Both estrogen receptors and androgen receptors contribute to testosterone-induced changes in the morphology of the medial amygdala and sexual arousal in male rats

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Abstract

In male rats, a steroid-sensitive circuit in the forebrain regulates mating behavior. The masculine phenotype in one component of the circuit, the posterodorsal nucleus of the medial amygdala (MePD), depends on the level of circulating androgens in the adult. To investigate which gonadal steroid receptor(s) mediate sexual arousal and MePD plasticity, adult male rats were castrated and given Silastic capsules containing the nonaromatizable androgen 5α-dihydrotestosterone (DHT), 17β-estradiol (E2), both steroids, or nothing. A fifth group was sham-castrated and treated with blank capsules. DHT treatment was necessary and sufficient to maintain the expression of noncontact penile erections and ultrasonic vocalizations in castrates. E2 had no significant effect on these measures. Both DHT and E2 increased olfactory investigation (“nosepokes”) during the noncontact penile erection test. E2, but not DHT, maintained intromission patterns, while either steroid, alone or in combination, maintained ejaculatory behavior. Regional volume and cell soma size of the MePD both decreased following castration. Additionally, MePD cell size was lateralized, with left hemisphere neurons larger than those on the right, an effect that appeared independent of steroid manipulations. DHT and E2 each maintained neuronal soma size. E2 maintained MePD regional volume more effectively in the left MePD than in the right, which may have been due to a greater sensitivity of the left to both castration and hormone treatment. Thus, both androgen receptors and estrogen receptors appear to participate in sexual behaviors that may be mediated by the MePD in adult rats, and both receptors contribute to the steroid-regulated structural plasticity in this brain region.

Keywords: Noncontact penile erection; Ultrasonic vocalization; Male copulation; Androgen receptor; Estrogen receptor; Hemispheric laterality

Testicular androgens can permanently masculinize aspects of the central nervous system during a critical period of brain development (Arnold and Breedlove, 1985; Breedlove, 1992; Cooke et al., 1998; Madeira and Lieberman, 1995). Yet there is increasing evidence that gonadal hormones also act throughout life to maintain masculine traits in the brain (McEwen, 1999). In rats, the sexual dimorphism in volume and neuronal soma size within the posterodorsal nucleus of the medial amygdala (MePD) depends on circulating androgens in the adult. Reversing the level of adult androgens in males and females will reverse the sex differences in MePD regional volume and soma size ( Cooke et al., 1999). Androgen-dependent plasticity in the MePD has also been observed in Syrian ( Gomez and Newman, 1991; Romeo and Sisk, 2001) and Siberian hamsters ( Cooke et al., 2001 ). Substance P, arginine vasopressin, and cholecystokinin expression in the MePD is also dependent on circulating androgens in adulthood (Shamma and De Vries, 1995; Malsbury and McKay, 1994; Micevych et al., 1988; Simerly et al., 1997). Together, these studies suggest that some masculine traits in the MePD reflect an animal’s current hormonal state and not an organizational effect of steroids early in life.

Understanding how gonadal hormones influence MePD neurons entails determining which gonadal steroid recep-
tor(s) mediate the effect. Considerable evidence suggests that estrogen receptors (ERs) have a role. The MePD densely expresses genes for ERs α and β (Shugrue et al., 1997; Simerly et al., 1990) and the enzyme aromatase (Jakab et al., 1993; Roselli et al., 1997), which converts androgens into estrogens. ERs are also necessary to maintain masculine levels of several neupeptides including substance P, arginine vasopressin, and cholecystokinin in the MePD of the adult rat (Micevych et al., 1994; Simerly et al., 1989; Wang and De Vries, 1995). Golgi-stained neurons in the MePD respond to 17β-estradiol (E2), but not the nonaromatizable androgen 5α-dihydrotestosterone (DHT), by increasing the size of their somata and the length and branching of their dendrites in castrate Syrian hamsters (Gomez and Newman, 1991). Additionally, the absolute refractory period of stria terminalis neurons, which are presumed to originate from the medial amygdala (MeA), is lengthened by castration, an effect that was reversed by E2 but not DHT (Kendrick and Drewett, 1980).

These results suggest that estrogen, derived from the aromatization of testosterone, acts upon ERs to maintain a masculine phenotype in the MePD and activate the steroid-dependent behaviors mediated by this brain region. However, there is evidence that the androgen receptor (AR) also influences the adult MePD. Castration leads rapidly to a drop in noncontact penile erections (NCEs), which are displayed when the male rat is exposed to airborne stimuli from an estrous female (Manzo et al., 1999). Bilateral MeA lesions abolish NCE expression in sexually experienced male rats, but leave reflexive erection from tactile stimulation unimpaired (Kondo et al., 1997). ARs appear to mediate NCEs since DHT, but not E2 treatment, is sufficient to restore NCE in castrated Wistar rats (Manzo et al., 1999). Together, these data suggest that AR-expressing neurons in the MeA mediate NCEs. Further evidence that ARs in the MeA have some role in male sexual behavior comes from Grêco et al. (1998), who find that noncontact sociosexual experience elicits c-fos expression in AR-expressing neurons in the MeA and limbic forebrain. Moreover, DHT implants into either the MeA or the septum enhance copulation in male rats (Baum et al., 1982).

This neurobehavioral evidence raises the possibility that ARs are also involved in maintaining the masculine features of MePD neurons. The purpose of the following experiment was to determine whether ARs and/or ERs contribute to the maintenance of a masculine phenotype in the MePD of adult male rats and the masculine sexual behaviors attributed to this brain region.

Materials and methods

Animals and surgery

Forty-nine adult male Long-Evans rats (Harlan) were selected from a pool of 60 for their display of at least two ejaculations during a preoperative copulation test. Animals were housed in standard rat cages (27 × 49 × 20 cm) with food and water freely available. Animals in different treatment conditions were distributed evenly among the cages, such that each treatment group received equivalent housing and social stimulation. Lights in the colony were set to a 12:12 schedule, with lights off at 1400 h and on at 200 h. Animal care and procedures followed standards set by the National Institutes of Health and were approved by the Animal Care and Use Committee of the University of California, Berkeley.

Following the preoperative sexual behavior test, animals were weighed, anesthetized with ketamine rat cocktail (90 mg/kg ketamine and 10 mg/kg xylazine, i.p.), and castrated via midline scrotal incisions. In sham-castrated animals (hereafter termed “shams”), testes were visualized but not removed. Immediately following castration, Silastic capsules (3.2 mm outside diameter, 1.6 mm inside diameter) containing either 50% 17β-estradiol (E2; No. E-8875, Sigma; diluted with cholesterol) or 100% 5α-androstan-17β-ol-3-one (DHT; No. A-8380, Sigma) were implanted subcutaneously in the interscapular region. DHT capsules were 3 cm long (effective release length) and two were implanted in each animal. E2 capsules were 5 mm long and only one was implanted in each animal. Castrates received either DHT alone plus one blank 5-mm capsule (n = 9), E2 alone plus two blank 3-cm capsules (n = 10), both steroids (n = 10), or three empty capsules (n = 10; hereafter termed “blanks”). A fifth group, shams (n = 8), received three empty capsules.

Prior to implantation, all capsules were rinsed in 100% ethanol and incubated at 37°C for 48 h in a saline solution (0.9%) to stabilize the steroid gradient across the capsule.

Behavioral studies

Ten adult Long-Evans females were used in the copulatory and NCE tests. They were housed in the same colony room as the experimental subjects, but were kept together three or four per cage on a separate rack, with food and water freely available. Two weeks before testing began, females were ovarioctomized and implanted subcutaneously with Silastic capsules (3.2 mm outside diameter, 1.6 mm inside diameter; 10 mm long) containing 10% E2, diluted with cholesterol. To induce sexual proceptivity, females were injected subcutaneously with 0.5 mg of progesterone (No. P-0130, Sigma) 5 h before the test. Each female was used in no more than five copulatory testing sessions per week.

We conducted NCE tests during the 30-day hormone treatment period to test the hypothesis that DHT is sufficient to maintain NCE following castration in Long-Evans rats, as it is in Wistar rats (Manzo et al., 1999). Each male was tested twice for NCE, beginning 12–14 days after surgery, with no more than 13 days between tests. One copulatory
behavior test was conducted 10 days after surgery, prior to NCE testing.

Behavior tests were conducted at least 1 h after lights out. Males were brought to the testing room, three cages at a time, and kept on a darkened shelf behind a paper screen. Mating behavior was observed within a rectangular glass aquarium (40.6 × 30.5 × 61.0 cm) containing clean pine sawdust on the floor. To begin the mating test, the male was placed into the aquarium and allowed to acclimate to the arena for 5–7 min before introduction of the female. A single observer, without knowledge of the hormonal condition of the male, recorded the latency to mount and the number and latency of intromissions and ejaculations. The tests ended when the male had achieved two ejaculations, had failed to mount or ejaculate within 30 min, or if the first postejaculatory interval exceeded 10 min.

Twelve to 14 days after surgery, the animals were brought to the testing room as described above and tested for NCEs. The NCE test box consisted of a pair of rectangular Plexiglas chambers (male’s chamber: 30 × 11 × 63 cm; female’s chamber: 30 × 11 × 31 cm), separated by two transparent partitions 3 cm apart, which prevented the male and female from physical contact. The dimensions of the chamber encouraged the male to stand in profile to the observer, which facilitated the viewing of NCEs. The partition on the male’s side contained a single hole (2.8 cm in diameter) from which the number and duration of nosepokes was recorded via two infrared movement sensors. Nosepokes were recorded because they may indicate the male’s interest in the stimulus female. By means of a small fan, airborne cues were drawn from the female’s chamber into the male’s chamber. Numerous holes in the external wall of the female’s chamber and a Plexiglas lid that fitted tightly over the top of the apparatus ensured that outside air was drawn exclusively from the female’s chamber into the male’s chamber. Sawdust covering the floor of the male’s chamber was replaced after every trial, whereas that in the female’s chamber was replaced once a day.

Each male was placed into the chamber and allowed to acclimate 5–7 min before introduction of the female into the other chamber. The latency and number of NCEs was recorded for 20 min by a single observer unaware of the male’s hormonal status. An NCE was recorded when an erect penis was observed and the male engaged in prolonged, exclusive grooming of its genitals. This was marked by a unique posture not seen in other grooming behavior. NCEs were frequent accompanied by an elevation of the hind paws and hip thrusts. At the end of the trial, the female was removed first, followed by the male.

An ultrasound transceiver (Petterson bat detector D 230) was tuned to 50 kHz and the microphone attached to the middle partition within 1 cm of the male’s snout during a typical nosepoke. Male and female rats emit ultrasonic calls prior to copulation (White et al., 1993), and 50-kHz calls by the male have been shown to increase the darting behavior of estrous females as well as the rate of copulation (Geyer et al., 1978). Ultrasonic calls may indicate communication between the male and the stimulus female that may reflect interest in further contact and thus serves as a useful index of sexual arousal. Calls were taped with a cassette recorder (Optimus CTR-109) and were quantified later by a single investigator unaware of the male’s hormonal status by using a hand counter. The investigator counted all audible calls, assuming that the partition would attenuate the female’s calls. No other attempt was made to discriminate between male and female calls.

Histology

Thirty days after surgery, animals were administered an overdose of sodium pentobarbital and perfused intracardially with phosphate-buffered saline (pH 7.4), followed by 10% buffered formalin for 20 min. Brains and seminal vesicles were removed and placed in 10% formalin. After at least 1 month of postfixation, the brains were placed over-night in 20% phosphate-buffered sucrose at 4°C prior to sectioning.

Each brain was scored along the right cortex to mark laterality, blocked coronally at the inferior colliculus and olfactory tubercle, and sliced coronally at 40 μm on a freezing microtome. One of three slices was mounted on coded, gelatin-coated slides, allowed to air-dry, and stained with thionin.

Microscope analysis

An investigator unaware of the treatment condition measured the volume of the MePD on both sides of the brain (Fig. 1A). To estimate the volume, the boundary of the MePD on each side was outlined by using a microprojector (25×) in all sections in which it appeared. The drawings were scanned and total area was calculated by using NIH Image. This value was then multiplied by the section thickness (40 μm) and the sampling ratio (3) to estimate total volume of the MePD per hemisphere.

An average of 30 neuronal somata in the MePD were drawn on each side from one section at 630× using a Zeiss compound microscope and camera lucida. Neurons were selected from the caudomedial portion of the MePD, which contains the densest expression of gonadal steroid receptors (Fig. 1B). Occasionally, minor tissue damage prevented the drawing of neurons within the sampling region, precluding the estimation of cell size for that hemisphere. Neurons were identified by the presence of a Nissl-stained cytoplasm and distinct nucleolus. Drawn cells were similarly digitized and the mean area of MePD neurons for each hemisphere of each animal was estimated. Final mean estimates of soma size were calculated based on the number of animals per group (N = 8–10/group).
Statistical analyses

Unequal variances and the large number of males displaying zero NCE precluded the use of analysis of variance (ANOVA) for that measure. Therefore, NCE incidence (the fraction of males displaying at least one NCE) was analyzed with $\chi^2$ ($df = 4$) and Fisher’s exact probability tests. All other variables were analyzed with ANOVA or regression. We first compared the isolated control group (shams) with blank-treated castrates (blanks) to determine whether circulating androgens had an effect on the measure of interest with two-way ANOVAs, using trial number as the repeated measure for behavioral tests and cerebral hemisphere as the repeated measure for brain measures. The effect of steroid treatment on castrates was assessed with mixed design, three-way ANOVAs, with one repeated measure (either trial number or cerebral hemisphere) and two independent variables, DHT (+ or −) and E2 (+ or −). A regression analysis was done to examine the relationship between MePD morphology and behavior, using average unilateral MePD volume or soma size from each subject as independent variables. Hemispheric laterality independent of treatment group was also evaluated separately using a matched-pairs $t$ test. Post hoc comparisons were based on Student’s $t$ tests. In all tests, a two-tailed probability value of 0.05 was used as the criterion for significance, and $N$ represents the number of animals.

Results

Body, brain, and seminal vesicle weights

Animal body weights were the same at the onset of the experiment and, as expected, untreated castrates gained less weight than did sham-operated controls ($P = 0.002$). Among the castrates, E2 treatment, also as expected (Wade, 1972), caused the animals to gain less weight during the experiment ($P = 0.01$), but DHT had no effect on body weight ($P = 0.6$; Table 1), and there was no statistical interaction of the two steroids on this measure. Although the mean brain weight of sham castrates was less than that of castrates, the difference was not significant ($P = 0.07$) and, because this difference was the opposite of the effects of castration on MePD volume, differences in brain weight could not account for regional volume differences reported below. Likewise, there was no effect of steroid treatment on brain weight among castrates (Table 1). Hence, the absolute volumes of the MeA were used in the analysis.

Seminal vesicles (including seminal fluid) were weighed at the time of brain sectioning. As expected, blank-treated castrates had significantly lighter seminal vesicles than did sham controls ($P < 0.0001$), and DHT treatment significantly maintained seminal vesicle weight in castrates ($P < 0.0001$). E2 given alone also maintained seminal vesicle weight ($P = 0.02$), although less effectively than DHT (Table 1).
Effects of hormone on sexual arousal and copulation

NCE

NCE incidence in test 1 was not reliably different between treatment groups \( (P = 0.1) \). However, there were significant differences in the second test \( (P = 0.0008) \) between sham castrates and blank-treated castrates \( (P = 0.001) \), which reflected the difference in the proportion of shams that displayed one or more NCEs (6 of 8) compared to the blank-treated castrates (0 of 9). More importantly, sham controls and DHT-treated animals did not differ in their display of NCEs, and the proportion of NCEs displayed by animals given E2 + blank was significantly less than either shams \( (P = 0.01) \) or those treated with DHT \( (P = 0.008) \), but did not differ from blank treated castrates. Twelve of 19 DHT-treated males displayed at least one NCE, whereas only 1 of the 10 E2-only males displayed a single NCE in test 2 (Fig. 2A).

Nosepokes

Repeated-measures ANOVA of nosepoke number among shams and castrates revealed a marginal effect of castration \( (P = 0.05) \). In contrast, cumulative nosepoke duration was greatly reduced by castration \( (P = 0.0005; \text{Fig. 2B}) \), suggesting this measure of attention to estrous females is more sensitive to circulating androgen than is nosepoke number. Further, there was an interaction between trial number and gonadal status \( (P = 0.01) \), due to shams increasing, and blanks decreasing, the time spent nosepoking between the first and second test. Among the castrate groups, ANOVA revealed a significant main effect of E2 and DHT on nosepoke duration \( (P = 0.02 \text{ and } P = 0.05, \text{respectively}) \). Both E2- and DHT-treated rats nosepoked longer than blank-treated castrates. ANOVA also revealed significant trial-by-steroid effects for both E2 \( (P = 0.01) \) and DHT \( (P = 0.003) \). However, this increase in the second trial seemed to be mostly due to increased duration of nosepokes from animals getting both steroids. Post hoc tests showed that by test 2, all steroid-treated animals nosepoked significantly longer than blanks \( (all \ P_s < 0.0004) \).

Table 1

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Preoperative body weight (g)</th>
<th>Final body weight (g)b,c</th>
<th>Brain weight (g)</th>
<th>Seminal vesicle weight (g)b,c,d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>315.3 (9.0)</td>
<td>401.1 (11.1)</td>
<td>2.06 (0.03)</td>
<td>1.84 (0.09)</td>
</tr>
<tr>
<td>Castrate + Blanks</td>
<td>316.3 (7.6)</td>
<td>380.0 (8.4)</td>
<td>2.15 (0.03)</td>
<td>0.10 (0.02)</td>
</tr>
<tr>
<td>Castrate + DHTd</td>
<td>311.5 (6.7)</td>
<td>381.8 (9.7)</td>
<td>2.13 (0.04)</td>
<td>1.57 (0.11)</td>
</tr>
<tr>
<td>Castrate + E2e</td>
<td>313.9 (7.5)</td>
<td>330.6 (6.5)</td>
<td>2.11 (0.02)</td>
<td>0.24 (0.02)</td>
</tr>
<tr>
<td>Castrate + DHT + E2</td>
<td>314.1 (9.0)</td>
<td>343.5 (9.6)</td>
<td>2.10 (0.03)</td>
<td>1.47 (0.07)</td>
</tr>
</tbody>
</table>

a Values are written as means \( \pm \) standard error of the mean (SEM). Letters indicate significant differences within column: b Effect of gonadectomy (sham versus untreated castrates); c Main effect of E2 treatment (ANOVA among castrates); d Main effect of DHT treatment (ANOVA among castrates). There were no significant interactions of E2 and DHT for any of these measures. All values of \( P \leq 0.02 \).

e DHT, 5α-dihydrotestosterone; E2, 17β-estradiol.
Mount latency was decreased by DHT (P = 0.01), whereas intromission latency was decreased by E2 (P = 0.01) and not DHT (P = 0.11). Accordingly, E2-treated rats achieved more intromissions than did blanks (P = 0.02), while DHT was without significant effect on intromissions. As expected, shams ejaculated significantly more often than blanks (P = 0.02). DHT and E2 each independently increased ejaculation number (P < 0.01). No interaction between the two steroids was detected for the number of ejaculations, but DHT + E2-treated rats achieved marginally more than those given either steroid alone (Table 2). Among castrates, mean ejaculation latency was also shortest in those receiving both steroids.

Hormone and laterality effects on MePD somata and regional volume

ANOVA of sham controls and blank-treated castrates revealed a significant effect of castration, with blanks having smaller MePD neurons (32%) than shams (P = 0.001; Fig. 4A). Unexpectedly, there was also a significant main effect of laterality (P = 0.001) with MePD neurons being larger in the left hemisphere than in the right in sham controls and untreated castrates. There was no interaction between laterality and gonadal status (P = 0.9).

Among castrates, steroid treatment yielded main effects of DHT (P = 0.0004) and E2 (P < 0.0001) on MePD soma size. The effect of laterality on MePD soma size remained significant (L > R, P = 0.001), but no interaction between the steroid treatment and laterality was detected (P = 0.3 for both steroids). Thus, both steroids maintained soma size and were just as effective on the right as on the left.

An L > R laterality across individuals from all groups was also revealed with a matched-pairs t test (P < 0.0001), with left hemisphere MePD neurons being 18% larger than those on the right. Thus, cell size was lateralized in the MePD independent of hormonal status. MePD volume was also found to be laterализed based on a matched-pairs t test across the groups (P = 0.04), with MePD volume greater on the right than on the left (Fig. 4B). Although ANOVA revealed that bilateral MePD volume decreased following castration (P = 0.02) and showed no

Table 2
Hormone effects on copulatory behaviors*

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Mount latency (\times 10^3)</th>
<th>Intromit latency (\times 10^3)</th>
<th>Intromit number</th>
<th>Ejaculation latency</th>
<th>Ejaculation number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (n = 8)</td>
<td>206 (3)</td>
<td>487 (255)</td>
<td>8.2 (2)</td>
<td>645 (208)</td>
<td>1.3 (0.3)</td>
</tr>
<tr>
<td>Castrate + Blanks (n = 10)</td>
<td>903 (260)</td>
<td>1241 (209)</td>
<td>2.9 (1.2)</td>
<td>1467 (213)</td>
<td>0.4 (0.2)</td>
</tr>
<tr>
<td>Castrate + DHT (n = 9)</td>
<td>78 (25)</td>
<td>527 (244)</td>
<td>6.5 (1.9)</td>
<td>922 (224)</td>
<td>1.2 (0.3)</td>
</tr>
<tr>
<td>Castrate + E2* (n = 10)</td>
<td>233 (175)</td>
<td>321 (173)</td>
<td>8.6 (1.9)</td>
<td>990 (186)</td>
<td>1.2 (0.3)</td>
</tr>
<tr>
<td>Castrate + DHT + E2 (n = 10)</td>
<td>181 (98)</td>
<td>419 (130)</td>
<td>8.0 (0.9)</td>
<td>814 (164)</td>
<td>1.8 (0.2)</td>
</tr>
</tbody>
</table>

* The mean number and latency of mating behaviors recorded 10 days after surgery. Values are means (± SEM). Latencies are recorded in seconds. Superscripts denote significant differences within the column. T tests revealed an effect of castration (difference between sham controls and blank-treated castrates) in all measures. Among castrates, analysis of variance revealed: \(^a\) a main effect of DHT, \(^b\) a main effect of E2, or \(^c\) an interaction between DHT and E2. All values of \(P \leq 0.04\). Intromission number refers to the number of intromissions required to achieve the first ejaculation.
significant interaction between castration and laterality ($P = 0.2$), it is evident that there was a greater reduction in MePD volume in the left hemisphere than in the right. This was verified with a $t$ test, which showed a significant effect of castration in the left hemisphere ($P = 0.04$), but not in the right ($P = 0.1$).

Repeated-measures ANOVA of the castrates did not detect any effects of steroid treatment on MePD volume, but did reveal a main effect of laterality ($R > L$, $P = 0.006$). There was a strong trend toward an interaction between E2 and laterality ($P = 0.06$) that prompted us to examine effects of steroid within each hemisphere. ANOVA revealed that E2 has a significant effect in the left MePD but not in the right ($P = 0.01$ and 0.6, respectively); DHT had no significant effect in either hemisphere. Thus, the laterality of MePD volume overall appears primarily due to a lateralized response to castration and E2 treatment.

**Correlations between MePD morphology and behavior**

In addition to behavioral differences found between groups, we asked whether there is also covariation between neural structure and behavior within various treatment groups. Among sham castrates, right hemisphere MePD soma size was positively correlated with the number of NCEs in test 1 ($r = 0.7$; $P = 0.02$) but not test 2. No other structural measures correlated with behavior in sham castrates. Further regressions were conducted only within groups in which steroid treatment affected a given behavior. DHT treatment increased MePD soma size bilaterally and nosepoke duration, yet we found a positive correlation between these two variables only in the left hemisphere ($r = 0.7$, $P = 0.03$) but not the right hemisphere ($r = 0.5$, $P = 0.12$). This was the only significant within-groups correlation detected.

**Discussion**

The overall objective of this work was to determine which gonadal steroid receptor is responsible for the steroid-dependent plasticity in the MePD of adult rats. In a former study, we found that after 30 days, blank-treated castrates had significantly smaller MePD regional volumes and somata than did sham castrates. Testosterone treatment was able to prevent the reductions in volume and soma size in castrate males, and significantly increased somal and volume measures in adult females (Cooke et al., 1999). In the present study, we gave castrated adult male rats DHT and/or E2 to assess the role of AR and ER activation in the maintenance of MePD cell size and regional volume after castration. We also assessed the role of those steroid receptors in various indices of sexual arousal. Our results confirm previous reports that ARs mediate the ability of male rats to display NCEs and we show for the first time that 50-kHz USVs also depend on AR but not ER activation. Furthermore, we found that ARs and ERs independently mediate significant growth within the MePD. Finally, we found evidence that cell size and regional volume are lateralized in the MePD. The laterality of cell size appeared independent of hormone manipulations, while MePD regional volume was due to greater sensitivity of the left hemisphere to castration compared to the right.

**Hormonal control of male sexual arousal and copulation**

Measures of body and seminal vesicle weight verified the efficacy of the steroid treatments. E2-treated castrates had significantly reduced body weights, and DHT treatment maintained seminal vesicle weight in castrates, as previously observed (Södersten, 1975; Wade, 1972). Our observations of NCEs, USVs, and nosepokes were used as indi-
ces of sexual arousal. NCE was examined because of its reported dependency on AR and the MeA (Kondo et al., 1997; Manzo et al., 1999). Preejaculatory USVs have been found to facilitate lordosis (Geyer et al., 1978) and, in the context of mating, are dependent on circulating androgens (Geyer et al., 1978). Nosepoking has been previously used as an index of male attention to a female (Manzo et al., 1999), but little is known about its hormonal or anatomical substrates.

NCE incidence in the first test was not reliably different among the groups, as shown by the overall $\chi^2$ and post hoc Fisher’s tests. This lack of effect in test 1 could have been due to unfamiliarity with the testing situation or to insufficient time elapsed since castration. However, as was found by Manzo et al. (1999), our blank-treated castrates failed to display any NCE in either test, making it less likely that residual testicular androgens accounted for the lack of reliable differences in test 1. Nevertheless, there was a significant correlation between the size of the MePD somata on the right side and the number of NCE in test 1 among the sham control males, which is consistent with the idea that these neurons participate in this behavior.

In test 2, there was a clear divergence between E2-treated males and those treated with DHT. The level of NCEs produced by E2 males approached that of blank-treated castrates, whereas NCE levels in DHT-treated males, either alone or with E2, approached that of shams. The near-normal level of NCE expression by those given DHT suggests that AR activation is necessary and sufficient for the expression of NCE. Because lesions in the MeA disrupt NCE production (Kondo et al., 1997) and the MePD is rich in ARs (Simerly et al., 1990), DHT may act directly in the MePD to facilitate NCE expression. DHT is also required for the expression of penile reflexes in spinally transected rats, and the hormone treatment given here undoubtably influenced spinal motoneurons (Forger et al., 1992; Hart et al., 1983) Thus, DHT acting on the neuromuscular system controlling penile reflexes may also play a role in the production of NCEs.

We confirmed that circulating gonadal steroids are required for 50-kHz USVs, as blank-treated castrates produced few USVs even in the first test. We also found that androgens per se were both necessary and sufficient for USV production since DHT maintained this behavior in castrates but E2 contributed little to its activation. This finding is consistent with that of Vagell and McGinnis (1998), who reported that hydroxyflutamide, an AR antagonist, reduced the production of USVs in male rats. However, they also reported that RU 58668, an ER antagonist, blocked USV production. Our results are discrepant with this latter finding, but we cannot rule out the possibility that we failed to detect a subtle effect of E2. Nevertheless, the importance of the AR in USV expression has been clearly demonstrated in both studies.

We found that cumulative nosepoke duration was more sensitive to castration than the number of nosepokes. Both E2 and DHT maintained nosepoke duration, although the additive effect of these two hormones was not evident until the second test.

Male rats may continue to show the ejaculatory pattern several weeks after gonadectomy (Davidson, 1966), yet we found several interesting results from the single copulation test, conducted only 10 days after gonadectomy. We found that DHT treatment was sufficient to reduce mount latency and to increase ejaculation number as compared to blank-treated castrates. DHT has previously been found to maintain or restore mounts in male rats (Butera and Czaja, 1989; Gladue, 1984; Södersten, 1975) and to restore ejaculations when given in high doses (Whalen and Luttge, 1971). E2 was more effective than DHT in reducing intromission latency and the number of intromissions prior to an ejaculation. As expected, ejaculation number dropped after castration, and either DHT or E2 helped to prevent this decline. The two steroids together appeared to be more effective than either alone, but this trend was not statistically significant.

**Neural plasticity in the MePD: Effects of DHT, E2, and laterality**

Our analysis of MePD somata and regional volume confirms that circulating androgens exert a trophic effect on this structure, as castration resulted in a significant reduction in both values. This is consistent with our work and that of others who have studied steroid-dependent MePD peptide expression (De Vries et al., 1985; Micevych et al., 1994; Simerly et al., 1989; Swann and Newman, 1992).

MePD somata respond to both estrogenic and androgenic metabolites of testosterone. While there is evidence that E2 exerts a trophic effect on neurons in the hamster MePD (Gomez and Newman, 1991), no one has, to our knowledge, reported that DHT treatment can also alter MePD neuronal morphology independently of ER stimulation. This finding supports the notion that ARs also play a role in the maintenance of a masculine MePD phenotype, and perhaps contribute to the expression of masculine behaviors. Evidence of an additive effect of DHT and E2 in the MePD further supports the notion that ARs and ERs have independent, trophic effects on MePD neurons. There is evidence that AR and ER are coexpressed within individual MePD neurons (Gréco et al., 1998), but our data do not permit us to determine whether DHT and E2 exerted their effects on the same or on distinct populations of cells. In fact, it is also possible that either steroid acts upon some other brain site to induce changes in the MePD.

E2 was the only steroid to influence MePD volume after castration, apparently in the left but not the right hemisphere. Since regional volume is presumed to reflect the aggregate morphology of perikarya and neuropil, the effect of E2 on volume suggests that E2, but not DHT, may have influenced MePD dendrites. This idea is supported by the work of Gomez and Newman (1991) and Lorenzo et al.
(1992), who demonstrated that E2 increases MeA dendrite length in Syrian hamsters and the rat, respectively. There is growing awareness of the role of ERs and ARs in regulating growth and plasticity in the brain. For example, gonadal steroids increase growth-associated membrane protein (GAP-43) in the limbic system of adult (Lustig et al., 1991) and neonatal rats (Shugrue and Dorsa, 1994). E2 treatment increases neurite extension of hypothalamic cells in vitro (Toran-Allerand, 1976). Not surprisingly, E2 increases the expression of tau and class II-β-tubulin, both of which are involved in process growth (Ferreira and Caceres, 1991; Rogers et al., 1993). Estradiol has also been found to upregulate n-cadherin expression in the adult hippocampus (Monks et al., 2001). Androgens regulate actin and the gap junction protein connexin 32 in adult motoneurons of the spinal nucleus of the bulbocavernosus, which displays steroid-dependent structural and synaptic plasticity (Breedlove and Arnold, 1981; Leedy et al., 1987; Matsumoto, 1997; Matsumota et al., 1992). Whether any or all of these proteins are involved in MePD plasticity remains unknown.

Intriguingly, two measures of MePD morphology, regional volume and soma size, were lateralized in opposing directions: somata were larger in the left hemisphere, but MePD volume was larger in the right hemisphere. Since our data provide no insight into the proximate mechanism that underlies MeA laterality, we can only speculate why we observed this pattern of effects. Von Ziegler and Lichtensteiger (1992) reported that the expression of aromatase is greater in the left MeA than the right of male rats just before birth. This early asymmetry may permanently alter the metabolic state of MePD cells, perhaps increasing the ability of the left MePD to metabolize androgens and synthesize proteins, both at a basal level and in response to steroid. It is also possible that ER density is greater in the left MePD, as it is in the cerebral cortex (Sandhu et al., 1986). This too would increase the ability of the left MePD to synthesize proteins in response to steroid.

The asymmetry in volume among castrated rats may be explained by postulating a greater steroid sensitivity of dendrites in the left MePD compared to those on the right. Although the difference did not reach statistical significance with repeated-measures ANOVA, the left MePD displayed a greater reduction in volume than the right following castration. Likewise, although no effect of steroid was detected with the overall analysis, the left hemisphere nonetheless showed an effect of E2 when each hemisphere was analyzed separately. This too is consistent with the notion that the left and right sides of the brain may be differentially sensitive to steroids.

Because DHT treatment maintained several behaviors related to sexual arousal (NCEs and USVs) and the size of neurons in the MePD, it is possible that the increased soma size in this region is directly responsible for the maintained behaviors. However, E2 treatment, which had no effect on NCEs or USVs also maintained soma size, which suggests that increased soma size per se is not sufficient to maintain the behaviors, or that different populations of neurons increase soma size depending on whether E2 or DHT is administered. In other words, there might be a subset of MePD neurons that respond solely to DHT and mediate some aspects of sexual arousal, while another subset of MePD neurons respond solely to E2 and do not contribute to NCEs or USVs. If different subsets were responding to the two steroids, one would expect that the mean size of neurons sampled from this region would be greatest in animals receiving both steroids, which is what we found (Fig. 4A). However, it is also possible that E2 and DHT affect the same population of neurons, and that the two steroids together increase the size of those neurons more than either alone.

Interestingly, both E2 and DHT treatments increased another index of male arousal (duration of nosepokes), so it is conceivable that the increase in MePD soma size induced by each steroid contributes to maintaining this behavior. There is also an interesting dissociation between these measures of sexual arousal and neural morphology, as the DHT treatment that maintained all three measures (NCE, USV, and nosepoke duration) had no discernible effect on the volume of the MePD. So it seems unlikely that DHT maintains these behaviors by acting upon MePD volume. On the other hand, E2 was the only treatment that maintained MePD volume, albeit only in the left hemisphere, so estrogenic maintenance of this structural characteristic may contribute to some male behaviors (e.g., see Table 2).

Individual differences in brain and behavior

Sham castrates with large somata in the right MePD displayed more NCEs in the first test than those with smaller somata. Larger neurons have a greater surface area and thus may integrate more sensory inputs than smaller neurons. This may have caused the difference in performance when the rats were unfamiliar with the testing chamber. By the second test, increased familiarity with the testing apparatus among the shams may have masked the contribution of individual differences in MePD morphology to the behavior.

Among the DHT-treated rats, within-group morphological differences predicted the duration of nosepokes. Larger somata among DHT-treated rats were associated with longer nosepoke duration. The effect of DHT on the behavior, and the correlation of a structural measure with a behavior in steroid-treated animals, suggest that MePD structure directly contributes to this measure of sexual interest. This association is also consistent with the role of the MePD in pheromonal communication, as nosepokes likely reflect the male’s interest specifically in airborne cues emanating from the estrous female.
Conclusion

Interconnections between the MeA and other sexually dimorphic circuits appear to underlie the expression of male sexual behavior in rodents (Newman, 1999). Having previously found that the morphology of one subdivision of the circuit, the MePD, was dependent upon circulating testosterone in adulthood, we now report that stimulation of both ARs and ERs appears to be required for testosterone to maintain MePD morphology fully. Furthermore, both androgenic and estrogenic stimulation appears to be necessary to maintain the entire complement of male sexual arousal thought to be mediated by this brain region. NCEs and 50-kHz USVs, two behaviors presumed to reflect sexual arousal and to involve the MeA, appear to depend solely on circulating androgens per se. But stimulation of either ARs or ERs can maintain the male’s interest in receiving airborne cues from estrous females. Because the same androgen and estrogen treatments that maintained these behaviors also maintained the neural morphology of the MePD, the stereoidal maintenance of MePD morphology may well mediate male sexual arousal in rats. Whether steroids act directly upon the MePD, or act upon some other target to indirectly affect MePD morphology, remains unknown.

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References


Swann, J.M., Newman, S.W., 1992. Testosterone regulates substance P within neurons of the medial nucleus of the amygdala, the bed nucleus of the stria terminalis and the medial preoptic area of the male golden hamster. Brain Res. 590, 18–28.