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Changes in Androgen Receptor, Estrogen Receptor alpha, and Sexual Behavior with Aging and Testosterone in Male Rats¹

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Abstract

Reproductive aging in males is characterized by a diminution in sexual behavior beginning in middle age. We investigated the relationships among testosterone, androgen receptor (AR) and estrogen receptor alpha (ER α) cell numbers in the hypothalamus, and their relationship to sexual performance in male rats. Young (3 months) and middle-aged (12 months) rats were given sexual behavior tests, then castrated and implanted with vehicle or testosterone capsules. Rats were tested again for sexual behavior. Numbers of AR and ER α immunoreactive cells were counted in the anteroventral periventricular nucleus and the medial preoptic nucleus, and serum hormones were measured. Middle-aged intact rats had significant impairments of all sexual behavior measures compared to young males. After castration and testosterone implantation, sexual behaviors in middle-aged males were largely comparable to those in the young males. In the hypothalamus, AR cell density was significantly (5-fold) higher, and ER α cell density significantly (6-fold) lower, in testosterone- than vehicle-treated males, with no age differences. Thus, restoration of serum testosterone to comparable levels in young and middle-aged rats resulted in similar preoptic AR and ER α cell density concomitant with a reinstatement of most behaviors. These data suggest that age-related differences in sexual behavior cannot be due to absolute levels of testosterone, and further, the middle-aged brain retains the capacity to respond to exogenous testosterone with changes in hypothalamic AR and ER α expression. Our finding that testosterone replacement in aging males has profound effects on hypothalamic receptors and behavior has potential medical implications for the treatment of age-related hypogonadism in men.

Keywords

androgen receptor; estrogen receptor alpha; aging; male rat; anteroventral periventricular nucleus (AVPV); medial preoptic nucleus (MPN)

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Introduction

In a variety of mammalian species including rats, monkeys and humans, there is an age-related decline in sexual performance that may be attributable to physiological or psychological changes (Chambers and Phoenix, 1983; Nicolosi et al., 2004; Smith et al., 1992). Much of the loss of male sexual function with aging has been correlated with a decline in serum testosterone. This change has been shown in humans (Harman et al., 2001), rhesus monkeys (Downs and Urbanski, 2006), and rats, including Sprague-Dawley (Roselli et al., 1986; Wu et al., 2009; Karpas et al., 1983), Wistar (Bernardi et al., 1998; Taylor et al., 1996), Brown Norway (Chen et al., 1994; Gruenewald et al., 2000), and Fischer 344 strains (Chambers et al., 1991; Luine et al., 2007).

However, not all studies show a clear relationship between testosterone, aging, and sexual behavior, a concept that is particularly important when considering the transitional life period of middle age. In fact, during this life stage, testosterone levels in rats may or may not differ from those in young animals depending up the absolute chronological age, strain, and/or other physiological, behavioral and experimental differences. Thus, whereas some studies have shown age-related declines in testosterone at middle age, others have not demonstrated such a loss (Gruenewald et al., 2000; Frankel and Mock, 1981; Wu and Gore, 2009). Furthermore, testosterone replacement to castrated aging rats does not effectively reinstate sexual performance as it does in young rats (Chambers and Phoenix, 1984; Chambers and Phoenix, 1986; Chambers et al., 1991; Sato et al., 1998), underscoring the point that absolute testosterone levels do not necessarily predict the capacity to perform sexual behavior, and that there are other changes to the aging brain that may explain this behavioral decline. Furthermore, much of this latter literature is primarily focused on old rats and information about effects of testosterone treatment to middle-aged rats is rather limited, raising the question of what hormonal and neurobiological changes may be occurring at this life stage.

We hypothesized that the loss of sexual behavior with age may not be due to levels of testosterone *per se* but rather to age-related changes in the actions of testosterone on its androgen receptor (AR) in brain regions involved in the control of masculine sexual behaviors and the hypothalamic-pituitary-gonadal axis (Vagell and McGinnis, 1998; Portillo et al., 2006). Circulating testosterone levels regulate expression of the AR, as in young males, castration can eliminate, and testosterone replacement can restore, AR expression in the brain (Iqbal et al., 1995; Lu et al., 1999; Lu et al., 1998; Wood and Newman, 1993). In comparing young and old male Fischer 344 rats (4 and 26 months, respectively), Chambers *et al.* showed that nuclear AR binding is very low in castrated rats, and elevated by testosterone treatment in both ages, albeit to a greater extent in the young group (Chambers et al., 1991). This suggests that the responsiveness of the aging brain to testosterone is diminished. We showed age-related increases in AR immunoreactive cells within several preoptic-hypothalamic regions between young and middle-aged rats (Wu and Gore, 2009; Wu et al., 2009). However, much more information is needed about the implications of this change at midlife.

It is also important to consider that some actions of testosterone on masculine behaviors and physiology are due to its aromatization to estradiol and subsequent actions on estrogen receptors (ER) (Clancy et al., 2000; Larsson et al., 1973; Morali et al., 1977; Vagell and McGinnis, 1997; Zumpe et al., 1993). Relatively little is known about the interactions among estradiol, aging, and behavior. Even the literature on serum estradiol is quite contradictory depending up rat strain and absolute age: levels of estradiol have been reported to undergo no change (Goya et al., 1990; Wu et al., 2009), decrease (an effect that was dependent upon prior sexual experience (Wu and Gore, 2009)), or increase (Fujita et al., 1990; Herath et al., 2001; Luine et al., 2007). The hypothalamic ER α may also change with age, although evidence to date shows little change in ER α gene expression or protein immunoreactivity with aging

(Bottner et al., 2007; Madeira et al., 2000; Wu and Gore, 2009; Wu et al., 2009). Still, this question merits investigation in the context of sexual behavior and aging.

In the current study, we investigated the effects of aging, testosterone treatment, and their interactions on sexual behavior and expression of AR and ER α in the hypothalamus, focusing on the transition between young and middle-aged rats. Together, these studies were intended to provide novel information about the potential roles of these hormone receptors, their regulation by testosterone, and their relationship to sexual behavior during reproductive aging.

Materials and Methods

Animals

Male rats—Male Sprague-Dawley rats were purchased at 2-3 months (young, N=18) and 10-11 months (middle-aged, N=38) from Harlan Sprague-Dawley Inc (Indianapolis, Indiana; Stock/Strain: Hsd:Sprague Dawley[®]™ SD[®]™). The middle-aged rats were retired breeders. Young rats were given sexual experience by placing them with a receptive female rat (rotated among males) twenty consecutive nights and checking for sperm in the females' vaginas the next morning. At the end of this period, all young males had mated on average 15 times, estimated to be similar to what middle-aged breeders had experienced in the breeding colony.

Female rats—Female Sprague-Dawley rats (same stock) were purchased at 2-3 months (N=37). They were ovariectomized and estradiol-implanted [one silastic capsule (1.98-mm I.D. \times 3.18-mm O.D. \times 5-mm length; Dow Corning Corporation, Midland, MI) packed with 5% crystalline 17 β -estradiol (Sigma-Aldrich, St. Louis, MO)]. These female rats received 500 μ g progesterone (Sigma-Aldrich, St. Louis, MO) in sesame oil (0.1 ml) injection three hours before the mating trials. Only females who showed lordosis with male rats were used in mating trials.

Rats were housed in an AAALAC-approved facility (two same-sex animals per cage, cage dimensions 47 \times 20 \times 25 cm) with Rat Sterilizable Diet (Harlan Teklad LM-485 7012, Madison, WI) and water available *ad libitum*. The light cycle was 12 h light, 12 h dark cycle (lights on 2300h), and temperature was 21 \pm 1 $^{\circ}$ C. All animal procedures were approved by the UT-Austin Institutional Animal Care and Use Committee (Protocol number: 08030101) and studies were performed following the *Guide for the Care and Use of Experimental Laboratory Animals*.

Pre-tests for Mating Behaviors

All the male rats were given two pre-tests (one day off between tests) to measure the baselines for each parameter of the mating behaviors, with different receptive females rotated among males. The experimental design is shown in Figure 1.

Mating tests were performed under dim red light, beginning 3 hours after lights out (1400h-1700h). Every female was habituated to the chamber for 10 minutes. Every male was habituated to the chamber for 20 minutes before the female was introduced. 20-minute mating trials took place in a transparent Plexiglas cage [76 cm (L) \times 32 cm (W) \times 46 cm (H)]. The following parameters of male sex behavior were recorded in each test (Agmo, 1997): latency to mount; frequencies of mount, intromission and ejaculation; and percentage of rats that intromit or ejaculate in the 20 minute test. The test was terminated at 20 minutes or after a second ejaculation, whichever came first. The choice of this 20-minute cut-off was based on the literature using trials of 15-30 minutes for similar measures (Damassa et al., 1977; Frankel, 1981; Chambers et al., 1991; Vagell & McGinnis 1998; Wu & Gore, 2009). Mating trials were recorded using a Sony Handycam Hi-Fi Stereo Video 8 XR camcorder (Sony Corporation of America, New York, NY). The videotaped trials were further analyzed by using JWatcher v1.0

computer software (www.jwatcher.ucla.edu, Dan Blumstein's Lab, UCLA & The Animal Behaviour Lab, Macquarie University, Sydney) to quantify the behavior of each experimental animal (Crews et al., 2007).

All the young males finished ejaculation in both of two pre-tests ($n = 18$). For middle-aged male rats, only those who ejaculated successfully in at least one of the two pre-tests were chosen to participate in further experiments ($n = 27$). Only these latter rats are described in the remaining sections, in order to focus comparisons on rats with residual capacity to mate and based on the paradigm of (Frankel 1981).

Castration and Hormone Implantation

The next day after the pretests, young and screened middle-aged males were randomly assigned to one of two experimental groups: gonadectomized with testosterone implants and gonadectomized with vehicle implants. Capsules were 1.5 c.m. Silastic tubing (1.98-mm I.D. \times 3.18-mm O.D. \times 5-mm length; Dow Corning Corporation, Midland, MI) filled with crystalline testosterone [4-Androsten-17 β -ol-3-one (Sigma-Aldrich, St. Louis, MO)] or cholesterol (Sigma-Aldrich, St. Louis, MO). Capsule size was based on Vagell & McGinnis (1997) and Damassa et al. (1977), and confirmed by a pilot study in our laboratory showing that such capsules produced circulating total testosterone concentrations of ~ 1 ng/ml in middle-aged male rats. The ends of implants were sealed with 100% silicone Household Adhesive (DAP Inc. Baltimore, MD). For the castration and implantation surgery, animals were anesthetized with isoflurane via a gas anesthesia machine. Bilateral orchidectomy was performed, after which a blood sample (~ 0.5 ml) was collected from the rat's tail lateral vein to get a pre-castration hormone level. Then each rat was implanted subcutaneously either with two testosterone capsules or two vehicle capsules. Rats were treated post-operatively with 5 mg/kg Rimadyl. They were allowed to recover for two weeks.

Post-tests for Mating Behaviors

Two weeks after castration and capsule implantation, rats were given two mating tests (post-tests) to measure each parameter of the mating behaviors after treatment, each time with different receptive females (Figure 1). Behaviors were scored as described for the pre-tests. There was one day off between the two tests.

Perfusion

One day after post-tests at 0800-1100 h (0-3 hours before lights out at 1100h) rats were deeply anesthetized with ketamine (40 mg) and xylazine (8 mg). A 24 hour post-mating time point was chosen based on published work in this arena (Fernández-Guasti et al., 2003) and to enable us to make direct comparisons with our previous paper (Wu & Gore, 2009). The thoracic cavity was opened and a blood sample (8-9 ml) was drawn from the ventricle for subsequent hormone assays. Animals were perfused (48 ml/min) sequentially with 0.9% saline (24 ml), 0.9% saline with 10% heparin (24 ml), and 1% paraformaldehyde with 3.75% acrolein (48 ml), followed by 4% paraformaldehyde (480 ml) (Wu et al., 2009). PBSA (phosphate-buffered saline A: 0.122 M PO₄, 0.077 M NaCl, pH=7.3) was used to dissolve all fixatives. We removed the brains from the skull and post-fixed them for 3 hours in 4% paraformaldehyde and then transferred the brains into PBSA with 0.05% sodium azide for storage at 4°C. Then brains were cut on a vibrating microtome (Leica VT 1000S, Leica Microsystems, Nussloch, Germany) to get tissue sections (40 μ m-thick) for further experiments. Sections were stored in PBSA at 4°C until use.

Immunohistochemistry

Antibodies—For AR immunohistochemistry, sections were incubated in the rabbit polyclonal AR antibody N-20 (1:1500; Santa Cruz Biotechnology, Inc.) raised against a peptide mapping at the N-terminus (amino acids 299–315) of AR of human origin [Genetic locus: AR (human) mapping to Xq11.2-q12; AR (mouse) mapping to 9 A5.3]. Previous work has shown high specificity of this antibody for the AR based on preabsorption of this antibody with the antigen, western blots, and other controls (Chlenski et al., 2001; McKinnell et al., 2001). We also recently published a paper with this antibody and in that report (Wu and Gore, 2009) along with the current one, specific nuclear labeling was detected only in those brain regions that express the AR (Simerly et al., 1990).

The ER α antibody (Upstate Biotechnology, Waltham, MA, Ab C1355, used at 1:20,000 dilution) was a purified rabbit polyclonal antibody generated against the final 14 C-terminal amino acids (TYYPPEAEGFPNTI) of the rat ER α . The validation of this antibody has been described in detail previously by others' laboratories and ours (Friend et al., 1997; Moffatt et al., 1998; Wu et al., 2009). Similar to the AR antibody, in this study and our previous ones (Chakraborty et al., 2003; Wu and Gore, 2009; Wu et al., 2009), labeling was exclusively nuclear and restricted to regions of the brain reported to express ER α (Simerly et al., 1990).

Immunohistochemistry procedure—All the sections were processed in two IHC runs per antibody. For each run, animals from each age and treatment group were equally represented. In order to make the experimenter blind to age and treatment group, sections IDs were then recoded. Sections containing the preoptic area were selected in a 1:2 series (AVPV region) or a 1:4 series (MPN region; see below for details) and rinsed in PBSB (phosphate-buffered saline B: 0.12 M PO₄: 0.154 M NaCl, pH=7.3). DAB immunohistochemistry was performed as described previously in detail using either the AR or ER α antibody (Wu and Gore, 2009; Wu et al., 2009). Sections were mounted on slides, dehydrated and coverslipped.

Rationale for brain regions of analysis—The current study focused on AR and ER α immunoreactivity in the anteroventral periventricular nucleus (AVPV) and the medial preoptic nucleus (MPN). These regions were studied based on our initial pilot studies suggesting that these regions had among the highest density of both of these nuclear steroid hormone receptors throughout the entire hypothalamus-preoptic area (Wu & Gore, unpublished). We also wished to follow up on our two published reports studying relationships among age, hormone levels, and AR- and ER α -immunoreactivity in these same regions (Wu and Gore, 2009; Wu et al., 2009). Of interest, one of these studies showed a significant negative correlation between serum testosterone levels and AR-immunoreactivity in both the AVPV and the MPN of aging intact males (Wu et al., 2009). These two regions are also sexually dimorphic (Bloch and Gorski, 1988; Simerly, 1998): the MPN is a hub for the regulation of masculine sexual behavior (Hull and Dominguez, 2006) and the AVPV is believed to be involved in GnRH/gonadotropin release, although those results are based on female rats (Wiegand and Terasawa, 1982; Wintermantel et al., 2006). Finally, it was also reported that sexual behavior alters the volume of the AVPV in male rats (Prince et al., 1998). Therefore, although there are many other potential regions of interest, we chose to focus on AVPV and MPN.

Stereological Analysis

A stereological analysis was performed according to methods described in detail previously (Chakraborty et al., 2005; Wu et al., 2009). For the AVPV, a 1:2 series (5-6 sections) and for MPN a 1:4 series (8-9 sections) was used. A wet-mount of fresh tissue showed that average tissue thickness was 40.89 μ m. The sections were carefully matched for rostral-caudal landmarks among all the animals, and the regions were identified in Nissl-stained sections by comparing anatomical landmarks to an atlas of the rat brain (Swanson, 1998). Quantitative

analyses were done as previously described (Wu et al., 2009). The number and density (# immunoreactive cells/volume of each nucleus) of AR-immunoreactive (AR-ir) or ER α -immunoreactive (ER α -ir) nuclei falling within the regions was quantified. The coefficients of error (Cruz-Orive/Geiser) and variation of the estimates were calculated as described by Schmitz and Hof (Schmitz and Hof, 2000) and were between 0.03 and 0.10 per group. Photomicrographs were taken to produce the figures, and images were subjected to only minor adjustments of contrast using Adobe Photoshop 7.0 (Adobe, San Jose, CA). In order to avoid any bias, any adjustments were applied equally to tissues from rats of different ages.

Serum Hormone Concentrations

Blood samples collected at the time of castration surgery or perfusion were centrifuged (8000 rpm, 5 min) and the serum collected and stored at -80°C for hormone assays.

Enzyme immunoassay (EIA) of serum testosterone—Testosterone concentrations were measured by a single testosterone enzyme immunoassay using the EIA kit DSL-10-4000 according to the method described by Diagnostic Systems Laboratories, Inc. (Webster, TX). Duplicate samples were run at a volume of 50 μl each. The minimum detectable level of testosterone was 0.04 ng/ml per tube. Intra-assay variability was 2.60%. The cross-reactivity percentage of the testosterone antiserum with 5 α -dihydrotestosterone is 6.6%. However, the relatively low concentration of 5 α -dihydrotestosterone in male rats predicts that it will have a minimal effect on assay results.

Enzyme immunoassay (EIA) of serum free testosterone—Free testosterone concentrations were measured by two testosterone enzyme immunoassays using the EIA kit DSL-10-4900 according to the method described by Diagnostic Systems Laboratories, Inc. Duplicate samples were run at a volume of 50 μl each. The minimum detectable level of testosterone was 0.19 pg/ml per tube. Intra-assay variability was 1.95% and 1.65%. Inter-assay variability was 8.82%. The cross-reactivity of free testosterone antiserum with 5 α -dihydrotestosterone is negligible.

Radioimmunoassay (RIA) of serum estradiol—Estradiol concentrations were measured by a single ultra-sensitive estradiol RIA using the DSL-4800 RIA kit according to the method described by Diagnostic Systems Laboratories, Inc. Duplicate samples were run at a volume of 200 μl each. The minimum detectable level of estradiol was 2.2 pg/ml per tube. Intra-assay variability was 1.86%. The estradiol antiserum has a high affinity for estradiol and low cross-reactivity to other naturally-occurring estrogens.

Statistical Analysis

Statistical analysis was done with each rat as the unit of analysis. Using SPSS statistical software (version 15.0; SPSS Inc., Chicago, Illinois), effects of age and treatments were evaluated on the following endpoints: regional AR-ir and ER α -ir density (calculated as cell numbers/volume), serum hormone levels, and behavioral parameters. First, datasets were tested for homogeneity of variance and normality. For datasets that met these criteria, comparisons were made ANOVA (see below for details) followed by post hoc analysis when indicated by a significant interaction. Otherwise data were square root transformed to normalize variation across a broad range of values and then analyzed. Repeated measures ANOVA was used for behavioral parameters, and two-way ANOVA was used for AR- and ER α -ir density. For calculation of percentage of males showing intromissions and ejaculations, Fisher's Exact Test was used (McGinnis et al., 1996; Vagell & McGinnis, 1998). The criterion for statistical significance was $p < 0.05$.

Results

Sexual Behavior of Male Rats

All of the young males ($n = 18$) mated to ejaculation in the two pre-tests. Twenty-seven of the original 38 MA males mated to ejaculation in at least one pre-test, and subsequent data shown are from those 27 males. The percentage of males intromitting or ejaculating in each trial (Figure 2A and 2B) was calculated, and comparisons were made using Fisher's Exact test ($df = 1, 45$ for all). For the percentage of males intromitting, the young-T group and MA-T groups were significantly higher than the MA-V group in both post-test 1 (young-T vs. MA-V: $P = 0.017$; MA-T vs. MA-V: $P = 0.00686$) and in post-test 2 (young-T vs. MA-V: $P = 0.00681$; MA-T vs. MA-V: $P = 0.0129$; Figure 2A). For the percentage of males ejaculating, the young group was significantly different from the MA group in both pre-test 1 ($P = 0.0316$) and in pre-test 2 ($P = 0.0085$). Following castration and hormone treatment, the percentage of ejaculations was significantly lower in the young-V and MA-V rats in both post-tests compared to the testosterone groups ($P < 0.001$ for all comparisons of vehicle to testosterone treated rats; Figure 2B).

The results on behavioral measures of mount frequency, intromission frequency and mount latency are shown in Figure 3 (panels A, B and C, respectively, and in Supplementary Table 1). Time was the within-subjects factor and age and treatment were the between-subjects factors in this repeated measures design. The effects of time were not significant for any of these parameters. However, the interaction effects of time and treatment were significant for mount and intromission frequencies (mount frequency: $F_{3,102} = 9.031$, $P < 0.001$, Figure 3A; intromission frequency: $F_{3,102} = 14.175$, $P < 0.001$, Figure 3B). The main effect of age was significant for mount latency ($F_{1,35} = 7.463$, $P < 0.05$; Figure 3C), mount frequency ($F_{1,34} = 13.757$, $P < 0.01$) and intromission frequency ($F_{1,34} = 21.693$, $P < 0.001$) with young groups having shorter mount latency and higher mount and intromission frequencies. A main effect of hormone treatment was detected for mount frequency ($F_{1,34} = 16.086$, $P < 0.001$) and intromission frequency ($F_{1,34} = 16.738$, $P < 0.001$). Testosterone-treated animals had significantly higher frequencies than V-treated groups for both mounts and intromissions. There were no significant interactions between treatment and age on the three measurements (Mount latency: $F_{1,35} = 2.483$, $P = 0.124$; Mount frequency: $F_{1,34} = 1.761$, $P = 0.194$; Intromission frequency: $F_{1,34} = 1.759$, $P = 0.194$).

AR Immunoreactivity in AVPV and MPN

Representative photomicrographs of AR-immunoreactivity in the AVPV and MPN are provided in Figure 4. Quantitative stereologic analyses of AR-ir cell densities were performed, and results shown in Figure 5. There was no effect of age on AR cell density. A significant main effect of treatment in both AVPV and MPN was found (AVPV: $F_{1,21} = 78.43$, $P < 0.0001$; MPN: $F_{1,21} = 91.97$, $P < 0.0001$), with significantly greater density of AR cells in the testosterone than the vehicle treated groups of both ages.

ER α Immunoreactivity in AVPV and MPN

Representative photomicrographs of ER α -immunoreactivity in the AVPV and MPN are shown in Figure 6. Stereologic analyses of ER α -ir cell densities were performed (Figure 7). There was a significant main effect of treatment on ER α cell density in AVPV ($F_{1,17} = 12.69$, $P < 0.01$) and MPN ($F_{1,17} = 125.97$, $P < 0.0001$), with ER α cell density being significantly lower in the testosterone than the vehicle groups of both ages. There was no effect of age on ER α cell density.

Serum Hormone Concentrations

In intact rats (Figure 8A), serum total testosterone levels tended to be higher in the middle-aged than the young group but this was not significant ($P = 0.113$). Seventeen days after castration and hormone (T or V) treatment, there was no effect of age ($P = 0.198$) but a significant effect of treatment ($P < 0.001$). Vehicle animals had significantly lower testosterone levels than the testosterone group. In addition, the testosterone levels in the T-replaced group were in a similar range to pre-castration levels (c.f. Figures 8A and 8B).

Free testosterone concentrations in intact rats did not differ between the middle-aged and the young group ($P = 0.087$; Figure 8C). Following castration and hormone (T or V) treatment, there was no effect of age ($P = 0.131$) and a significant effect of treatment ($P < 0.001$), with free testosterone levels higher in the testosterone than the vehicle groups.

Serum estradiol levels were measured in intact rats, with levels similar between the young and middle-aged groups (Young: 8.96 ± 1.62 ; MA: 6.77 ± 1.33). Due to a technical error, serum estradiol levels could not be measured in the terminal sample.

Discussion

In these studies, we examined the effects of testosterone on masculine sexual behavior and on androgen receptor and estrogen receptor α expression in the preoptic area of the brain in young and middle-aged male rats. We made the following observations: 1) Aging was associated with a significant decline in sexual behavior in intact males, a finding that is in agreement with previous studies (Chambers and Phoenix, 1983; Smith et al., 1992). 2) Testosterone given to young or middle-aged rats at the time of castration was associated with the maintenance or even improvement of most masculine sexual behaviors, at similar levels to these behaviors in the young pre-test (intact) rats. The exceptions were mount and intromission frequencies, which were consistently lower in the middle-aged than the young group. 3) Rats receiving the vehicle at the time of castration had a loss of sexual behaviors, with the exception of mount latency in the young group. 4) In the POA of gonadectomized adult male rats, AR density was significantly higher, and ER α density lower, in testosterone than vehicle-treated males, an effect that was similar in the two age groups. 5) Serum testosterone and estradiol levels, in general, were similar between the two ages. As a whole, these data show that absolute serum hormone levels cannot explain age differences in sexual behavior. Further, the aging brain has the continued capacity to respond to exogenous testosterone treatment with increased AR, and decreased ER α , in a manner similar to the young brain.

Masculine Sexual Behavior, Hormones, and Aging

In the current study, we observed an age-associated decline in sexual behavior, but little difference in testosterone concentrations. Although testosterone is important for masculine sexual behavior, a causal relationship between testosterone concentrations and sexual behavior is controversial, and our results and others' suggest that at least some of these processes are dissociated. For example, it was reported in young rats that administration of less than one-third the normal intact levels of testosterone is needed in castrated male rats to maintain normal sexuality (Damassa et al., 1977). Intact male rats that could complete copulatory behavior showed no correlation between the behavioral measures and testosterone levels (Smith et al., 1992). In other words, more testosterone is not "better" than less testosterone in maintaining masculine sexual behaviors, as long as some threshold level of testosterone is achieved (Damassa et al., 1977; Frankel, 1981).

The ability of testosterone to regulate sexual behavior becomes even further dissociated with aging. Declines in plasma testosterone concentrations have typically been associated with a

loss of sexual performance in aging males across many species, although most of this work utilized males of more advanced age than the middle-aged rats in the current study (Bernardi et al., 1998; Chambers et al., 1991; Chen et al., 1994; Downs and Urbanski, 2006; Gruenewald et al., 2000; Harman et al., 2001; Luine et al., 2007; Roselli et al., 1986; Taylor et al., 1996; Wu et al., 2009). When testosterone levels in aging male rats were replaced to levels similar to those in young rats, the decline of sexual arousal (Gray et al., 1981) and copulatory activity [current results and (Chambers et al., 1991; Hsu et al., 1986)] could not be restored or maintained. Together, these findings indicate that low levels of serum testosterone in aging males are not exclusively responsible for the diminution of sexual behaviors.

The one behavior that was not regulated in a predictable way by testosterone was mount latency. Castrated young male rats had similar mount latencies whether replaced with vehicle or testosterone. Middle-aged rats had longer mount latencies in the pre-test, and after castration, this behavior was facilitated by testosterone but not vehicle. These results suggest that mount latency is testosterone-independent only at the young age, and indicates a potential age-related change in the neural circuitry underlying this motivational behavior. Previously, mount latency was shown to be modulated by testosterone in mice (James and Nyby, 2002). Beyer et al. (1981) reported that effects of hormone treatment (either testosterone or estradiol) in castrated male rats differed for mounts compared to intromissions and ejaculations, with a greater restorative effect in the former than the latter. Thus, the neural mechanisms controlling mounting behavior probably differ somewhat from those involved in intromission and ejaculation (reviewed in Hull and Dominguez, 2007). It is also notable that there were only two behaviors that differed between the two ages in the post-tests: mount frequency and intromission frequency, both of which were lower in middle-aged than young rats. Again, because hormone levels were largely comparable between these age groups, the differences are presumably due to changes in neural circuits with aging.

Steroid Hormones in Intact Aging Male Rats

It may initially seem puzzling that testosterone and free testosterone concentrations did not differ significantly between the young and middle-aged intact males of the present study, as many previous publications showing an age-related *decline* in serum testosterone (Bernardi et al., 1998; Chambers et al., 1991; Chen et al., 1994; Downs and Urbanski, 2006; Gruenewald et al., 2000; Harman et al., 2001; Luine et al., 2007; Roselli et al., 1986; Taylor et al., 1996; Wu et al., 2009). By contrast, there is a body of literature that demonstrates little or no difference in total testosterone concentrations between middle-aged and young male rats (Frankel, 1981; Frankel and Mock, 1981; Wu and Gore, 2009; Gruenewald et al., 2000). It is difficult to reconcile these disparate results, but testosterone concentrations are known to vary based on rat strain, time of day (Gruenewald et al., 2000), and sexual experience (Wu and Gore, 2009; Edinger and Frye, 2007). In addition, testosterone is released in a pulsatile manner, with more than 5-fold fluctuations from nadir to peak (Steiner et al., 1984). When these factors are put into the context of chronological age, they further complicate the interpretation of the results. Our concentrations in young males are in a similar range (from 1-10 ng/ml) as reported in the literature (Sprague-Dawley: Steiner et al., 1984; Frankel 1981; Karpas et al, 1983; Wu et al., 2009; Wu and Gore, 2009; Fischer 344: Chambers et al., 1991; Brown-Norway: Gruenewald et al., 2000; Gruenewald & Matsumoto 1991; Long-Evans: Damassa et al., 1977; Wistar: Herrera-Pérez et al., 2008; Fernández-Guasti et al., 2003; Romano-Torres et al 2006; Taylor et al., 1996). For middle-aged rats, testosterone levels have been reported to range from 0.5 to 6 ng/ml, with results from the literature overlapping with our current findings (Sprague-Dawley: Frankel 1981; Frankel & Mock 1981; Wu et al., 2009; Wu and Gore, 2009; Brown-Norway: Gruenewald et al., 2000; Wistar: Herrera-Pérez et al., 2008).

Free testosterone concentrations, which represent the bioavailable pool, were similar between our young and middle-aged rats. We previously reported that free testosterone increased between young and middle-aged rats (Wu and Gore, 2009). Few published studies have addressed levels of free testosterone in an aging rat model. Andersen et al. (2002) reported that free testosterone levels in Wistar rats decreased from 3 to 22 months in Wistar rats, but no intermediate ages were studied. Shen et al. (2000) found that free testosterone concentrations were similar in Sprague-Dawley rats at 2- and 13-months of age, similar to our current results. Thus, future studies would help to resolve some differences in results.

We also found no difference in serum estradiol concentrations in our intact young and middle-aged animals. The relationship between serum estradiol and aging is unclear, as the literature in rats reports increases (Luine et al., 2007; Herath et al., 2001), decreases (Wu and Gore, 2009; Herrera-Pérez et al., 2008), or no change (Wu et al., 2009; Goya et al., 1990; Fujita et al., 1990) depending upon the model and the ages studied. Of these reports, to our knowledge only four include middle-aged animals: in two there was a decrease with aging (Wu and Gore, 2009; Herrera-Pérez et al., 2008) and in two there was no change (Wu et al., 2009; Fujita et al., 1990). There are experimental differences in rat strains, the ages of analysis, and the specific assay used to measure hormone levels. The range of estradiol levels in the current study, 5-10 pg/ml, is similar to that in the previously published literature from our laboratory [Sprague-Dawley rats, range 5-35 pg/ml; (Wu and Gore, 2009)], and others [young Wistar rats, range 10-15 pg/ml; (Phillips-Farfán et al., 2007); Wistar rats: young (19 pg/ml) and middle-aged (4 pg/ml) (Herrera-Pérez et al., 2008)]. Again, future work is needed to provide much-needed reconciliation of these results.

Role of Sexual Experience and/or Satiety

The behavioral status, sexual experience, or satiety of the experimental animals may have an effect on hormones and their hypothalamic receptors. In the current study, the middle-aged rats were retired breeders and had not bred for several months, whereas young rats were given extensive experience and then used in our tests shortly thereafter. Several studies in male rats (both young and aging) has shown that animals that have mated have higher testosterone levels than non-mated counterparts (Frankel, 1984). Edinger and Frye (2007) showed that young male rats with sexual experience had higher testosterone concentrations than naïve males. A recent study from our laboratory also supports and extends that result by showing that animals with sexual experience, irrespective of age, have higher total and free testosterone than naïve counterparts (Wu and Gore, 2009). The timing and amount of the latest sexual activity may also influence the results (reviewed in Fernández-Guasti and Rodríguez-Manzo, 2003). We chose a 24-hour post-testing interval for euthanasia for comparison with our previous study (Wu & Gore, 2009) and that of other laboratories (Phillips-Farfán et al., 2007). In experienced young male rats, testosterone concentrations did not differ with time relative to the last sexual experience (Romano-Torres et al., 2007) or with sexual exhaustion (Fernández-Guasti et al., 2003). Estradiol levels also did not differ between experienced rats given a range of sexual behavior (Phillips-Farfán et al., 2007).

Steroid Hormones and Behavior in Castrated Male Rats

Our study showed that castration and testosterone implantation resulted in concentrations of total and free testosterone that were in a similar range as intact levels. Nevertheless, exogenous testosterone maintained or even facilitated aspects of sexual behavior. This is interpreted to mean that exogenous testosterone may be more behaviorally effective than the same mean level of endogenous testosterone in intact males, especially in shortening mount and intromission latencies. There are three interpretations for these differences between serum endogenous and exogenous testosterone: 1) circulating testosterone concentrations may not be completely representative of the testosterone concentration in regions of the brain, including

preoptic area, that control sexual behavior; 2) Circulating serum testosterone in intact males includes peaks and troughs (Bartke et al., 1973) whereas testosterone capsules probably provide more continuous release. The consequences of pulsatile vs. continuous testosterone may include a difference in neural actions of testosterone. In support of this, Gruenewald *et al.* showed that whereas basal testosterone concentrations did not differ between young and middle-aged rats, the circadian rhythm of testosterone release was dampened with age (Gruenewald et al., 2000). 3) Our rats were given testosterone implantation at the time of castration, thereby maintaining hormone levels. Other studies have evaluated effects of testosterone treatment following a longer post-castration delay (Chambers and Phoenix, 1984; Chambers et al., 1991; Sato et al., 1998). These varying experimental paradigms may differentially up- or down-regulate neural steroid hormone receptors, thereby producing behavioral differences. This hypothesis is consistent with the observation that the antiandrogen, Sch 16423, was able to block the restoration of masculine sexual behavior but had a comparatively weak effect on the maintenance of copulatory behavior (McGinnis and Mirth, 1986). Moreover, considerably higher amounts of testosterone are required for restoration of sexual behavior in castrates, as opposed to its maintenance when treatment is initiated immediately (Damassa et al., 1977).

AR and ER α Immunoreactivity in Aging Males

The expression of AR mRNA and protein in the hypothalamus varies with age and hormone status, with most published work focused on developing or young adult animals (Iqbal et al., 1995; Lu et al., 1999; Lu et al., 1998; Walker et al., 2009; Wood and Newman, 1993). We previously reported that AR immunoreactive cell density increased with age in the preoptic area of intact male rats (Wu and Gore, 2009; Wu et al., 2009). By contrast, Chambers et al. (1991) showed that androgen binding was decreased in the preoptic area of aged compared to young male rats, a difference that may be attributable to the more advanced age of the animals of Chambers et al. (1991) compared to our rats. A relationship between testosterone concentrations and hypothalamic AR expression is well-established: castration diminishes AR expression while testosterone treatment can restore it (Krey and McGinnis, 1990; Wood and Newman, 1993). Our current results add to both of these lines of research. They show that the age difference in AR is not detectable in castrated male rats; further, we show that testosterone treatment at the time of castration elevates AR immunoreactive cell density similarly in young and middle-aged rats. We conclude that the preoptic area continues to be responsive to testosterone as manifested by expression of AR protein immunoreactivity in middle aged animals.

Some effects of testosterone on sexual behavior may be mediated through its aromatization to estradiol and subsequent actions on estrogen receptors. Further, estradiol facilitates masculine sexual behavior (Baum and Vreeburg, 1973; Larsson et al., 1976; Vagell and McGinnis, 1997). Effects of castration, hormone treatment, and aging on the expression of the ER α in male rats have been investigated, although not within a single study. Castration was reported to increase ER α mRNA in the MPN of young male rats (Handa et al., 1996). By direct contrast, Roselli and Resko (1993) reported that estradiol binding in the preoptic area was significantly lower in castrated compared to intact male rats at both young and old ages. Treatment of male rats with hormones also affects ER α binding and immunoreactivity. Clancy *et al.* (2000) reported that counts of ER-immunoreactive cells in the medial preoptic area were significantly lower in estradiol- compared to control-treated male rat, and this was region specific, as a difference was not detected in the bed nucleus of the stria terminalis or the lateral septum. Effects of testosterone treatment on ER α expression were studied in the current study: in the MPN, ER α cell density was substantially higher in the vehicle compared to the testosterone treated rats of both ages. In the AVPV, testosterone also decreased ER α cell density but to a lesser extent. Finally, the effects of aging on estrogen receptor have been investigated. No

significant effect of age in AVPV and MPN was found in this study; similarly, Madeira et al. (2000) showed no difference in ER α immunoreactive cell numbers in the MPN. In further support, Bottner et al. (2007) showed no difference in ER α mRNA levels in the preoptic area. We speculate that testosterone replacement in the current study may regulate ER α levels either indirectly through binding to AR-expressing cells, or directly through its aromatization to estradiol. As some cells may co-express AR and ER α (Greco et al., 1998), these actions are not necessarily mutually exclusive.

Sexual experience and/or satiety may influence levels of hypothalamic hormone receptors. Phillips-Farfán et al. (2007) showed that ER α immunoreactive cell density was increased in the medial preoptic area of rats that mated to satiety, but not rats given a single ejaculation, compared to unmated controls. We previously found no difference in either AR- or ER α immunoreactive cell density in the AVPV or MPN of sexually naïve or experienced male rats (Wu and Gore, 2009). In experienced male rats, time after mating to satiety was related to AR density in several hypothalamic and preoptic brain regions (Romano-Torres et al., 2007). In the MPN, AR density was decreased 24 hours after males were allowed to mate to one ejaculation and further decreased in males mated to exhaustion (Fernández-Guasti et al., 2003). In comparing young males that were sorted into copulators or non-copulators, Portillo et al. (2006) reported no difference between these groups in AR density in the medial preoptic area, and lower numbers of ER α cells in non-copulators compared to copulators. As a whole, these data show the importance of consideration of behavioral status in relationship to the endpoint of AR and ER α cells, as aging, experience, time since sexual activity, and amount of activity, are important factors in their expression.

Conclusion

We had initially hypothesized that the age-related behavioral decline in masculine behaviors may be due to changes in androgen and estrogen receptor numbers in the hypothalamus, and/or a diminished responsiveness of these receptors to testosterone treatment in animals of increasing age. The current results do not support that hypothesis, as both young and middle-aged castrated males responded similarly to testosterone replacement with an up-regulation of the AR and a down-regulation of the ER α . Therefore, we postulate that other neurotransmitter systems involved in the control of sexual behaviors in male rats are responsible for the pre-castration differences between the two age groups. In addition, other brain regions involved in the neural circuitry that controls masculine behaviors merit investigation. Future studies are necessary to delineate the nature of these circuits in middle-aged males.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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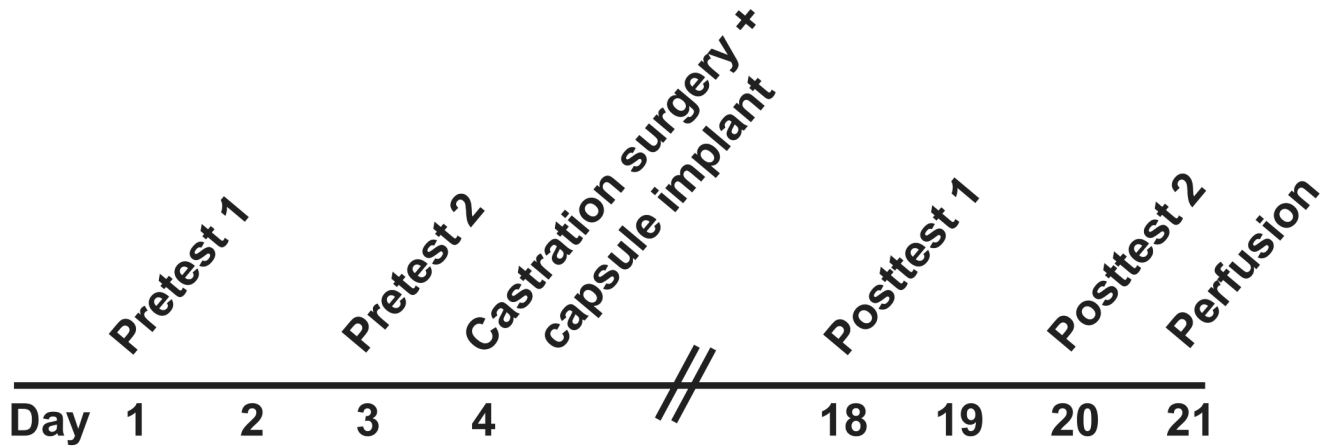
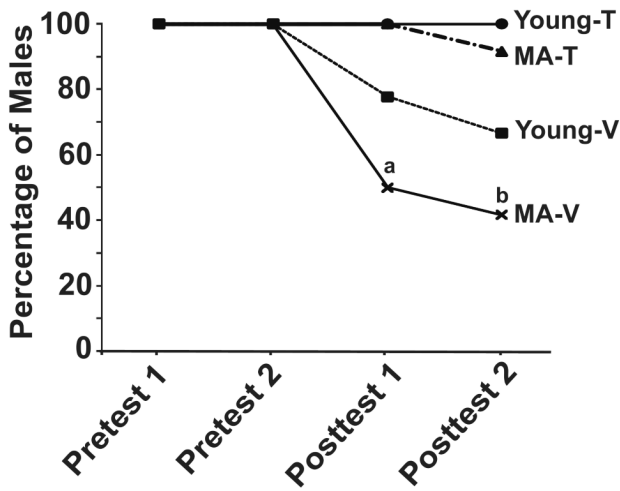


Figure 1.

An experimental flowchart is shown. Young and middle-aged rats were given two pretests 2 days apart. One day later, they were anesthetized, and a blood sample was drawn to enable measurement of intact serum hormone levels. Then, rats were bilaterally castrated, and implanted with a Silastic capsule containing either testosterone or vehicle. Two weeks later, the post-tests were performed two days apart. One day after testing, rats were euthanized by perfusion; a blood sample collected at that time was used for measuring hormone levels following the castration and hormone treatment.

A. Percentage of Males Intromitting



B. Percentage of Males Ejaculating

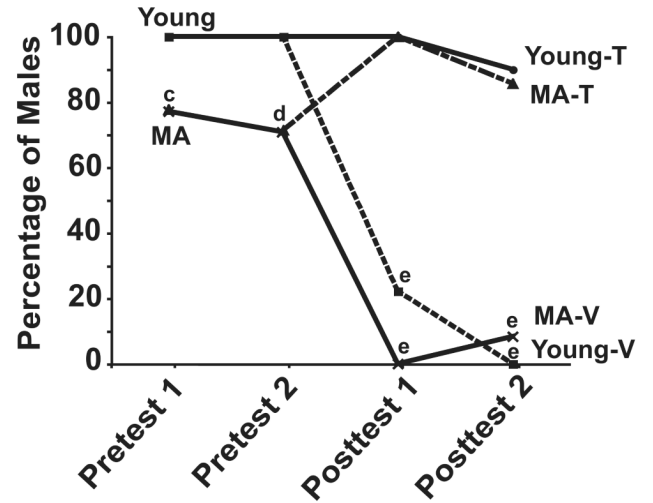


Figure 2.

Behavioral analyses of the two pre-tests and two post-tests are shown. The mean percentages of males intromitting and ejaculating are shown in panel A and B. Data for the pre-tests are shown combined for each age. For the post-tests, data for the four groups (young-T, young-V, MA-T, MA-V) are shown separately. In post-test 1 and post-test 2, the percentage of males intromitting were significantly higher in the young-T and MA-T compared to the MA-V group (a: $P < 0.05$ and $P < 0.001$ compared to the corresponding young-T group and MA-T respectively; b: $P < 0.001$ and $P < 0.05$ compared to the corresponding young-T group and MAT respectively). The percent of males ejaculating was significantly higher for young than middle-aged males (c: $P < 0.05$; d: $P < 0.01$). In Post-test 1 and Post-test 2, Fisher's Exact test was used with the young-T and MA-T groups as the bases of comparison. These groups were significantly different than the young-V and MA-V groups (e: $P < 0.001$ for all comparisons). Sample sizes were: Young-T (n=9), Young-V (n=9), MA-T (n=14), and MA-V (n=13). Abbreviations: MA, middle-aged; T, testosterone; V, vehicle.

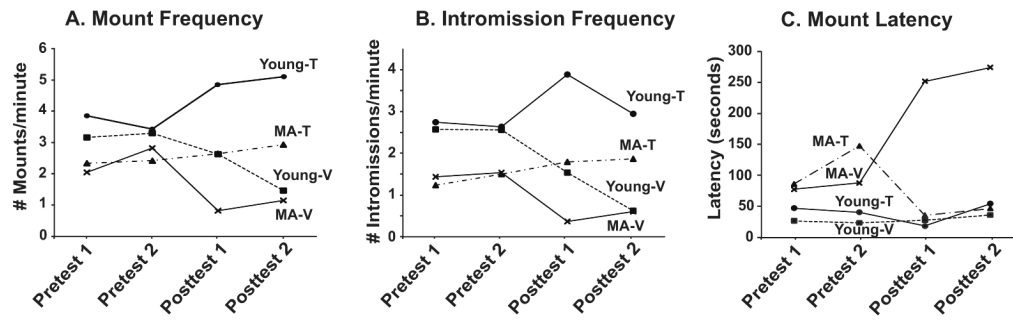


Figure 3. Mean mount frequency (A), intromission frequency (B) and mount latency (C) are shown. Repeated measures analyses showed significant effects of age (mount frequency: $P < 0.01$; intromission frequency: $P < 0.001$; mount latency: $P < 0.05$) and treatment (mount frequency: $P < 0.001$; intromission frequency: $P < 0.001$). See text for further analytical details on statistical results, which could not be plotted due to the repeated measures design, and Supplementary Table 1 for reporting of the means \pm standard errors.

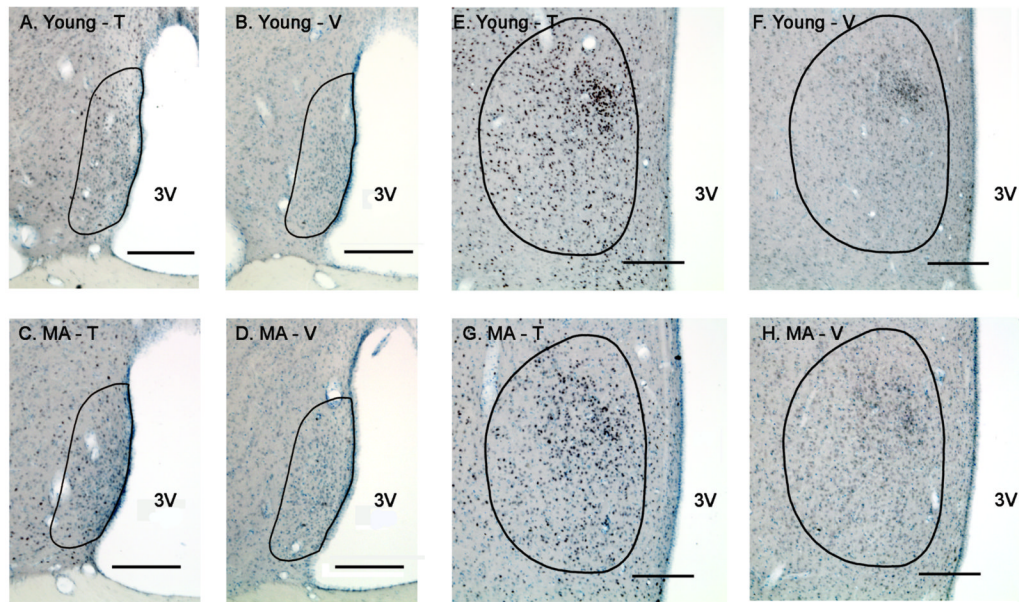


Figure 4.

Photomicrographs of AR immunoreactivity in the AVPV (left) and MPN (right) of young and middle-aged rats are shown. Rats were perfused at the completion of the two post-tests, 17 days after castration and hormone (testosterone or vehicle) treatment. Representative sections are shown for the AVPV and MPN, respectively, of young-T (A, E), young-V (B, F), middle-aged-T (C, G) and middle-aged-V (D, H) male rats. The contours of the AVPV or MPN are drawn based on Nissl staining and according to Swanson's rat brain atlas (1998). Scale bar = 200 μ m. Abbreviations: MA, middle-aged; T, testosterone; V, vehicle; 3V, third ventricle.

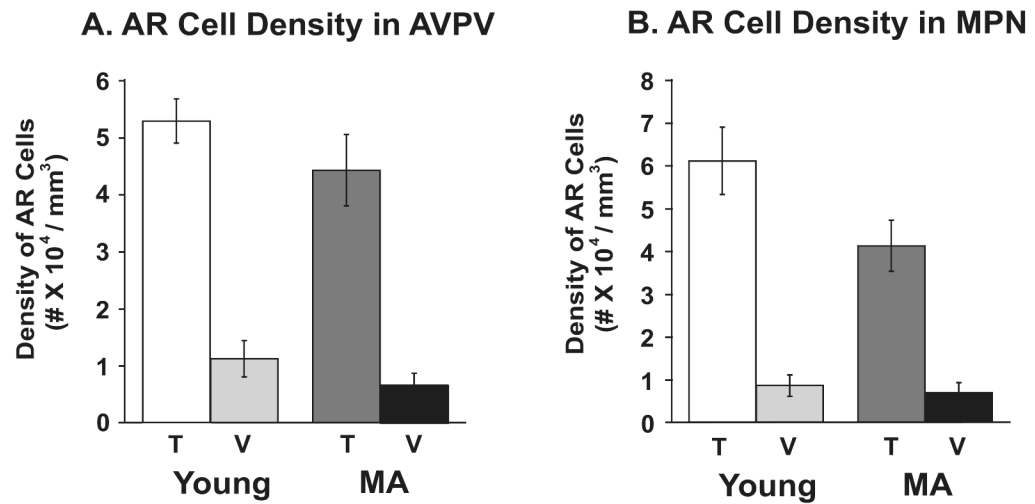


Figure 5.

Stereologic analysis results of AR-immunoreactive cell density (number of immunoreactive cells/regional volume) in the AVPV (panel A, n=8 rats per group) and MPN (panel B, n=8 rats per group). The bars represent the mean \pm SEM. AR cell density was significantly higher in T- than in V-treated males of both ages ($P < 0.0001$). Abbreviations: AVPV, anteroventral periventricular nucleus; MPN, medial preoptic nucleus; T, testosterone; V, vehicle; MA, middle-aged.

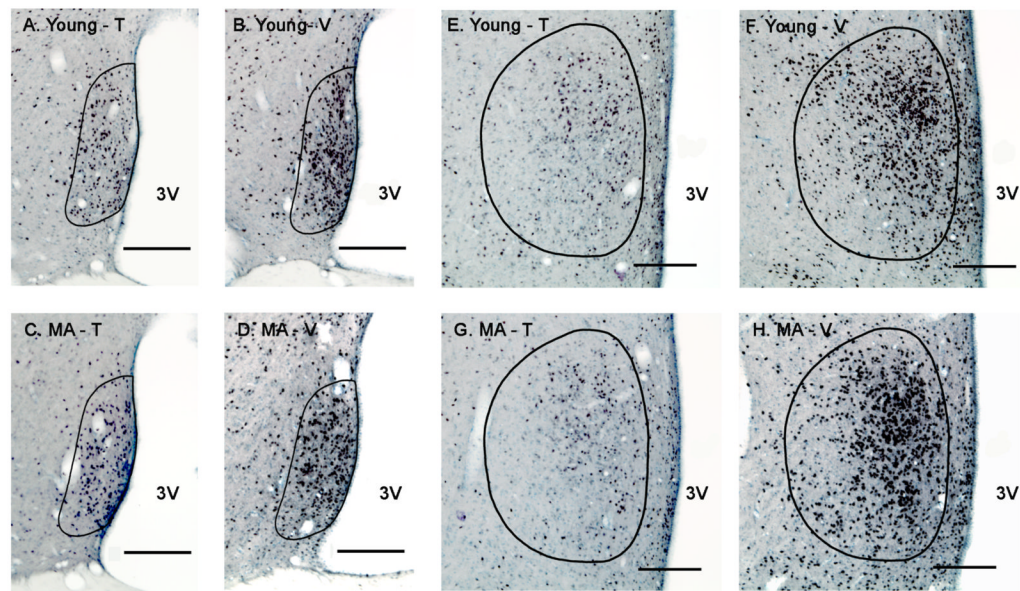


Figure 6. Photomicrographs of ER α immunoreactivity in the AVPV (left) and MPN (right) of the same young and middle-aged rats are shown. Representative sections from the AVPV or MPN, respectively, of young-T (A, E), young-V (B, F), middle-aged-T (C, G) and middle-aged-V (D, H) male rats are presented. The contours of the AVPV or MPN are drawn based on Nissl staining and according to Swanson's rat brain atlas (1998). Scale bar = 200 μ m. Abbreviations: MA, middle-aged; T, testosterone; V, vehicle; 3V, third ventricle.

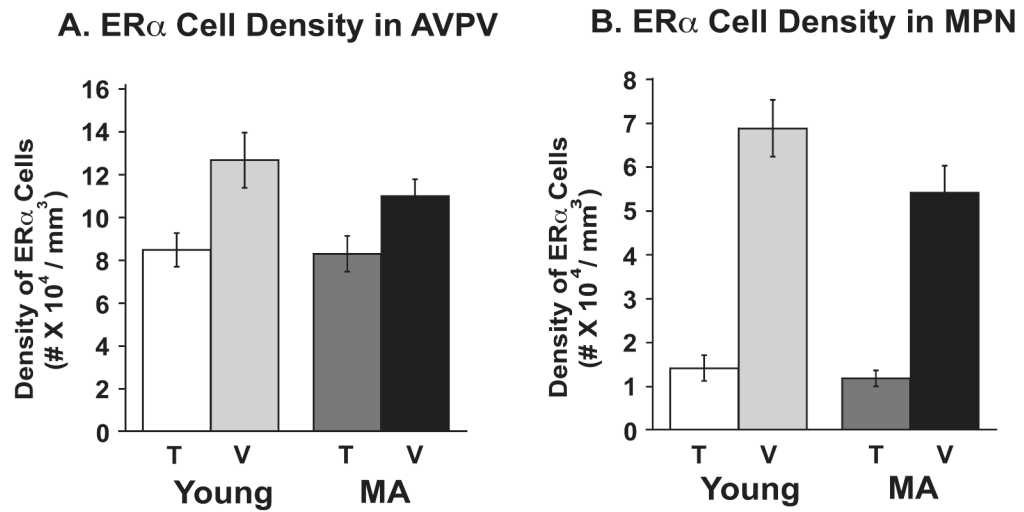


Figure 7.

Stereologic analysis results of ER α -immunoreactive cell density (number of immunoreactive cells/regional volume) are shown for the AVPV (panel A, n=6 rats per group) and the MPN (panel B, n=6 rats per group). The bars represent the mean \pm SEM. ER α -cell density was significantly lower in T-treated rats of both ages in AVPV ($P < 0.01$) and MPN ($P < 0.0001$). Abbreviations: AVPV, anteroventral periventricular nucleus; MPN, medial preoptic nucleus; T, testosterone; V, vehicle; MA, middle-aged.

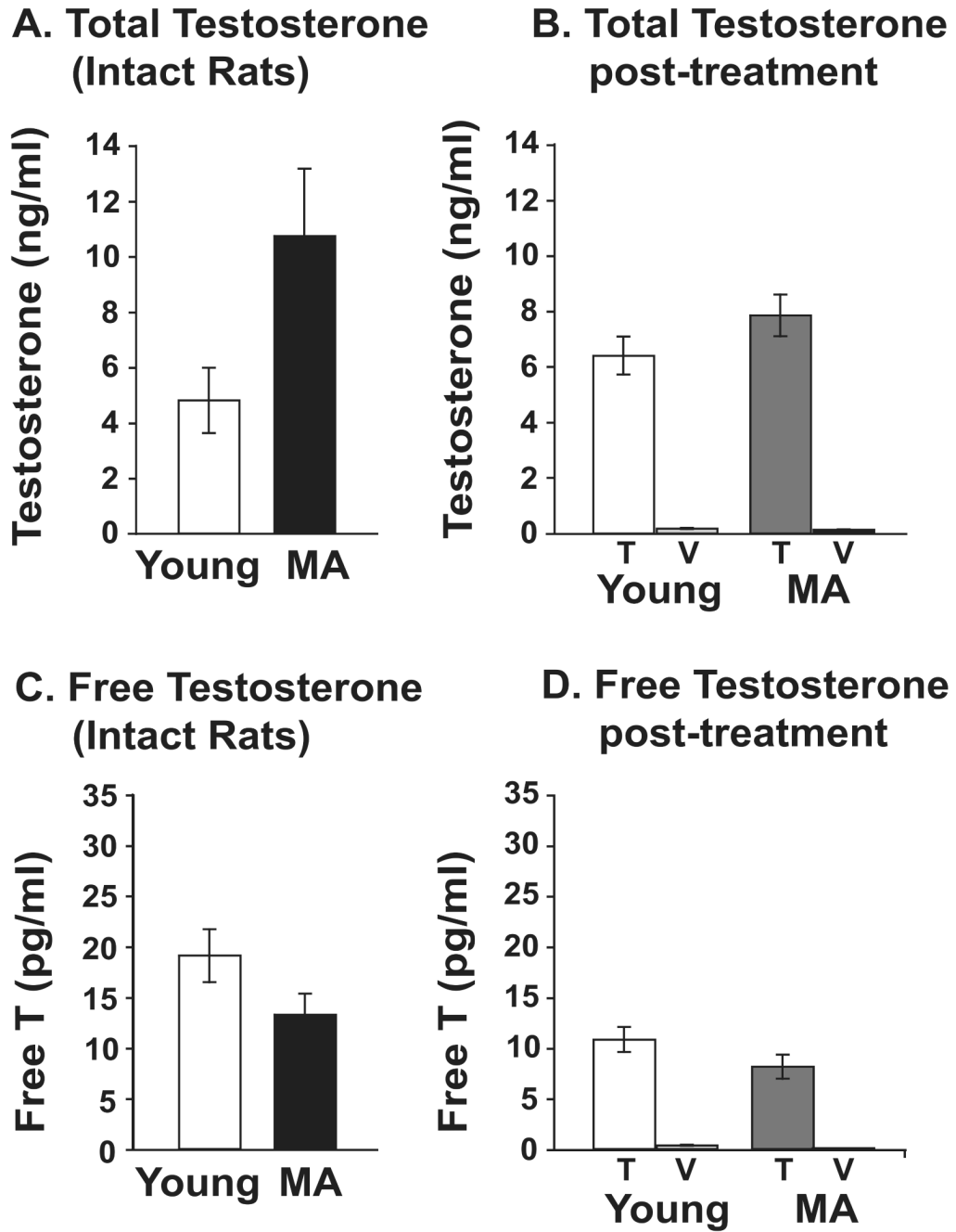


Figure 8. Serum total and free testosterone were assayed in young and middle-aged male rats before and after castration and hormone treatment. Hormones from intact young and middle-aged rats are shown in panels A and C. Hormones in the four groups 17 days after castration and treatment are shown in panels B and D. There was a significant main effect of testosterone treatment on total and free testosterone in samples collected at euthanasia ($P < 0.001$ for both). The bars represent the mean \pm SEM. Abbreviations: T, testosterone; V, vehicle; MA, middle-aged.