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Masculine Sexual Behavior Is Disrupted in Male and Female Mice Lacking a Functional Estrogen Receptor α Gene

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Masculine sexual behavior is regulated by testosterone (T). However, T can be metabolized to form estrogens or other androgens, which then activate their own receptors. We used knockout mice lacking a functional estrogen receptor α (ERα) gene to test the hypothesis that, following aromatization, T acts via the ERα to activate normal masculine sexual behavior. After gonadectomy and T replacement, wild-type (WT) male and female mice displayed masculine behavior. However, given the same T treatment, little masculine behavior was displayed by mice of either sex that lack a normal copy of the ERα gene. In particular, the latency to display masculine sex behavior and the number of mount attempts per trial were significantly reduced in the ERα^- mice compared to WT littermates ($P < 0.05$). In addition, we found that in both sexes, ERα^- mice have a smaller cluster of androgen receptor immunoreactivity in the bed nucleus of the stria terminalis. Using adult ERα^- mice we were unable to determine whether these genotypic differences are due to organizational or activational effects. However, it is clear that the ERα plays a key role in the expression of masculine sexual behavior and in the regulation of androgen receptors in a neuronal cell population involved in the display of motivated behaviors. © 1997 Academic Press

Sex steroids control the expression of masculine sexual behavior. Castration eliminates or drastically reduces display of sexual behavior in all mammals studied (Meisel and Sachs, 1994). The primary sex steroid secreted by the testes is testosterone (T). Treatment with T after castration reinstates expression of masculine sexual behavior (Meisel and Sachs, 1994). Testosterone acts through several receptors. It can activate the androgen receptor (AR) directly or after conversion to dihydrotestosterone (DHT) by 5α-reductase (Mooradian, Morely, and Korenman, 1987). Alternatively, T can activate estrogen receptors (ER) after conversion to estradiol (E_2) by the aromatase enzyme (Mooradian *et al.*, 1987). A wealth of research has demonstrated that in male rodents, T stimulates masculine sexual behavior, at least in part, by activation of neural ER (Meisel and Sachs, 1994; Davidson, 1969). In female mice and rats, T treatment will stimulate mounting of receptive females and pelvic thrusting at frequencies equivalent to that exhibited by males (Edwards and Burge, 1971; Manning and McGill, 1974; Baum, Sodersten, and Vreeburg, 1974). It is unclear, however, whether T activates this masculine sexual behavior in females via ER.

Until recently, it was assumed that there was only a single ER, which, after binding to its ligand, altered transcription of specific target genes. This ER has been cloned, sequenced, and well characterized in several species (e.g., Green, Walter, Kumar, Krust, Bornert, Argos, and Chambon, 1986). However, a second ER (designated ERβ) has been discovered (Kuiper, Enmark, Pelto-Huikko, Nilsson, and Gustafson, 1996; Mosselman, Polman, and Dijkema, 1996) and, in the rat, is present in brain (Kuiper *et al.*, 1996; Li, Schwartz, and Rissman, 1997). Several isoforms of the ERα have been identified (Friend, Ang, and Shupnik, 1995; Skipper, Young, Bergeron, Tetzlaff, Osborn, and Crews, 1993). Finally, E_2 can exert very

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rapid effects in some brain regions, suggesting the presence of a membrane-bound ER (Ramirez, 1992). We used knockout mice with a disrupted ERα gene (Lubahn, Moyer, Golding, Couse, Korach, and Smithies, 1993) to test the hypothesis that the ERα is required for the expression of masculine sexual behaviors in male and female mice. In addition, we quantified androgen receptor (AR) immunoreactivity in the brains of WT and ERα^- mice to assess possible indirect effects of the lack of ERα on the expression of AR.

MATERIALS AND METHODS

Animals

Mice were generated by pairing heterozygotic animals and genotyping their offspring by PCR analysis of tail DNA. Mice were genotyped using a modification of previously published methods (Lubahn *et al.,* 1993). Homozygotic mice, either wild-type (WT, +/+) or knockout mice which possessed two copies of the disrupted ERα gene (ERα^-, −/−), were used in these studies. At weaning, each mouse was housed individually. Mice received *ad lib* water and Purina mouse chow and were maintained on a standard LD 12:12 cycle (lights off at 1300 EST).

Between 45 and 50 days of age mice were gonadectomized under general anesthesia (100 mg/kg ketamine and 10 mg/kg xylazine). At the time of surgery a 4- to 5-mm-long Silastic-R (Dow Corning) implant (0.98-mm id, 2.08-mm od) filled with T and cholesterol (diluted 1:1) was implanted subcutaneously (sc) in the back of the neck between the shoulders. The plasma levels of T achieved with these implants is at the low end of the physiological range for a WT male (Wersinger and Rissman, unpublished observations).

Behavioral Testing

Two weeks later, sex behavior tests were conducted during the dark phase of the light/dark cycle. The behavior tests were conducted in an 18 × 30-cm clear Plexiglas testing cage. The cage was placed on a ventral viewing stand so intromissions could be accurately quantified. All tests were conducted under red light illumination (between 1600 and 0100 h). Stimulus females were ovariectomized heterozygotes implanted sc with estradiol (50 μg/0.025 ml sesame oil in a Silastic implant; 1.98-mm id, 3.17-mm od). Three to five hours before testing stimulus females received a sc injection of 500 μg progesterone.

All the subjects were sexually naive at the time of the first test. Each subject was placed alone in the testing cage and allowed to acclimate for 1 h. The stimulus females were placed in the cage and the latency to mount and number of mounts, mounts with thrusts, intromissions, and ejaculations were recorded. Males ($n = 8$ −/− and 10 +/+) were given a series of 4-h tests conducted at three-day intervals until they ejaculated or until they had received four tests (whichever occurred first). If a male never exhibited a given behavior, the latency was recorded as 960 min (240 min/test × four tests). Females ($n = 10$ −/− and 10 +/+) were given a series of 1-h tests at a similar interval until they exhibited mounting behavior with pelvic thrusting or until they had completed four tests (whichever occurred first). The maximum latency was 240 min for females.

Immunocytochemistry

To examine the distribution and density of AR immunoreactivity in the brains of WT and ERα-minus mice a second group of male ($n = 5$ −/− and 6 +/+) and female ($n = 6$ −/− and 5 +/+) mice was gonadectomized as above. At the time of surgery a 3-mm-long Silastic implant (0.98-mm id, 2.08-mm od) filled with T and cholesterol (diluted 1:1) was implanted (sc). Three weeks after gonadectomy and implantation, the mice were deeply anesthetized and perfused briefly with heparinized saline followed by 5% acrolein in 0.1 M sodium phosphate buffer. The brains were quickly removed, cryoprotected in 20% sucrose overnight, and frozen in chilled *N*-methyl butane. Fifty-micrometer transverse sections were cut in the coronal plane in a freezing microtome and collected in 0.05 M Tris buffer (Sigma) and 0.15 M NaCl with 1.5% polyvinylpyrrolidone (PVP; average mol wt 40,000; Sigma), pH 7.6.

Every other section was processed for androgen receptors using rabbit anti-androgen receptor IgG (PG21; gift of Geoffrey Greene, University of Chicago) at 0.47 μg/ml (1:2000 diluted) in 0.5% Triton X-100 in TBS (Tris–Triton) with 0.1% gelatin and 2% normal goat serum for 1.5 h at 37°C. Anti-androgen receptor IgG was visualized using VECTASTAIN Elite ABC solutions prepared according to the instructions of the Elite ABC Kit (Vector Laboratories; Burlingame, CA), followed by the glucose oxidase, nickel–diaminobenzidine method, which yielded a black reaction product.

Image Analysis

The area covered by AR-immunoreactive (AR-ir) nuclei in different regions in the brain was estimated in coded sections using computerized image analysis with NIH IMAGE (Version 1.57, developed by Dr. Rasband at the National Institutes of Health). The light intensity and camera settings were kept constant across sections. Areas that were significantly darker than background were visualized using gray-level thresholding. The density of AR immunoreactivity was expressed as the ratio of the area covered by immunoreactive product to the total sampling area (a 0.15 × 0.15-mm square centered in areas with homogenous clusters of AR immunoreactivity). Four consecutive sections were used for the lateral septum, three for the medial preoptic area, and one for the bed nucleus of the accessory olfactory tract.

The density of AR immunoreactivity in the bed nucleus of the stria terminalis (BNST) was too high for a similar analysis because it contained a large cluster of AR immunoreactivity in which the AR-ir nuclei overlapped. Instead, because the AR-ir nuclei could not be distinguished individually, the thresholded image was outlined and measured in four successive sections that spanned the BNST. The volume of this cluster was estimated by multiplying the sum of the area values of the outlined areas by 0.1 mm (the distance between each successive section).

Statistics

Fisher exact probability tests or a Mann–Whitney U test was performed on the behavioral data. Since the length of the sex behavior tests differed between males and females, the behavioral data for each sex were analyzed separately. The histological data were analyzed using two-way ANOVAs.

RESULTS

As shown in Fig. 1, nearly all the WT males mounted, performed pelvic thrusts, intromitted, and ejaculated. By contrast, only 40% of ERα^- males mounted, 25% showed pelvic thrusting, 10% achieved intromission, and none ejaculated (all differences were significant, $P < 0.05$). Similar deficits were seen in T-treated females tested with a receptive female. Nine of the ten WT females exhibited mounting behavior including pelvic thrusting. Yet only 40% of ERα-minus females mounted the stimulus animals, and none showed thrusting behavior (all comparisons were significantly different, $P < 0.05$).

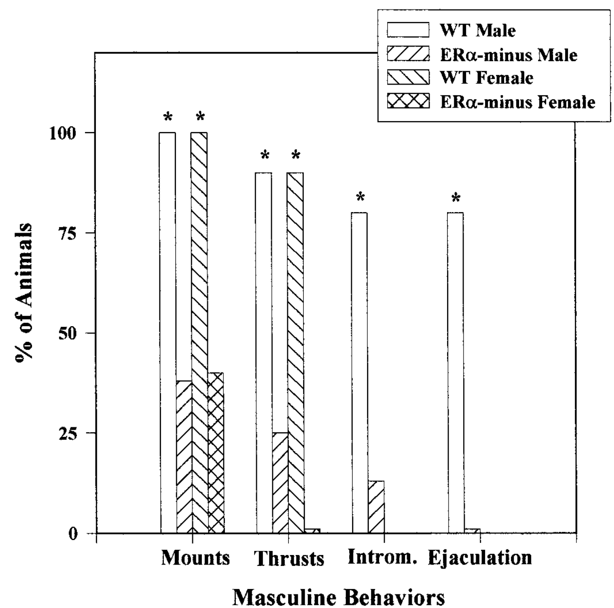

FIG. 1. The percentage of subjects that exhibited mounts, mounts with thrusts (thrusts), intromissions, and ejaculations of male and female wild-type and ERα^- mice. *Significantly different from same-sex ERα^- mice, $P < 0.05$.

The latency to the first episode of mounting ($T = 3.36$, $P < 0.01$ for males; $T = 134.0$, $P < 0.05$ for females), thrusting behavior ($T = 110.0$, $P < 0.01$ for males, $T = 150.0$, $P < 0.01$ for females), intromission ($T = 107.0$, $P < 0.01$), and ejaculation ($T = 108.0$, $P < 0.01$) was significantly longer in ERα^- animals of both sexes than in WT mice (Fig. 2). Likewise, the number of mounts ($T = -2.92$, $P < 0.01$ for males; $T = 69.5$, $P < 0.01$ for females), mounts with thrusts ($T = 46.5$, $P < 0.01$ for males; $T = 60.0$, $P < 0.01$ for females), intromissions ($T = 50.0$, $P < 0.01$), and ejaculations ($T = 108.0$, $P < 0.01$) per test (Fig. 3) was significantly lower in the ERα^- than in WT mice. These data show clearly that masculine sexual behavior, displayed by members of either sex, is severely attenuated by disruption of the ERα gene.

Because activation of both AR and ER is involved in the expression of masculine sexual behavior (Baum and Vreeburg, 1973; Meisel and Sachs, 1994), we examined the neuronal distribution and density of AR-ir. There were no differences in the density of AR-ir in any of the major AR-ir containing regions of the brain, including the lateral septum, medial preoptic area, and the bed nucleus of the anterior olfactory tract among our

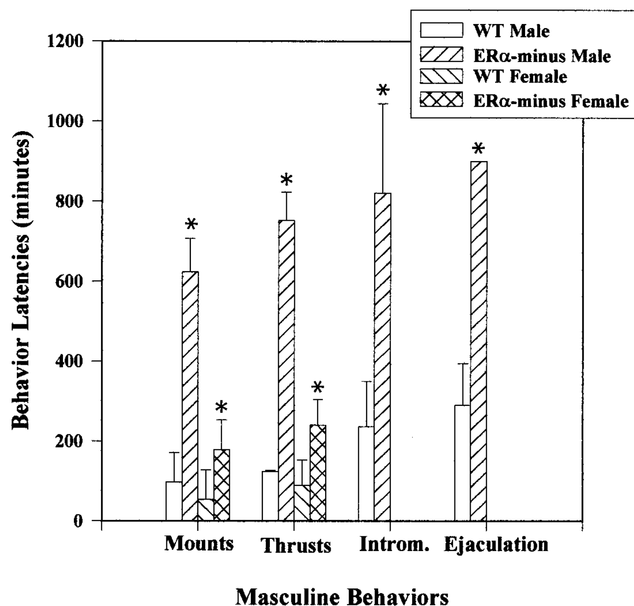

FIG. 2. The latencies (data expressed as mean number of minutes ± SEM) for wild-type (WT) subjects to exhibit masculine sexual behavior in response to a receptive stimulus female of male and female WT and $ER\alpha^-$ mice. *Significantly different from same-sex WT mice, $P < 0.05$.

groups of mice (see Table 1). Although the density of the cluster of AR-ir nuclei in the BNST was too high to be analyzed, there were significant differences in the volume of this cluster. The cluster of AR-ir nuclei was significantly smaller in the BNST of $ER\alpha^-$ animals than of WT littermates ($P < 0.001$, $F(1, 21) = 34.68$). In addition, this cluster was larger in males than in females ($P < 0.001$, $F(1, 21) = 53.61$). This sex difference persisted regardless of genotype (Fig. 4). The mean volumes ± SEM were 0.075 ± 0.002 mm^3 and 0.060 ± 0.002 mm^3 for WT male and female mice and 0.064 ± 0.004 mm^3 and 0.041 ± 0.002 mm^3 for $ER\alpha^-$ male and female mice, respectively.

DISCUSSION

Our data show that the $ER\alpha$ gene is required for the display of masculine sexual behaviors in both male and female mice. Although there are likely to be performance deficits in these mice also (Ogawa, Lubahn, Korach, and Pfaff, 1997; Eddy, Washburn, Bunch, Goulding, Gladen, Lubahn, and Korach, 1996), we propose that the behavioral deficits displayed by the $ER\alpha^-$ mice result primarily from a lack of sexual motivation. When rodents are motivated to mate, but for whatever reason display poor sexual performance, they initially attempt to mate. Yet, when sexual behavior is not reinforced by intromissions or ejaculations, their attempts gradually extinguish (Meisel and Sachs, 1994). In our study, the few $ER\alpha^-$ mice that attempted to mate took significantly longer to display the behavior and showed dramatically less behavior than did the WT animals, suggesting that they are less motivated. Previous lack of reinforcement cannot have biased our results since we used sexually naive animals. When male $ER\alpha^-$ and WT mice are tested for association preferences, $ER\alpha^-$ males spend less time with a female than do WT males (Rissman, Wersinger, Taylor, and Lubahn, 1997). In addition, unlike WT males, $ER\alpha^-$ males do not prefer hormone-primed stimulus females over unreceptive ovariectomized females (Wersinger and Rissman, unpublished observations). Future studies using operant tasks to assess motivation will determine more directly whether the deficit in masculine sexual behavior is due to decreased sexual motivation in the $ER\alpha^-$ mice.

Although we did not include gonadectomized animals without steroid replacement in this study, we can infer from a vast body of research that, especially in sexually naive animals, such a group would show very little masculine sexual behavior (Meisel and Sachs,

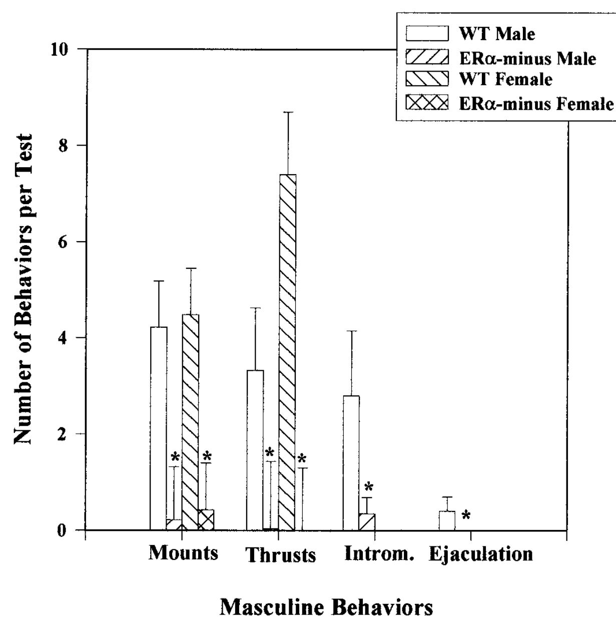

FIG. 3. The mean number (±SEM) of mounts, mounts with thrusts, and intromissions per test of male and female wild-type (WT) and $ER\alpha^-$ mice. *Significantly different from same-sex WT mice, $P < 0.05$.

TABLE 1
Density of Androgen Receptor Immunoreactivity

Region	+/+ Male	−/− Male	+/+ Female	−/− Female
Lateral septum	0.227 ± 0.012	0.230 ± 0.014	0.231 ± 0.008	0.226 ± 0.011
Bed nucleus of the accessory olfactory tract	0.307 ± 0.016	0.326 ± 0.006	0.295 ± 0.010	0.291 ± 0.013
Medial preoptic area	0.331 ± 0.019	0.333 ± 0.029	0.337 ± 0.010	0.363 ± 0.019

Note. Numbers express the ratio of the area covered by androgen immunoreactivity and the total sampling area. There were no significant group differences.

1994) and that the sexual behavior we observed is due primarily to the activational effects of testosterone. Many studies have shown, using pharmacological means, that estrogenic action via the aromatization of testosterone is necessary for testosterone to activate masculine sexual behavior (reviewed in Meisel and Sachs, 1994 and Baum, Carroll, Cherry, and Tobet, 1990; e.g., Christensen and Clemens, 1975; Vagell and McGinnis, 1997). Our data further demonstrate the importance of estrogenic action.

In another study, ERα^- male mice with intact gonads mounted as often as, and latencies similar to those of, WT males (Ogawa *et al.,* 1997). Several major methodological differences between this study and our own may account for the discrepancy in the data. First, gonad-intact, male and female ERα^- mice have significantly elevated levels of circulating hormone levels compared with WT mice (Rissman *et al.,* 1997; Eddy *et al.,* 1997). One reason we selected to test gonadectomized, T-replaced animals was to avoid the confound present in these gonad-intact animals with markedly different levels of circulating hormones. Second, we conducted our behavioral tests in large, neutral test cages, not in home cages. We believe that our testing conditions maximize the detection of genotypic differences in sexual motivation (for a fuller discussion of the differences, see Rissman *et al.,* 1997).

Since the ERα^- mice lack a functional ERα during both development and in adulthood, we do not know whether the differences in behavior and AR immunoreactivity we observe are a result of disrupting estrogen's ''activational,'' and/or ''organizational'' effects. One possible mechanism by which E_2 activates sexual behavior is that it acts directly on neurons that underlie the motivational components of sexual behavior. Indeed, ERα is present in all of the forebrain regions in which the intracranial implantation of T or E_2 activates masculine sexual behavior in rats (Simerly, Chang, Muramatsu, and Swanson, 1990; Christenson and Clemens, 1974; Davis and Barfield, 1979). Another possibility is that ERα up-regulates other steroid receptors, the activation of these receptors could stimulate masculine sexual behavior. Data showing that treatment of castrated male rats with E_2 and DHT, or T, is more effective at restoring sexual behavior than E_2 alone (Baum and Vreeburg, 1973) support this hypothesis. Androgen receptors appear to be induced by E_2 in the brain (Burgess and Handa, 1993; Handa, Roselli, Horton, and Resko, 1987) and many regions that express ERα, including the BNST, also express AR (Wood, Brabec, Swann, and Newman, 1992). Interestingly, about 32% of steroid receptor-containing neurons in the BNST of the male Syrian hamster are immunoreactive for both ER and AR (Wood and Newman, 1995). If ERα induces production of AR, the lack of ERα may account for the reduction in AR immunoreactivity that we noted in the BNST.

The lack of functional ERα during development undoubtedly has effects on the organization of the brain. It is possible that the neural substrate that underlies masculine sexual behavior does not develop normally in the absence of estrogenic action via the ERα and that this abnormal neural substrate does not support the display of masculine sexual behavior. It is unlikely that the reduction in the volume of AR immunoreactivity in the BNST is simply due to a general deficit of the ability of the ERα^- animals to synthesize androgen receptors since the ERα^- animals had normal levels of androgen receptors in other brain regions. Therefore, the absence of ERα during development may have altered the number of cells in the BNST that are capable of making androgen receptors (via apoptosis, for example), rather than changing the level of AR in individual cells in the adult BNST.

The reduction of AR expression in the BNST may contribute to the deficits in masculine behavior noted in the ERα^- animals. Sexual stimuli induce Fos-like immunoreactivity (Fos-ir) in the BNST of rats and mice (Baum and Everitt, 1992; Baum, Brown, Kica, Rubin, Johnson, and Papaioannov, 1994). Mating also aug-

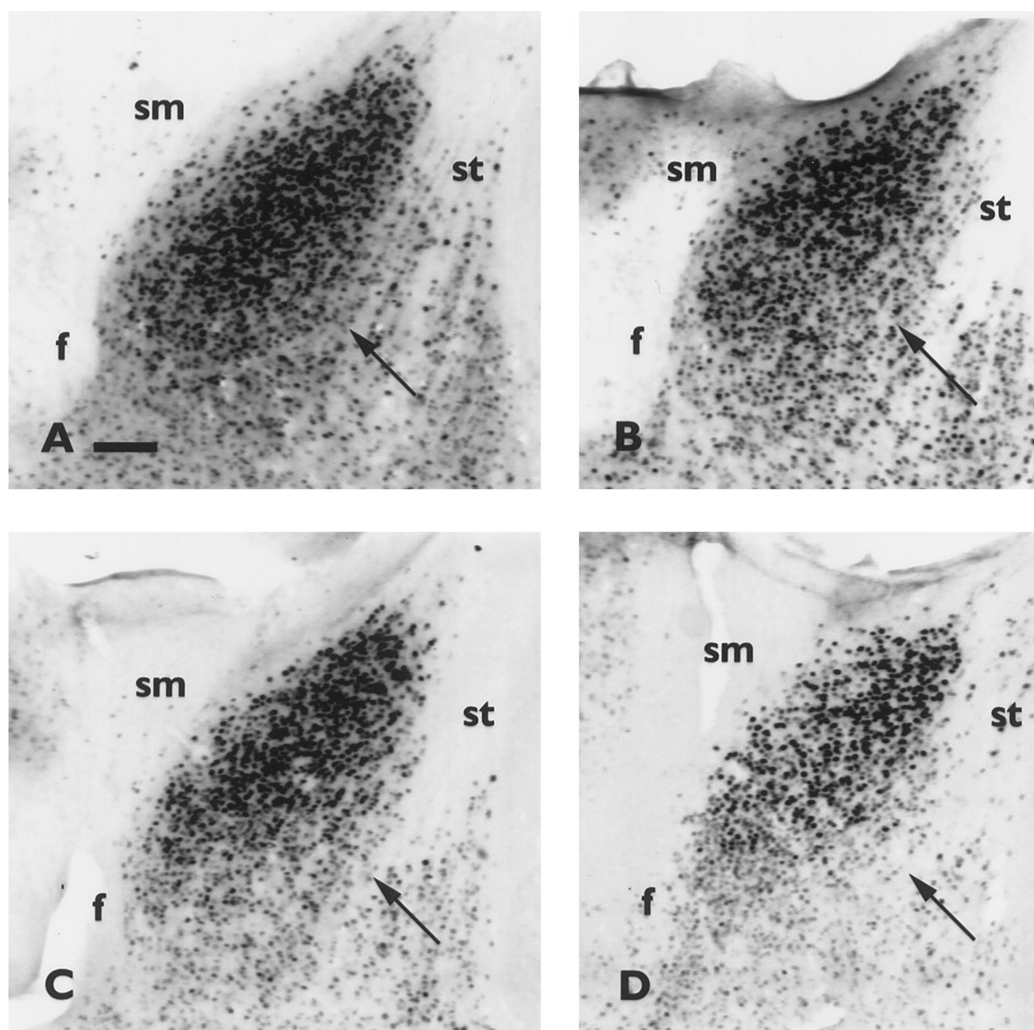

FIG. 4. Clusters of androgen receptor immunoreactive nuclei in the BNST (arrows) of a WT male (A) and female (B) and an ERα$^-$ male (C) and female (D). f, fornix; sm, stria medullaris; st, stria terminalis. Bar, 100 μm. The volumes of these clusters were significantly greater in WT subjects than in ERα$^-$ mice ($P < 0.0001$). It was also greater in male versus female mice ($P < 0.0001$), but there was no significant interaction between sex and genotype.

ments Fos-ir in AR-containing neurons throughout the Syrian hamster brain, including in the BNST (Wood and Newman, 1993). Together, these observations suggest that the BNST may play a role in the steroidal regulation of sex behavior. Perhaps one of the mechanisms by which ERα regulates masculine sexual behavior is via the regulation of AR expression in the BNST.

In contrast to several other mammalian species, female rats and mice show very high levels of masculine sexual behavior, both spontaneously and in response to adult T treatment. It has been suggested that this may be a result of their *in utero* exposure to high levels of androgens, possibly derived from adjacent male fetuses (vom Saal and Bronson, 1980). In our study it is clear that the presence of the ERα has a profound effect on the level of masculine sexual behavior displayed. Neonatal exposure to androgens affects aggressive behavior and masculine sexual behavior in female mice of many (including C57BL and BALB/c strains) but not all strains (Swiss Webster) of mice (Edwards and Burge, 1971; Vale *et al.*, 1973; Rines and vom Saal, 1984). Future studies comparing the level of masculine sexual behavior in steroid-free females of both genotypes treated with either oil or T perinatally will provide insight as to

whether disruption of the ERα prevents organizational and/or activational effects of T.

We have shown that the ERα is essential for expression of masculine sexual behavior in male and in female mice. Our data suggest that the motivational component of masculine sexual behavior relies on normal expression of the ERα. Since only a small percentage of ERα^- animals showed masculine sexual behavior, we could not systematically compare the performance component of masculine sexual behavior. However, the development and function of motoneurons in the spinal cord that innervate the penile musculature are dependent on AR not ER (Breedlove and Arnold, 1981), and preliminary data (Freeman and Rissman, unpublished data) show that ERα^- males have normal penile musculature. Future studies examining *ex copula* penile reflexes could accurately assess sexual performance in males. Finally, we have provided further evidence that the ERα plays a role in the regulation of AR in the brain.

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