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Actions of Testosterone in Prepubertal and Postpubertal Male Hamsters: Dissociation of Effects on Reproductive Behavior and Brain Androgen Receptor Immunoreactivity

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This study was conducted to determine whether there is a increase in responsiveness to the activating effects of testosterone on male reproductive behavior during puberty in male golden hamsters and whether responsiveness to behavioral actions of testosterone is correlated with the ability of testosterone to upregulate brain androgen receptor immunoreactivity (AR-ir). Sexually naive male hamsters were castrated at 21 or 42 days of age and implanted subcutaneously with a pellet containing 0, 2.5, or 5 mg of testosterone. One week later, males were given a 10-min mating test with a receptive female. Animals were euthanized 1 hr after the behavioral test, and blood samples and brains were collected. Plasma testosterone levels were equivalent in prepubertal and adult males that had been administered the same dose of testosterone. However, adult males exhibited more mounts, intromissions, and ejaculations than prepubertal males, demonstrating that postpubertal males are more responsive than prepubertal males to the effects of testosterone on sexual behavior. In both age groups, testosterone increased the number of AR-ir cells per unit area in several brain regions involved in male sexual behavior, including the medial preoptic nucleus (MPN), medial amygdala, posteromedial bed nucleus of the stria terminalis, and magnocellular preoptic nucleus (MPNmag). Surprisingly, testosterone increased AR-ir in the latter three regions to a greater extent in prepubertal males than in adults. Thus, prepubertal males are more responsive to the effects of testosterone on AR-ir in these regions. In a separate experiment, a pubertal increase in the number of AR-ir cells per unit area was found in both the MPN and MPNmag of intact male hamsters. These results indicate that a testosterone-dependent increase in brain AR during puberty may be necessary, but is not sufficient, to induce an increase in behavioral responsiveness to testosterone.

One hallmark of puberty in males is the appearance of steroid-dependent reproductive behaviors. Clearly, one factor contributing to this behavioral maturation is the pubertal rise in testosterone secretion from the testes. However, an increase in responsiveness of the nervous system to the actions of testosterone appears to be a second mechanism involved in pubertal maturation of sexual behavior. Evidence for a pubertal increase in responsiveness to the behavioral actions of steroid hormones comes from studies demonstrating that even when juvenile males are treated with doses of testosterone that activate male reproductive behavior in adults, they do not express equivalent levels of male reproductive behavior (Baum, 1972; Larsson, 1967; Sisk, Berglund, Tang, and Venier, 1992; Sodersten, Damassa, and Smith, 1977). The cellular mechanisms contributing to the pubertal increase in behavioral responsiveness to testosterone are not known.

Although testosterone is the predominant circulating steroid hormone in adult male rodents, activation of many components of male reproductive behavior is due to the action of estradiol, which is formed locally in the brain by the aromatization of testosterone. However, several lines of evidence indicate that testosterone or an androgenic metabolite acts in concert with estrogen

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to stimulate the full repertoire of male reproductive behavior (reviewed in Meisel and Sachs, 1994). In addition, androgens play an indirect role in estrogenic activation of behavior, because activity of the aromatase enzyme in the preoptic area, a known target site for steroid activation of male reproductive behavior, is regulated by androgens (Roselli and Resko, 1984). Cellular responses to steroid hormones are mediated by specific steroid receptors that, when activated by a ligand, alter transcription of steroid-regulated genes within target cells. Thus, regulation of the abundance of androgen receptor within the neural circuitry mediating male reproductive behavior is a potential mechanism by which behavioral responsiveness to testosterone could be modulated during puberty.

In male ferrets, a pubertal increase in androgen receptor immunoreactivity (AR-ir) occurs within two brain areas that are components of the neural circuit mediating male reproductive behavior, the medial preoptic area and the medial amygdala (Kashon and Sisk, 1995; Kashon and Sisk, 1994). Furthermore, administration of a large dose of testosterone to prepubertal ferrets increases AR-ir in these brain areas (Kashon, Hayes, Shek, and Sisk, 1995). These findings raise the interesting possibility that the pubertal rise in testosterone may contribute to the pubertal increase in behavioral responsiveness to testosterone by upregulating androgen receptor in relevant brain regions.

The present experiments were conducted to investigate further the relationship between pubertal maturation of male reproductive behaviors and pubertal regulation of androgen receptor by testosterone, using the male golden hamster as an animal model. The experiments were designed to determine: (1) whether there is a pubertal increase in responsiveness to behavioral actions of testosterone in the golden hamster; (2) if there is a pubertal increase in AR-ir within the neural circuit known to mediate sexual behavior in male hamsters and, if so, whether testosterone upregulates AR-ir in the same brain regions; and (3) whether responsiveness to behavioral actions of testosterone is correlated with the ability of testosterone to upregulate brain AR-ir in male hamsters.

METHODS

Two experiments were conducted. In Experiment 1, the effects of varying doses of testosterone on male reproductive behavior and on brain AR-ir were examined in castrated prepubertal and postpubertal male hamsters. Experiment 2 was conducted to determine whether there is a pubertal increase in brain AR-ir; in this experiment, the immunocytochemical protocol was modified from that used in Experiment 1.

Subjects and Treatment

Male golden hamsters (*Mesocricetus auratus*) were bred at Michigan State University (E. Lansing, MI). All animals were housed in clear polycarbonate cages (37.5 × 33 × 17 cm) with wood chips (Aspen Chip Laboratory Bedding, Warrensburg, NY) and bedding (cotton upholstery batting). Following weaning at 21 days of age, animals were housed in same-sex sibling groups until treatment, at which time they were singly housed. Room temperature was maintained at 21 ± 2°C and the light–dark schedule was 14 hr light/10 hr dark (lights on at 2300 hr EST). Food (Teklad Rodent Diet No. 8640, Harlan, Madison, WI) and water were available *ad libitum.*

Experiment 1. Hamsters were castrated under methoxyflurane anesthesia at either 21 or 42 days of age and were implanted with a 3-week timed-release pellet (Innovative Research of America, Sarasota, FL) containing either 0, 2.5, or 5 mg of testosterone (n = 5–6 per age and treatment group). Two additional groups of hamsters received a sham castration and a 0-mg pellet implant at either 21 (n = 6) or 42 (n = 6) days of age. One week following treatment (either 28 or 49 days of age), all subjects were paired with a hormone-primed estrous adult female for a 10-min mating behavior test (see below). One hour after the onset of the mating test, hamsters were euthanized and perfused as described below.

Experiment 2. Twenty-one-day-old (n = 3), 28-day-old (n = 6), and 49-day-old (n = 6) intact male hamsters were used in this experiment. These animals received no experimental manipulation or behavioral test, but were sacrificed at approximately the same time of day as were animals in Experiment 1 as described below.

Tests for Male Reproductive Behavior (Experiment 1 only)

Immediately prior to the onset of the dark portion of the light/dark schedule, hamsters were moved in their home cages to a testing room. All behavioral tests occurred between 10 min and 3 hr after lights out. The male was placed in a clean 10-gal. aquarium (51 × 26 × 31.5 cm) with no bedding and was allowed 10 min to acclimate to the novel testing environment before the introduction of a receptive female hamster. Stimulus females were ovariectomized adults made estrous by

sequential injections of estradiol benzoate (10 μg in 0.1 ml sesame oil, sc, 48 hr prior to testing) and progesterone (0.1 mg in 0.1 ml sesame oil, sc, 4 hr prior to testing).

The behavior tests were videotaped under red light (three 25-W bulbs) with a Panasonic Color Video Camera (WV 3250). The testing aquarium was mounted on a stand over a slanted mirror, which provided a ventral view of the animals so that intromissions and ejaculations were clearly visible. Videotapes were later scored to determine the amount of time spent in anogenital investigation of the female, the frequency of vaginally oriented mounts, intromissions, and ejaculations.

Anogenital investigation was scored when the male investigated the perianal region of the female, including the flanks. A vaginal mount was scored when the male mounted with an orientation that would permit intromission. An intromission was scored if the male achieved vaginal penetration. An ejaculation was scored if, after a series of intromissions separated by short inter-intromission intervals, the male became temporarily uninterested in the female (20 sec–1 min) and engaged in extensive genital grooming. We had previously determined that this pattern of behavior was always correlated with sperm present in the vagina of the female and that sperm were not present when this behavior pattern was not observed.

Blood and Tissue Collection

Hamsters were euthanized with an overdose of Equithesin anesthetic (8 ml/kg, ip) and a 0.5-ml blood sample was obtained via cardiac puncture. Blood samples were centrifuged and plasma was removed and stored at −20°C until radioimmunoassay was performed. Body weight and flank gland length were recorded, and the seminal vesicles were removed and weighed after expression of the seminal fluid. Animals were perfused intracardially with 150 ml of Sorenson's phosphate buffer (pH 7.4) containing 0.8% NaCl, 0.8% sucrose, 0.4% β-D-(+)-glucose and 3 IU/ml heparin, followed by 250 ml of ice-cold fixative containing 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.5). Brains were removed, postfixed in 4% paraformaldehyde for 4 hr, and then stored in 20% sucrose in phosphate-buffered saline (PBS; 0.9% saline) at 4°C until sectioning approximately 48 hr later. Brains were cut into 40-μm-thick sections that were stored in a polyethylene-based cryoprotectant at −20°C until immunocytochemistry was performed.

Androgen Receptor Immunocytochemistry

Every third section from each brain was used for immunocytochemistry. Tissue from all treatment groups within an experiment was processed simultaneously. In Experiment 1, the following protocol was used. Sections were rinsed 10× in 0.1 *M* phosphate-buffered saline to remove the cryoprotectant. Sections were then incubated sequentially in 0.1 *M* glycine in 0.1 *M* PBS (30 min), 0.3% H_2O_2 in PBS (10 min), 4% normal goat serum (Vectastain ABC Elite kit, Burlingame, CA) in 0.3% Triton X-100 in PBS (PBS-TX; 1 hr), and 0.5 mg/ml rabbit anti-AR in PBS-TX (PG-21-18A, obtained from G. S. Prins, Michael Reese Hospital, Chicago, IL; 48 hr). Sections were then incubated in secondary antibody (goat anti-rabbit immunoglobulins, Vectastain ABC Elite kit, 1:200 in PBS-TX; 24 hr), followed by incubation in avidin–biotin–HRP complex (Vectastain ABC Elite kit, 1:50 in PBS-TX; 2 hr). For the chromogen reaction, sections were incubated for 6 min in 1% 3,3′-diaminobenzadine (DAB) containing 2% β-D-(+)-glucose, 0.04% NH_4Cl, 0.038% imidazole, 50 ml/ml 250 mM $NiCl_2$, and 0.0075% H_2O_2 in 0.05 *M* Tris-buffered saline (TBS). Sections were rinsed 3 times in PBS between incubations in each reagent. All incubations were at room temperature except for that with primary antiserum, which was at 4°C. After the DAB reaction, all sections were rinsed 5 times in distilled water, mounted onto gelatin-coated slides, dried, dehydrated in increasing concentrations of alcohols, cleared in Hemo-De, and coverslipped. For Experiment 2, the immunocytochemistry protocol was the same, except that the concentrations for primary antiserum, $NiCl_2$, and H_2O_2 were reduced to 0.25 mg/ml, 125 m*M*, and 0.00375%, respectively. Processing tissue in the absence of the primary antiserum resulted in no detectable immunostaining by the secondary antibody.

Analysis of Androgen Receptor Immunoreactivity

The areal density (cells per unit area) of AR-ir cell profiles (cross sections) was quantified in the medial preoptic nucleus (MPN), medial amygdala (MeAMY), lateral septum (LSept), bed nucleus of the stria terminalis, posteromedial subdivision (BNSTpm), and in the magnocellular region of the medial preoptic nucleus (MPNmag). These brain regions were selected for analysis because they are components of the steroid-sensitive neural circuit controlling male reproductive behaviors (Baum, Tobet, Starr, and Bradshaw, 1982; Lehman and Winans, 1982; Powers, Newman, and Bergondy, 1987; Wood and Newman, 1993b). Brain sections were inspected under bright-field microscopy and regions were located according to Kollack and Newman (1992). Two sections through each brain nucleus, separated by 120 mm and anatomically matched across animals,

were used for analysis. Each brain nucleus was centered in the field of view at 10×, and magnification was then increased to 60×. The microscopic images were captured by a video camera (Sony XC-77) and displayed on a computer monitor using NIH Image 1.56 software. These images, which represented an area of 100 by 130 μm at 60X, were then sharpened and printed out on a laser printer (Hewlett–Packard 5MP). All AR-ir profile counts were made from these printouts. A profile was considered immunopositive if blue–black reaction product was visible and of the shape and size of a cell nucleus. For each brain region examined, the average number of immunopositive nuclear profiles in four images (bilateral counts in each of two tissue sections) was computed for each animal, and data for experimental groups were expressed as mean number of AR-ir profiles/13,000 μm^2. A single experimenter blind to the age and treatment of the animals performed the microscopic analysis.

Testosterone Radioimmunoassay

Plasma concentrations of testosterone were measured with reagents in the Coat-A-Count Total Testosterone kit (Diagnostic Products, Los Angeles, CA) in two different assays. The lower limits of detectability of the assays were 0.1 and 0.08 ng/ml, and the intraassay coefficients of variation (CV) were 5.5 and 6.7%, respectively. The interassay CV was 6.1%.

Statistical Analysis

In Experiment 1, *t* tests were used to compare behavioral, physiological, and anatomical measures in prevs postpubertal sham castrates. For testosterone-treated castrated males, the effects of age and treatment on plasma testosterone concentrations and behavioral variables were analyzed by two-way ANOVAs (dose by age). Significant interactions were further analyzed with Tukey HSD tests, and pairwise comparisons of main effects were made with Fisher's PLSD tests. Group differences in the percentage of animals displaying at least one occurrence of a behavior were analyzed by χ^2 tests. In Experiment 2, one-way ANOVAs or *t* tests were used to examine the effect of age on physiological variables and AR-ir profile density in intact male hamsters. For all statistical tests, differences were considered significant when $P < 0.05$.

TABLE 1
Mean Plasma Testosterone Concentration, Seminal Vesicle Weight, and Flank Gland Length

Age (days)–endocrine status	Plasma testosterone (ng/ml)	Seminal vesicle (g)	Flank gland (mm)
Experiment 1 (sham-castrated, mated animals)			
28–intact	0.59 ± 0.18	0.05 ± 0.00	5.15 ± 1.06
49–intact	7.38 ± 0.65	0.32 ± 0.02	8.00 ± 0.22
Experiment 1 (castrated, testosterone-treated, mated animals)			
28–0 mg T	0.08 ± 0.00	0.02 ± 0.00	3.64 ± 0.95
28–2.5 mg T	2.17 ± 0.24	0.11 ± 0.01	3.82 ± 0.16
28–5 mg T	3.94 ± 0.63	0.13 ± 0.01	3.22 ± 1.48
49–0 mg T	0.08 ± 0.00	0.16 ± 0.03	6.97 ± 0.89
49–2.5 mg T	1.65 ± 0.44	0.35 ± 0.02	8.46 ± 0.52
49–5 mg T	4.84 ± 1.13	0.36 ± 0.03	7.94 ± 0.23
Experiment 2 (unmated, intact animals)			
21–intact	0.11 ± 0.01	0.01 ± 0.00	Not measurable
28–intact	0.34 ± 0.17	0.03 ± 0.00	6.82 ± 0.52
49–intact	5.54 ± 0.89	0.37 ± 0.03	8.42 ± 0.45

RESULTS

Experiment 1: Dose–Response Curves to Testosterone in Prepubertal and Adult Males

Behavior and Peripheral Measures

Sham-castrated, mated animals. Sham-castrated adults had significantly higher levels of circulating T than the prepubertal sham castrates ($t = 100.188$, $P < 0.05$, Table 1). Furthermore, seminal vesicle weights were heavier ($t = 349.212$, $P < 0.05$), and flank glands were longer ($t = 6.877$, $P < 0.05$), in adults than in prepubertal animals (Table 1).

Prepubertal and adult sham-castrates did not differ in the amount of time spent in anogenital investigation of the female (Table 2). Adult males exhibited significantly more vaginal mounts than their prepubertal counterparts, who never displayed any mounting behavior ($t = 5.177$, $P < 0.05$, Table 2). There were no significant differences between adult and juvenile males in the number of intromissions performed (Table 2). However, no prepubertal males intromitted, whereas one-third of the adults displayed one or more intromissions ($\chi^2 = 39.5$, $P < 0.05$). No ejaculations were observed in either group of animals.

Testosterone-treated castrates. ANOVA revealed a significant main effect of testosterone treatment on cir-

TABLE 2
Levels of Reproductive Behavior and AR-ir Areal Densities for Sham Castrates in Experiment 1

Reproductive behavior				Areal density of AR-ir cells (profiles/13,000 μm^2)				
Age (days)	Anogenital investigation (sec)	Mounts	Intromissions	MPN	MeAMY	BNSTpm	MPNmag	LSept
28	88.8 ± 21.1	0	0	36.2 ± 6.0	40.8 ± 4.2	89.6 ± 7.9	44.9 ± 3.5	68.0 ± 6.1
49	155.8 ± 22.9	3.2 ± 1.5*	1.0 ± 0.68	39.1 ± 5.3	46.8 ± 6.1	81.9 ± 4.2	51.1 ± 2.4	59.8 ± 5.9

* Significantly different from 28-day-old males.

culating plasma testosterone levels ($F = 23.918$, $P < 0.05$; Table 1). Castrates that received the 2.5-mg pellet had higher plasma testosterone levels than animals implanted with the blank pellet, in which plasma testosterone was undetectable. Similarly, castrates that received a 5-mg pellet had higher plasma testosterone levels than groups that received either the blank or the 2.5-mg pellet. No interaction existed between age and treatment on plasma testosterone; that is, testosterone pellets of a given dose produced similar levels of circulating testosterone in castrated males regardless of age.

Testosterone treatment significantly affected the duration of anogenital investigation ($F = 7.998$, $P < 0.05$, Fig. 1A), but there was neither an effect of age nor an interaction between testosterone treatment and age. Fisher's PLSD revealed that animals treated with either the 2.5- or 5-mg testosterone pellet displayed significantly more anogenital investigation than animals treated with the blank pellet, regardless of age.

There was a significant interaction between age and testosterone treatment on the frequency of vaginal mounts ($F = 4.314$, $P < 0.05$, Fig. 1B). In adults, both the 2.5- and 5-mg doses of testosterone elicited more mounts compared with adults receiving a blank pellet. This effect of testosterone on vaginal mounts was not observed in the prepubertal animals. Adults engaged in more vaginal mounting at the 2.5-mg testosterone dose compared with their juvenile counterparts.

Age significantly affected the frequency of intromissions ($F = 6.218$, $P < 0.05$, Fig. 1C) such that overall, adults engaged in more intromissions than prepubertal animals. Although there was no interaction between testosterone treatment and age on the frequency of occurrence of intromissions, only one prepubertal animal receiving the 2.5-mg testosterone dose displayed one intromission, whereas half of the adults treated with this dose displayed multiple intromissions ($\chi^2 = 26.12$, $P < 0.05$).

A two-way ANOVA revealed a significant interaction between age and testosterone treatment on the number of ejaculations ($F = 3.295$, $P < 0.05$, Fig. 1D). The adults that were implanted with the 2.5-mg testosterone pellet engaged in a greater number of ejaculations than the adults that had received either the 0- or 5-mg testosterone pellet ($P < 0.05$). This effect of testosterone on ejaculation was not observed in the juvenile animals. Adults displayed more ejaculations at the 2.5-mg testosterone dose than juvenile males receiving that dose of testosterone.

Androgen Receptor Immunoreactivity

Sham-castrated, mated animals. There were no significant differences in the number of AR-ir profiles per unit area between juvenile and adult sham castrates in any of the brain regions examined (Table 2).

Testosterone-treated castrates. Two-way ANOVAs revealed significant main effects of testosterone dose on the number of AR-ir profiles/13,000 μm^2 in the MPN and LSept ($F = 31.599$, $F = 31.814$, respectively, both $P < 0.05$), indicating similar effects of testosterone in both age groups. In both brain regions, the areal density of AR-ir profiles was higher in animals treated with either the 2.5- or 5-mg testosterone pellet than in the groups treated with the blank pellet, and areal density of AR-ir profiles did not differ between animals treated with either 2.5 or 5 mg of testosterone (Fig. 2).

In the MeAMY, there were significant main effects of both age ($F = 4.652$, $P < 0.05$) and testosterone ($F = 36.328$, $P < 0.05$), but no interaction (Fig. 2). Thus, collapsed across doses of testosterone, the number of AR-ir profiles/13,000 μm^2 was greater in juveniles than in adults. Similarly, across both age groups, the areal density of AR-ir profiles was significantly increased with each increasing dose of tesoterone.

Two-way ANOVAs revealed significant interactions between testosterone treatment and age on the number of AR-ir profiles/13,000 μm^2 in both the BNSTpm and

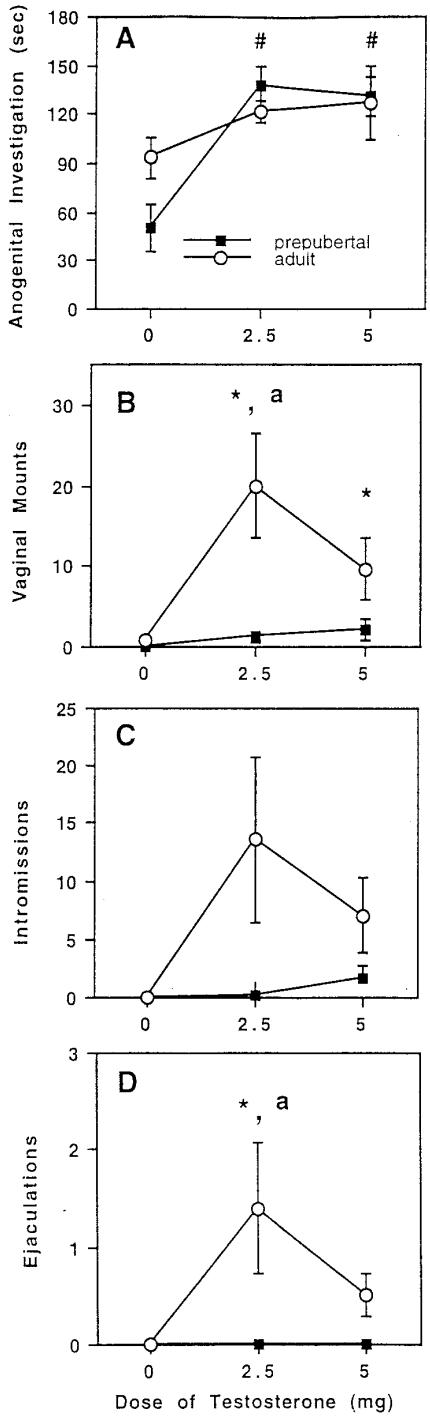

FIG. 1. Number of seconds engaged in anogenital investigation (A), number of vaginal mounts (B), number of intromissions (C), and number of ejaculations (D) in prepubertal and adult male hamsters given a 10-min test with a sexually receptive female. Males were castrated 1 week prior to testing and were implanted with a pellet containing 0, 2.5, or 5 mg of testosterone. "#" indicates significantly different from 0 mg testosterone for both age groups. Asterisk indicates significantly different from 0 mg testosterone for adults only. "a" indicates a significant difference between age groups for a given dose of testosterone. All values are means ± SEM.

MPNmag ($F = 4.30$ and $F = 4.097$, respectively, $P < 0.05$). For both age groups, testosterone treatment increased the areal density of AR-ir profiles in both the BNSTpm and MPNmag (Fig. 2). However, the increase in the number of AR-ir profiles/13,000 μm^2 was of a greater magnitude in juvenile males than in adults at both the 2.5- and 5-mg doses of testosterone. Photomicrographs illustrating the effects of testosterone on the areal density of AR-ir profiles in the BNSTpm at the two ages are shown in Fig. 3.

Experiment 2: Pubertal Development of Brain Androgen Receptor Immunoreactivity

One-way ANOVAs and subsequent posthoc tests revealed that 49-day-old intact males had higher plasma testosterone levels ($F = 29.771$, $P < 0.05$), seminal vesicle weights ($F = 123.22$, $P < 0.05$), and flank gland lengths ($F = 75.676$, $P < 0.05$) than either the 28- or 21-day-old intact males (Table 1). In addition, 28-day-old males had significantly longer flank glands than 21-day-old males ($P < 0.05$).

Analysis of AR-ir profiles in BNSTpm and MPNmag could not be performed in 21-day-old males due to poor tissue quality of sections containing these regions. In all brain regions examined, there was a trend toward an increased number of AR-ir profiles/13,000 μm^2 with increasing age (Fig. 4). However, in just two brain regions was there a statistically significant effect of age. Within MPN, areal density of AR-ir profiles was greater in 49-day-old compared with either the 28- or 21-day-old animals ($F = 11.627$, $P < 0.05$). In MPNmag, the number of AR-ir profiles/13,000 μm^2 was greater in 49-day-old males than in 28-day-old males ($t = 5.224$, $P < 0.05$). Figure 5 shows representative examples of AR immunopositive nuclei in the MPN of juvenile (28-day-old) and adult (49-day-old) males.

DISCUSSION

Relationship between Behavioral Responsiveness and AR-ir

The behavioral dose response curves to testosterone for prepubertal and postpubertal hamsters in Experi-

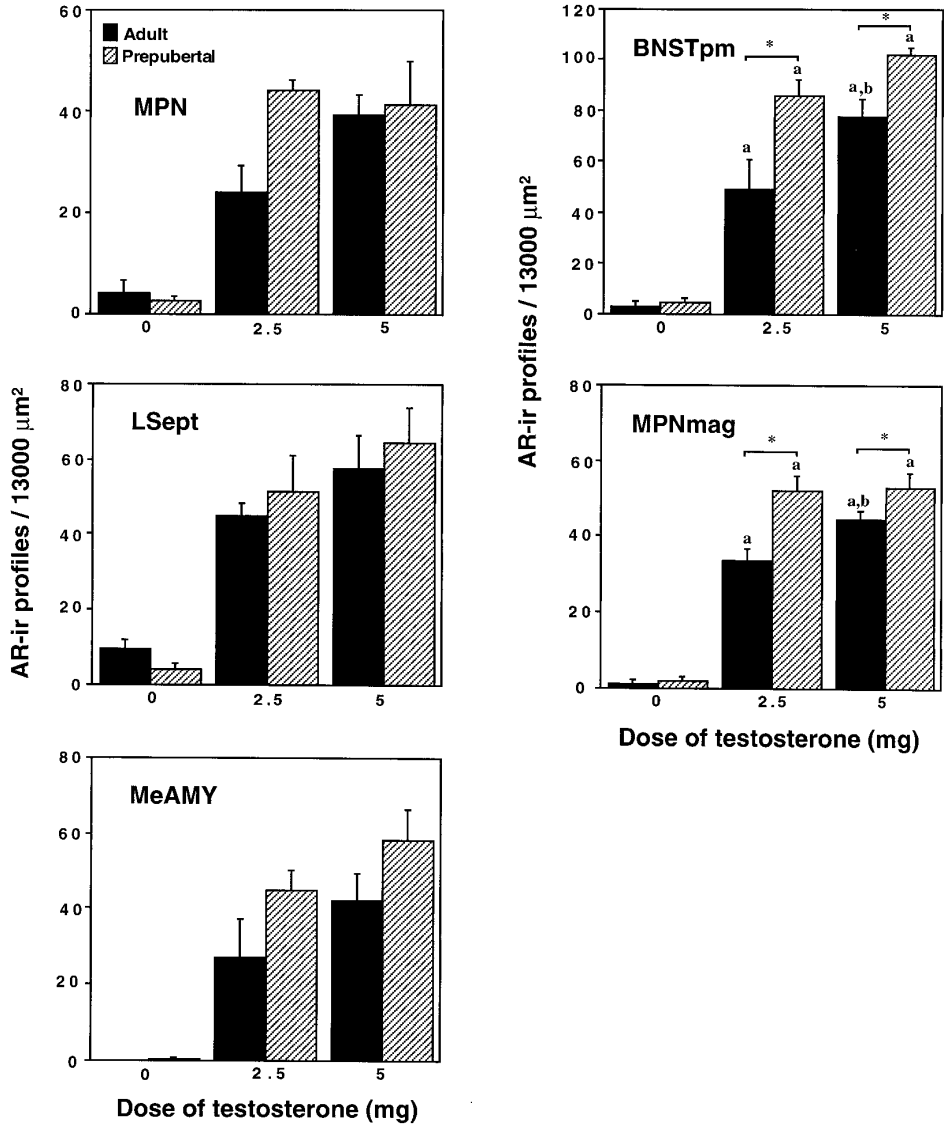

FIG. 2. AR-ir profiles/13000 μm^2 in the MPN, LSept, MeAMY, BNSTpm, and MPNmag of prepubertal (28 days old) and adult (49 days old) male hamsters that were castrated 1 week prior to sacrifice and received a pellet containing 0, 2.5, or 5 mg of testosterone. ''a'' indicates a significant difference from 0 mg testosterone within an age group. ''b'' indicates a significant difference from 2.5 mg testosterone within an age group. Asterisk indicates a significant difference between ages within a dose of testosterone. All values are means ± SEM.

ment 1 provide clear evidence that prepubertal males are less sensitive and less responsive to the effects of testosterone on male reproductive behavior than are postpubertal males. Doses of exogenous testosterone that were effective in activating male reproductive behavior in adults elicited little or no behavior in juveniles, even though plasma concentrations of testosterone were equivalent in the two age groups. In intact males not exposed to a female, AR-ir increased with age in brain regions that are part of the neural circuitry mediating male sexual behavior (MPN and MPNmag; Experiment 2). In addition, testosterone can increase AR-ir in these same brain regions (Experiment 1), suggesting that the pubertal rise in testosterone upregulates AR within the behavioral circuitry and in this way may consequently contribute to the pubertal increase in responsiveness to behavioral actions of testosterone. However, Experiment 1 shows that an increase in AR-ir within the behavioral circuit is not sufficient, even though it may be necessary, to induce an increase in

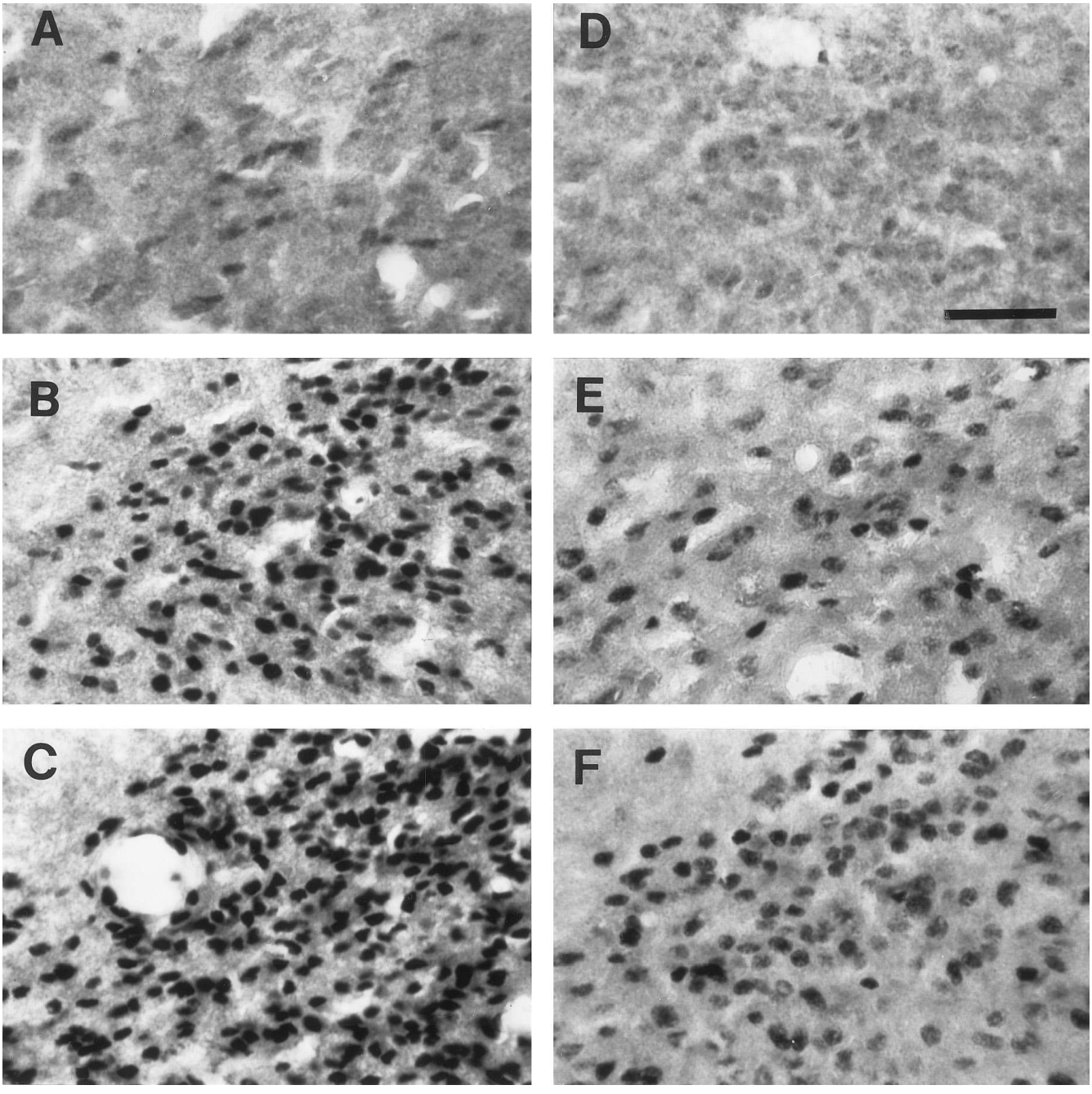

FIG. 3. Photomicrographs of AR-ir cells in the BNSTpm of testosterone-treated prepubertal and postpubertal male hamsters. (A–C) Prepubertal males that received testosterone pellets of 0, 2.5, and 5 mg, respectively. (D–F) Adults that received testosterone pellets of 0, 2.5, and 5 mg, respectively. Note that the area of each of these photographs is larger than the area used for quantifying areal density (36,000 vs 13,000 μm^2). Bar, 50 μm.

behavioral responsiveness. In all brain regions examined, the AR-ir response to testosterone in prepubertal males was equivalent to or greater than that of postpubertal males. Thus, by assessing both behavioral and AR-ir responses to testosterone in the same individual, the present experiments extend earlier investigations of behavioral maturation and pubertal regulation of AR-ir in the European ferret (Kashon and Sisk, 1995, 1994;

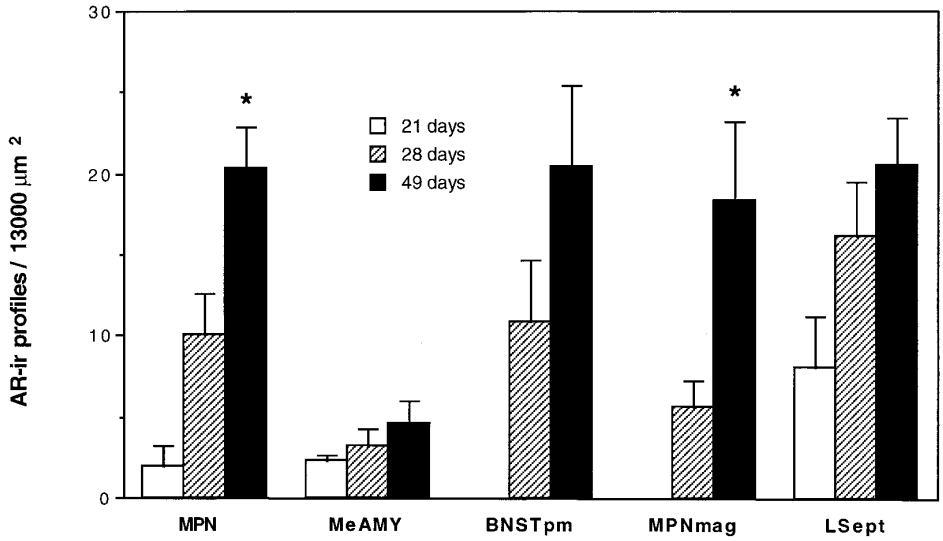

FIG. 4. AR-ir profiles/13000 μm^2 in the MPN, MeAmy, BNSTpm, MPNmag, and LSept of 21-day-old (prepubertal), 28-day-old (prepubertal), and 49-day-old (adult) male hamsters. Asterisk indicates a significant difference from other age groups for a given brain region. Tissue sections through BNSTpm and MPNmag were not available for 21-day-old males. All values are means ± SEM.

Sisk *et al.*, 1992) and clearly do not support the hypothesis that lack of behavioral responsiveness to testosterone is due to a relative inability of testosterone to upregulate AR in the behavioral neural circuitry of prepubertal males.

It appears, then, that other pubertal processes, perhaps in concert with a testosterone-induced increase in AR, cause steroid hormones to be more effective in activating male reproductive behavior. Androgens act together with aromatized estrogenic metabolites to activate male reproductive behavior (reviewed in Meisel and Sachs, 1994). Androgens also regulate activity of the aromatase enzyme in the preoptic area via activation of the androgen receptor (Roselli and Resko, 1984). Thus, it is possible that testosterone is more effective in increasing aromatase activity in adults due to pubertal changes in mechanisms that couple AR with the aromatase enzyme. Alternatively, a pubertal increase in estrogen receptor may be an important component of the neural mechanism underlying enhanced behavioral responsiveness in adult males. In any case, the pattern and/or duration of testosterone treatment given to prepubertal males in the present study apparently did not provide the appropriate stimulus for increased aromatase activity, upregulation of estrogen receptor, or other processes that may be necessary to increase behavioral responsiveness to steroid hormones during puberty.

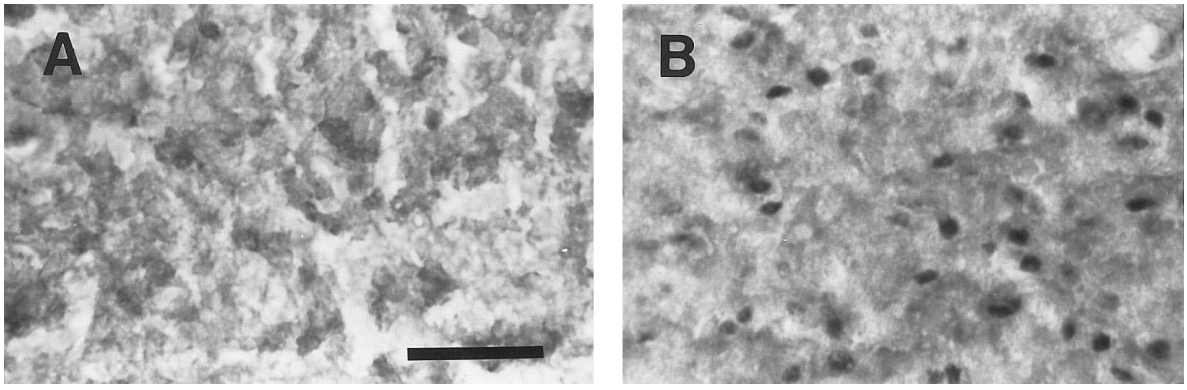

FIG. 5. Photomicrographs of AR-ir cells in the MPN of an intact 28-day-old prepubertal (A) and a 49-day-old adult (B) male hamster. Bar, 50 μm.

Testosterone-independent pubertal processes may also contribute to increased behavioral responsiveness to steroid hormones. Chemosensory cues from the female are an important factor in the activation of male reproductive behavior in hamsters. The salience of these cues or the way in which the information they contain is integrated with steroid-dependent cellular processes may change during pubertal maturation. Thus, age-dependent changes in the processing of chemosensory or somatosensory information may also be an important component of the maturation of male reproductive behavior.

One obvious difference between juveniles and adults in the present study was the length of time that their nervous systems had been exposed to endogenous testosterone prior to treatment. However, in male rats, 1 week of treatment with subcutaneous capsules that produced circulating levels of 2–5 ng/ml was sufficient to activate sexual behavior in adults that had been castrated 3–4 weeks earlier (McGinnis, Mirth, Zebrowski, and Dreifuss, 1989). Thus, 1 week of testosterone treatment should have resulted in expression of male reproductive behavior in juveniles if their nervous systems were capable of adult-like responses. It also does not appear that differential treatment of juvenile and adult males by the stimulus females can explain the age difference in responsiveness to testosterone. Regardless of the age of the male, the latency for the female to assume the lordosis posture was less than 30 sec, and the average amount of time the female spent in lordosis during the 10-min test was not different for juvenile and adult males. Adult female hamsters are generally larger than adult males, and of course the size difference is exaggerated when the female is paired with a juvenile male. However, it seems unlikely that a body size difference was a major factor in the relative lack of occurrence of mounts and intromissions in juvenile males because some juveniles were able to perform these behaviors.

The fact that the AR-ir responses to testosterone in BNSTpm, MPNmag, and MeAMY of prepubertal males were of greater magnitude than those of postpubertal males is surprising and intriguing. In all three of these brain regions, the immediate early gene product fos is expressed after a male hamster is exposed to female hamster vaginal pheromones (Fernandez-Fewell and Meredith, 1994; Fiber, Adames, and Swann, 1993). In addition, interest in female odors is a behavior that appears to be regulated by testosterone directly (not via estrogen; Powers and Bergondy, 1983) and that can be activated in both prepubertal males and adult females by testosterone (unlike full copulatory behavior; Johnston and Coplin, 1979; Powers and Bergondy, 1983). The robust AR-ir response to testosterone within BNSTpm, MPNmag, and MeAMY in prepubertal males may therefore be related to the fact that anogenital investigation was the only component of male sexual behavior examined in this study that was activated equally by testosterone in prepubertal and postpubertal males.

Alternatively, the underlying assumption that increases in steroid hormone receptor lead to increases in behavioral responses may need to be reevaluated. For example, if AR-mediated cellular responses were in some way inhibitory to mounting, then enhanced AR responsiveness to testosterone in BNSTpm, MPNmag, or MeAMY could underlie lack of activation of mounting by testosterone in prepubertal males. Finally, it is worth noting that prepubertal hamsters are more responsive to steroid inhibition of gonadotropin secretion compared with adult males (Sisk and Turek, 1983). If target cells for steroid-negative feedback regulation of gonadotropin secretion are intermingled with target cells for steroid activation of behavior, it is possible that the enhanced AR-ir response to testosterone in one or more of these three regions is related to enhanced responsiveness to steroid negative feedback in juvenile males.

The dissociation between testosterone's effects on sexual behavior and on AR-ir is also apparent in adult hamsters. In adults, the 5-mg dose of testosterone was required to restore AR-ir to levels observed in age-matched sham castrates, yet full restoration of sexual behavior was accomplished by the 2.5-mg dose in these same animals. In addition, the AR-ir response to testosterone was generally graded in adults, i.e., AR-ir increased with increasing dose of testosterone, while a maximal behavioral response was observed at the lowest dose administered. In these respects, the relationship between testosterone and AR-ir in adult hamsters appears to be more like that between testosterone and aggressive behavior rather than between testosterone and sexual behavior. Albert, Jonik, Watson, Gorzalka, and Walsh (1990) compared dose response curves to testosterone for activation of sexual and aggressive behaviors in adult male rats and found that extremely low levels of testosterone were sufficient to fully activate sexual behavior, while aggressive behaviors were proportional to the dose of testosterone.

Methodological Considerations

These experiments demonstrate that modification of immunocytochemical protocol can be used to reveal age-related differences in AR-ir within cells. In Experi-

ment 1, no effect of age was found in the areal density of AR-ir profiles in any brain region examined. This result was initially perplexing, because earlier pilot work had indicated that brain AR-ir in adult males was greater than that in prepubertal males, a finding that in fact had provided the impetus to conduct Experiment 1. In the interim between the pilot experiment and Experiment 1, however, immunocytochemical conditions had been changed to optimize AR immunostaining by increasing the concentrations of primary antibody, $NiCl_2$, and H_2O_2. Thus, this optimization could obscure age differences in AR-ir cell numbers by increasing the sensitivity for detection of AR immunopositive nuclei. Experimental manipulation of primary antibody concentration has been used to reveal changes in fos expression in neurons after steroid treatment (Auger and Blaustein, 1995). Therefore, in Experiment 2, lower concentrations of primary antibody, as well as $NiCl_2$ and H_2O_2, were used. Under these conditions, a pubertal increase in areal density of AR-ir profiles was observed in certain brain areas. We conclude that the immunocytochemical conditions used in Experiment 1 were sensitive enough to stain cells that express even very low levels of AR so that the number of AR-ir profiles per unit area was not different in pre- and postpubertal males. In contrast, the conditions used in Experiment 2 were such that cells expressing low levels of AR would not be detectable, and thus only cells with a relatively high abundance of AR were stained. Thus, it appears that the pubertal increase in AR-ir in Experiment 2 does not reflect a pubertal change in cell phenotype, but instead reflects an increase in AR expression within cells that, prior to puberty, expressed relatively little AR.

Another difference between the sham castrates in Experiment 1 and the intact males in Experiment 2 was the occurrence of a mating test 1 hr prior to sacrifice (Experiment 1) or not (Experiment 2). It is possible that some aspect of the mating experience, such as exposure to the female or to her scent, may rapidly increase testosterone and consequently AR-ir. However, the difference in plasma testosterone levels between gonad-intact prepubertal males in Experiments 1 and 2 was not large, and it is more likely that the different immunocytochemical conditions are responsible for the differences in the number of AR-ir profiles observed in the two experiments. Finally, the more sensitive immunocytochemical protocol used in Experiment 1 did not preclude an ability to detect group differences in the number of AR-ir profiles, as there were clear effects of castration, testosterone, and age by testosterone interactions on the areal density of AR-ir profiles in Experiment 1.

The mechanism by which testosterone increases AR-ir is not known, but it is probably a combination of at least two separate processes. First is a relatively rapid ligand-induced translocation of AR to the nucleus, followed by a second and more protracted response that results in a greater abundance of intracellular AR, through either an increase in AR protein synthesis or an increase in the half-life of AR. Several laboratories have documented that testosterone induces an increase in AR-ir within 15–30 min (Prins and Birch, 1993; Sar, Lubahn, French, and Wilson, 1990; Zhou, Blaustein, and De Vries, 1994), which is too rapid to reflect an increase in *de novo* synthesis of AR. However, longer term treatment with testosterone increases AR-ir even further (Kashon, Arbogast, and Sisk, 1996), and androgens administered to gonad-intact male rats increase AR-ir (Menard and Harlan, 1993). This latter finding cannot be explained on the basis of ligand-induced translocation of AR to the nucleus, because endogenous androgens are present and receptor is presumably already within the nucleus. The ability of testosterone to increase AR protein half-life (Kemppainen, Lane, Sar, and Wilson, 1992; Syms, Norris, Panko, and Smith, 1985) or AR mRNA or protein (Gonzalez-Cadavid, Vernet, Navarro, Rodriguez, Swerdloff, and Rajfer, 1993; Kerr, Allore, Beck, and Handa, 1995; Shan, Hardy, Catterall, and Hardy, 1995) has been documented under certain conditions in androgen-sensitive peripheral tissues and brain. The immunocytochemical data indicating that testosterone can upregulate the AR protein in brain are corroborated by androgen binding experiments, in which nuclear AR concentration in brain homogenates is increased by treatment with androgen at time points later than that required for saturation of the androgen receptor (Bittman and Krey, 1988; Krey and McGinnis, 1990; Prins, Bartke, and Steger, 1990). Thus, we believe the effects of testosterone on brain AR-ir in the present study reflect true increases in intracellular levels of AR.

In all groups of males in Experiment 1, the frequency of occurrence of sexual behaviors was relatively low, and within-group variability on the behavioral measures was relatively high. The most likely explanation for these characteristics of the data is that all animals were sexually naive prior to the behavioral tests. Because the effect of sexual experience on responsiveness to testosterone is unclear, and experience could not be easily equated in juvenile and adult males, we elected to use sexually inexperienced animals for this study. Similarly, it is virtually impossible to equate general social experience in juvenile and adult males because adults by definition will have lived longer under particular housing conditions. In this experiment, all males

were housed alone for 1 week prior to behavioral testing, but prior to that, prepubertal males were housed with their mothers and littermates, whereas adults were housed from weaning with same-sex siblings. Although this difference in social experience could conceivably contribute to a difference in responsiveness to testosterone between the two age groups, other data from this laboratory indicate that postweaning experience with conspecifics per se does not result in an increase in responsiveness to testosterone. Juvenile male ferrets that had been housed with another male for 7 weeks after weaning were still relatively unresponsive to the effects of testosterone on activation of male reproductive behavior, and the behavioral dose response curves for prepubertal and postpubertal ferrets with similar postweaning housing conditions were remarkably comparable to those of the present study for hamsters (Sisk *et al.*, 1992). Thus we believe that some aspect of pubertal maturation, and not the amount of time housed with male conspecifics, is responsible for the differential responsiveness to testosterone in prepubertal and adult hamsters.

Species Comparisons

The present data are in general agreement with previous studies in the rat (Freeman, Padgett, Prins, and Breedlove, 1995; Menard and Harlan, 1993), hamster (Wood and Newman, 1993a), ferret (Kashon *et al.*, 1995; 1996), and opossum (Iqbal, Swanson, Prins, and Jacobson, 1995), all of which used the PG-21 antibody to examine regulation of brain AR-ir by testosterone. In all of these reports, castration decreased and testosterone treatment restored brain AR-ir. It should be noted that when other antibodies to AR have been used to examine brain AR-ir, castration did not reduce AR immunostaining (Choate and Resko, 1992; Clancy, Whitman, Michael, and Albers, 1994), suggesting that AR conformation changes with the presence and absence of testosterone. In studies in which quantitative analysis of AR-ir cells was performed, the magnitude of regulation of AR-ir by androgen was brain region-dependent (Kashon *et al.*, 1995; 1996; Menard and Harlan, 1993). Although we did not observe dramatic regional differences in the extent to which AR-ir is regulated by testosterone in the present study, comparisons of results across studies are difficult because of differences in hormone treatment paradigms and species. We did, however, observe brain region differences in the extent to which pubertal changes in AR-ir occur, and presumably, the pubertal increase in testosterone was one factor that contributed to the age-related increase in AR-ir in the preoptic area. The factors that contribute to brain region-specific effects of androgen on AR-ir under physiological conditions are unknown, but undoubtedly include parameters of hormone secretion and cell group differences in afferent input and neurochemical phenotype.

Adult golden hamsters are seasonal breeders and reproductive competence is influenced by photoperiod. Testicular regression occurs in males exposed to short daylengths, and along with the ensuing decline in testosterone production, there is a decrease in responsiveness to behavioral effects of testosterone (Campbell, Finkelstein, and Turek, 1978; Morin and Zucker, 1978; Powers, Steel, Hutchison, Hastings, Herbert, and Walkers, 1989). The decreased behavioral responsiveness in short days is correlated with a decrease in androgen binding sites in the POA of male hamsters kept in short days (Bittman and Krey, 1988) and with decreased brain AR-ir (Wood and Newman, 1993a). If pubertal and seasonal changes in responsiveness to behavioral effects of testosterone are mediated by similar neural mechanisms, the present experiment would predict that seasonal changes in behavioral responsiveness cannot be explained solely on the basis of seasonal changes in the ability of testosterone to upregulate brain AR-ir.

Summary

This set of experiments has documented a pubertal increase both in behavioral responsiveness to testosterone and in AR-ir in brain regions important for male reproductive behavior in male golden hamsters. Testosterone-induced increases in AR would presumably result in enhanced responsiveness to testosterone. However, the lack of response of prepubertal males to the behavioral actions of testosterone cannot be explained on the basis of an inability of testosterone to upregulate brain AR-ir. This finding requires us to reformulate our assumptions about the relationship between abundance of AR and behavioral responses to testosterone, or to conclude that a testosterone-induced increase in AR is not sufficient (although it may be necessary) to increase behavioral responsiveness to testosterone in prepubertal male hamsters, or both.

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