus than adult males that had been castrated after puberty. There were no significant differences in ERα-ir cell number in any of the brain regions examined. The demonstration that exposure to gonadal hormones during pubertal development is associated with reduced AR-ir in the medial preoptic nucleus indicates that puberty is a period of neural development during which hormones shape steroid-sensitive neural circuits. © 2000 John Wiley & Sons, Inc. J Neurobiol 44: 361–368, 2000

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Perinatal development is often viewed as the major window of time for the organization of neural circuits by steroids (MacLusky and Naftolin, 1981). In males, testosterone titers increase markedly during late prenatal and early postnatal development (Erskine and Baum, 1982; Weisz and Ward, 1980). This transient increase in steroid production is responsible for the masculinization and organization of neural structures that mediate the effects of steroid hormones on reproductive behaviors and the hypothalamic-pituitary-gonadal axis in adulthood (Becú-Villalobos et al., 1997; Resko and Roselli, 1997). However, the perinatal period is not likely the only period of development during which the sensitivity of the central nervous system to steroid hormones can be determined or influenced. Arnold and Breedlove (1985) suggest that endocrine changes that occur well after neonatal development may have profound effects on the organization of neural circuits. A hallmark of pubertal development is the increased production and secretion of gonadal steroid hormones (Foster, 1994; Ojeda and Urbanski, 1994; Plant, 1994). Therefore, in addition to the perinatal period, puberty may also be associated...
with steroid-dependent neural development required for the maturation of adult physiology and behavior. There is good evidence for organizational effects of steroid hormones during puberty on social interaction (SI) in a novel environment and open-field ambulation, two behaviors used as inverse indices of anxiety. Adult male rats show less SI (Primus and Kellogg, 1989) and less open-field ambulation (Slob et al., 1986) than prepubertal males. If animals are gonadectomized before puberty and tested in adulthood, they display high levels of SI and open-field ambulation, similar to those displayed by juveniles (Brand and Slob, 1988; Primus and Kellogg, 1990). Testosterone replacement at the time of gonadectomy permits the normal pubertal change in these behaviors. In contrast, animals gonadectomized after puberty engage in the same amount of SI and open-field ambulation as intact adults (Brand and Slob, 1988; Primus and Kellogg, 1990). Thus, the presence of gonadal steroids during puberty results in a behavioral change that does not require the continued presence of steroids, an effect that fits the traditional definition of organizational effects of steroid hormones. Estrogen formed from the aromatization of testosterone mediates the puberty-related decrease in SI (Kellogg and Lundin, 1999). Taken together, these studies demonstrate that behavioral potentials can be recast during pubertal development by androgenic and estrogenic steroids.

In the present experiment, we explored the effects of gonadal hormones during puberty on steroid-sensitive limbic circuits. Specifically, we compared the number of androgen receptor- and estrogen receptor \( \alpha \)-immunoreactive (AR-ir and ER\( \alpha \)-ir, respectively) cells in brain regions that mediate steroid-dependent behavioral and neuroendocrine responses (Wood and Newman, 1995a; Wood and Newman, 1995b) in adult males that were gonadectomized either before or after puberty. If puberty is a developmental stage during which gonadal hormones shape the hormone responsiveness of steroid-sensitive neural circuits, then the number of AR-ir or ER\( \alpha \)-ir cells could be affected by the age at which gonadectomy occurs. We report here that AR-ir within the preoptic area of adults is indeed influenced by the presence of gonadal hormones during pubertal development.

**METHODS**

**Animals and Housing**

Twenty-four male Syrian hamsters (Mesocricetus auratus) obtained from Charles River Laboratories (Kingston, NY) were used in the present experiment. All animals arrived on the same day and were each housed separately in clear polycarbonate cages (37.5 \( \times \) 33 \( \times \) 17 cm) with wood chips (Aspen Chip Laboratory Bedding, Warrensburg, NY), and had ad libitum access to rodent chow (Teklad Rodent Diet No. 8640, Harlan, Madison, WI) and tap water. The vivarium was maintained at 21 \( \pm \) 2°C with a light/dark schedule of 14 h light/10 h dark (lights on at 0700 h EST). All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Michigan State University All-University Committee for Animal Use and Care.

**Experimental Design**

Twelve prepubertal (21 days of age) and 12 postpubertal (60 days of age) male hamsters were gonadectomized under methoxyflurane anesthesia (metofane, Schering-Plough, Union, NJ) on the day of arrival. Twenty-eight days after castration (i.e., at either 49 or 88 days of age), half the animals in each age group were given an injection of testosterone (2.5 mg/kg, \( sc \); Sigma, St. Louis, MO) or the sesame oil vehicle (\( n = 6/treatment and age \)) 4 h before tissue collection. This design generated 4 groups of animals (Fig. 1):

1. Males gonadectomized before puberty and given a single injection of oil in adulthood (GdxBP+Oil)
2. Males gonadectomized before puberty and given a single injection of testosterone in adulthood (GdxBP+T)
3. Males gonadectomized after puberty and given a single injection of oil in adulthood (GdxAP+Oil)
4. Males gonadectomized after puberty and given a single injection of testosterone in adulthood (GdxAP+T).

Testosterone was administered shortly before tissue collection to cause translocation of the ARs to the
nucleus of the cell and to maximize visualization of AR-ir (Kashon et al., 1996; Wood and Newman, 1999). Only brain sections from animals treated with testosterone were processed for AR-ir. The ERα antibody H222 was used in the present study. Since H222 staining intensity is generally decreased by the presence of estrogen (Blaustein, 1993; Sisk and DonCarlos, 1995), brain sections from the oil-treated males only were processed for ERα, because of the possibility that estrogen derived from the aromatization of the injected testosterone would reduce ERα-ir.

**Tissue Collection**

Four h after the injection of oil or testosterone, animals were given an overdose of sodium pentobarbital (130 mg/kg, ip), and blood samples were obtained via cardiac puncture for measurement of plasma testosterone and luteinizing hormone (LH) concentrations. Animals were then intracardially perfused with 100 mL of buffered saline rinse followed by 150 mL of 4% paraformaldehyde. Brains were removed from the skull and postfixed for 1 h in 4% paraformaldehyde and then transferred to a 20% sucrose solution in phosphate buffered saline (PBS). At least 48 h later, 40 μm coronal sections were made on a cryostat and stored in cryoprotectant at −20°C until the AR or ERα immunocytochemistry was performed, or until sections were mounted and stained with thionin to delineate the boundaries for cross-sectional area measurements.

**AR and ERα Immunocytochemistry**

Every fourth section from each brain was processed simultaneously during a single immunocytochemical run for either AR or ERα. For AR immunocytochemistry, free-floating sections were rinsed 5 times in 0.1 M PBS to remove the cryoprotectant. Sections were then incubated sequentially in 0.1 M glycine in 0.1 M PBS (30 min), 0.3% H₂O₂ in PBS (10 min), 4% normal goat serum (Vectastain ABC Kit, Burlingame, CA) in 0.3% Triton X-100 in PBS (PBS-TX; 1 h), and 0.25 μg/mL rabbit anti-AR in PBS-TX (PG-21-18a, obtained from G. S. Prins, Michael Reese Hospital, Chicago, IL; 48 h). Sections were then incubated in secondary antibody (goat-antirabbit, Vectastain Elite Kit, 1:200 in PBS-TX; 24 h), followed by incubation in avidin-biotin-HRP (Horseradish Peroxidase) complex (Vectastain ABC Elite Kit, 1:50 in PBS-TX; 2 h). For the chromogen reaction, sections were incubated for 1 h in 0.0125% diaminobenzidine (DAB), 0.01875% NiCl₂, and 0.05% H₂O₂. Sections were rinsed 3 times in PBS between incubations in each reagent. All incubations were at room temperature (RT) except for that with primary antibody, which was at 4°C. Tissue was reacted in DAB for 1 h to maximize visualization of AR-ir cells in long term castrates (Wood and Newman, 1999).

The protocol used for ERα immunocytochemistry was similar to that previously published by our laboratory (Romeo et al., 1999). Briefly, sections were incubated for 10 min in 1% sodium borohydride in Tris buffered saline (TBS) and blocked in 1% H₂O₂, 25% normal rabbit serum, and 1% bovine serum albumin in TBS for 20 min at RT, followed by incubation in H222 (1 μg of H222/mL of TBS, 0.5% Triton X-100, 0.1% gelatin, and 0.02% sodium azide, 72 h, 4°C). Tissue was then incubated in rabbit antirabbit secondary antibody (Vectastain Elite Kit, 1:200 in TBS), followed by an avidin-biotin horseradish peroxidase incubation (Vectastain ABC Kit, 1:50 in TBS), both for 90 min at RT. Tissue was then reacted in 0.05% DAB and 0.05% H₂O₂ for 10 min.

After the immunocytochemical procedure, sections were mounted on gelatin coated slides, dehydrated in increasing concentrations of alcohols, cleared, and coverslipped. To test for nonspecific AR or ERα staining, sections were processed as described above for the respective receptor, but in the absence of either primary or secondary antibody. Omission of either antibody from either protocol eliminated all detectable immunoreactivity.

**Microscopic Analysis**

The areal density (cells per unit area) of AR-ir and ERα-ir cells was determined for the medial preoptic nucleus (MPN), the magnocellular region of the medial preoptic nucleus (MPNmag), the posteromedial subdivision of the bed nucleus of the stria terminalis (BNSTpm), the ventral medial hypothalamus (VMH), the anterior subdivision of the medial amygdala (MeA), and the posterior subdivision of the medial amygdala (MeP). These areas were chosen for examination because these nuclei are known to contain numerous AR-ir and ERα-ir cells, and to mediate behavioral or neuroendocrine responses to steroids in adulthood (Wood and Newman, 1995a; Wood and Newman, 1995b). Bilateral counts were made in 2 sections for each nucleus, except for the BNSTpm and the MPNmag, in which 1 bilateral count was made in anatomically matched sections. When 2 brain sections were analyzed, they were separated by 160 μm and anatomically matched across animals. Slides were coded during microscopic analysis so that the experimenter was blind to the treatment of the animal.
Using bright-field illumination microscopy, nuclei were centered using a 10× objective, and the magnification was then increased to 40×. Cells that fell within the area (62,500 μm²) of an ocular grid centered in the eyepiece were counted. All data are expressed as the mean number of AR-ir or ERα-ir cells/62,500 μm². As this was not a stereological analysis, the cell counts do not represent the absolute number of AR-containing cells in the brain regions that were quantified.

The cross-sectional areas of MPN, MPNmag, BN-STpm, VMH, MeA, and MeP were measured on a separate adjacent set of Nissl-stained sections from the animals that were gonadectomized either before or after puberty and treated with oil in adulthood (e.g., GdxBP1 Oil and GdxAP1 Oil, respectively). Measurements were made by capturing the nucleus of interest with a 4× objective using a Sony XC-77 video camera and displaying the image on a monitor using NIH Image 1.56 software (National Institutes of Health, Bethesda, MD). The nucleus was outlined and the area (μm²) was computed by NIH Image 1.56 software. As before, slides were coded to keep the experimenter blind to the treatment condition of the animals.

Radioimmunoassays

**Testosterone Radioimmunoassay** Plasma testosterone concentrations were measured using the Coat-A-Count Total Testosterone Kit (Diagnostic Products, Los Angeles, CA). This assay has been previously validated in our laboratory for the measurement of plasma testosterone concentrations in Syrian hamsters. The lower limit of detectability of the assay was 0.09 ng/mL and the intra-assay CV was 6.5%.

**Luteinizing Hormone Radioimmunoassay** Plasma LH concentrations were measured using reagents in the rat LH kit obtained through Dr. A. F. Parlow and the National Hormone and Pituitary Program at the National Institute of Diabetes, Digestive, and Kidney Diseases (NIDDK). Values are reported as nanogram equivalents of NIDK-rLH-RP-3. This assay has been validated in our laboratory for the measurement of plasma LH concentrations in Syrian hamsters. The lower limit of detectability was 0.66 ng/mL, and the intra-assay CV was 8.6%.

**Statistical Analysis**

For groups gonadectomized before or after puberty and given testosterone, AR-ir cell number, hormonal, and somatic data were analyzed by t tests. Similarly, t tests were run on ERα-IR cell number, cross-sectional area, hormonal, and somatic data in the oil-treated groups that were gonadectomized before or after pubertal development. Differences were considered significant if p > .05. All values are reported as means ± SEM.

**RESULTS**

**Hormonal Measures**

As expected, testosterone was undetectable, or nearly so, in oil-treated males, and plasma LH levels were high and characteristic of long-term gonadectomized males (Table 1). Testosterone injections resulted in similar levels of circulating testosterone in GdxBP and GdxAP animals (Table 1). These testosterone concentrations are slightly higher than those found in intact adult males (Meek et al., 1997). Plasma LH levels were not significantly different in the GdxBP and GdxAP males receiving the testosterone 4 h prior to perfusion, and were approximately half those of the oil-treated groups (Table 1). Thus, a 4 h period of exposure to testosterone was sufficient to suppress plasma LH concentrations.

**AR- and ERα-ir Cell Number and Nuclear Cross-Sectional Area**

**AR-ir** Statistical analysis revealed that GdxBP+T males had a significantly greater number of AR-ir
cells in the MPN compared to the GdxAP+T males (Fig. 2). A trend toward increased AR-ir cell number in GdxBP+T males was also observed in the 3 other hypothalamic brain regions examined (i.e., MPNmag, BNSTpm, and VMH; Fig. 2). In contrast, the number of AR-ir cells in the amygdaloid regions was similar in the GdxBP and GdxAP groups (Fig. 2). Figure 3 shows a photomicrograph depicting the greater number of AR-ir cells in the MPN of GdxBP+T males compared with GdxAP+T males.

**ERα-ir and Cross-Sectional Area** ERα-ir cell number was equivalent in all nuclei examined in the GdxBP and GdxAP groups treated with oil (Figure 4). Furthermore, there were no significant differences in cross-sectional area in any of the nuclei measured (Table 2).

**DISCUSSION**

This experiment demonstrates that the number of AR-ir cells in the MPN is influenced by the presence of gonadal hormones during pubertal development. Specifically, adult males gonadectomized before puberty had a greater number of AR-ir cells in the MPN compared to adult males gonadectomized after puberty. A similar trend was observed in other hypothalamic regions but not in the amygdaloid regions, indicating that, during puberty, brain-region specificity determines the effectiveness of gonadal hormones on AR-ir. These data provide support for the concept that puberty is a developmental stage during which hormones shape steroid-sensitive brain regions, and sug-
suggest that a hormone-dependent change in androgen sensitivity or responsiveness is one outcome of normal pubertal development.

The difference in chronological age between the GdxBP and GdxAP groups is not likely to account for the reduced amount of AR-ir in the GdxAP males. Although hypothalamic AR levels are significantly reduced in aged males (approximately 20 months) (Chambers et al., 1991; Greenstein, 1979; Haji et al., 1980), the 49- and 88-day-old animals in the present study (both less than 3 months old) are well within the period of young adulthood. Therefore, our interpretation of the present data is that the decrease in AR-ir cell number during pubertal development is modulated by exposure to gonadal hormones during the time of puberty, and not by chronological aging between 49 and 88 days of age.

The presence of gonadal hormones during puberty may influence the expression or processing of AR protein within androgen-responsive cells without altering the absolute number of AR-containing cells. Both AR synthesis and half-life are increased by androgen within 4 h (Kempfainen et al., 1992; Syms et al., 1985). Therefore, the difference in the number of detectable AR-ir cells in testosterone-treated GdxBP and GdxAP males could reflect a difference in the interaction of testosterone with its own receptor, leading to lower and less readily detectable levels of intracellular AR in GdxAP males. The testosterone given 4 h before tissue collection and the 1 h incubation in nickel-intensified DAB were designed to maximize nuclear AR staining and the detection of extremely low levels of intracellular AR (Kashon et al., 1996; Wood and Newman, 1999). Therefore, the lower number of AR-ir cells in the GdxAP males may instead reflect a change in cell phenotype for a population of cells that no longer synthesize AR. Regardless of whether the reduced number of AR-ir cells in males exposed to gonadal hormones during puberty is due to reduced AR expression per cell or to reduced numbers of AR-expressing cells, the functional outcome is a change in response to testosterone. Thus, the hormonal events associated with puberty may

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>GdxBP+Oil</th>
<th>GdxAP+Oil</th>
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<tbody>
<tr>
<td>MPN</td>
<td>36798 ± 14716</td>
<td>398648 ± 19265</td>
</tr>
<tr>
<td>MPNmag</td>
<td>226021 ± 13644</td>
<td>23566 ± 14267</td>
</tr>
<tr>
<td>BNSTpm</td>
<td>348130 ± 23179</td>
<td>391906 ± 30334</td>
</tr>
<tr>
<td>VMH</td>
<td>390544 ± 18234</td>
<td>402371 ± 15121</td>
</tr>
<tr>
<td>MeA</td>
<td>147117 ± 5357</td>
<td>167736 ± 9012</td>
</tr>
<tr>
<td>MeP</td>
<td>273557 ± 21039</td>
<td>293349 ± 6876</td>
</tr>
</tbody>
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Normally act on target cells to modify their response to steroids later in life.

Previous data from this laboratory and others support the assertion that the interaction between testosterone and AR is altered by exposure of the brain to gonadal hormones during puberty. First, we have shown that the number of AR-ir cells in several hypothalamic nuclei is greater in gonadectomized, testosterone-treated prepubertal animals compared to their adult counterparts (Meek et al., 1997). Thus, animals that have not yet experienced the pubertal rise in gonadal steroids have a greater number of hypothalamic AR-ir cells compared with males that have experienced normal puberty. In contrast, the regulation of AR by testosterone appears to be similar in adult male hamsters housed in either long or short photoperiods. Like pubertal males, short-day housed adults have regressed testes and reduced testosterone and AR levels (Berndtson and Desjardins, 1974; Prins et al., 1990). Yet if long-day and short-day housed adults are experimentally treated to equate circulating levels of testosterone, AR levels in the hypothalamus are equivalent in the 2 groups of adults (Prins et al., 1990). Thus, despite the similarities between prepubertal males and short-day adults with respect to gonadal function, differences between the 2 groups with respect to testosterone regulation of hypothalamic AR may be related to the fact that the nervous system of the short-day housed adult has been impacted by steroids during puberty, while the prepubertal nervous system has not.

An alternative explanation for the decreased number of AR-ir cells in the MPN of males exposed to gonadal hormones during puberty is a steroid-dependent loss of AR-containing cells. Such putative cell death would appear to be specific to the AR-containing cells, as we did not observe an equivalent decrease in ERα-ir cell number, which is in agreement with our previous finding that prepubertal and adult male castrates have similar numbers of ERα-ir cells in hypothalamic and amygdaloid nuclei (Romeo et al., 1999). Furthermore, since about half of the AR-containing cells in the MPN are also ERα-positive (Wood and Newman, 1995a), any loss of AR-ir cells is likely to be specific to the subpopulation of neurons that contain only ARs. The lack of a difference in the cross-sectional area of the MPN of GdxBP and GdxAP males suggests that if the decrease in AR-ir cell number in the MPN is indeed due to a decrease in the absolute number of AR-containing cells, then the loss of cells is not of sufficient magnitude to reduce the area of the nuclei. However, the lack of a significant change in cross-sectional area of the nuclei measured in the present study cannot rule out the possibility that
The possibility that puberty is a period of development during which some AR-containing cells are selectively lost would be substantiated by the identification of apoptotic AR-ir cells in the MPN during pubertal development. It is perhaps unorthodox to view puberty as a period of neural development during which cell death is a mechanism for the refinement of neural circuits. However, synaptic pruning has been raised as an explanation for the decrease in cortical gray matter observed in boys and girls during adolescence (Giedd et al., 1999). The occurrence of fundamental developmental processes such as apoptosis and synaptic pruning during puberty would support the concept of puberty as a significant and perhaps critical period of neural development.

The functional consequence of reduced AR per cell or fewer AR-responsive cells after pubertal development is presently unknown. We have previously shown that in gonad intact, untreated males, an age-related increase in AR-ir cells in the MPN is associated with increased behavioral sensitivity to testosterone after puberty (Meek et al., 1997). However, as mentioned earlier, when prepubertal and adult males are treated with equal doses of testosterone, the number of AR-ir cells in the prepubertal males is greater than that found in adults, even though the testosterone activates mating behavior only in the adults (Meek et al., 1997). Taken together with the present data, our studies indicate that the relationship between AR and the facilitation of male mating behavior is not a simple or linear one, but instead involves complex interactions with other transcription factors, signalling pathways, and cellular processes.

In sum, this experiment provides evidence that the presence of gonadal hormones during the time of puberty results in alterations in the regulation of AR within hypothalamic cell groups related to reproductive function and behavior. These results show that pubertal processes remodel neural circuits previously established during earlier periods of development, suggesting that puberty is another important, perhaps critical, period of development during which gonadal hormones shape nervous system structure and function.

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REFERENCES


