Published in final edited form as:

Horm Behav. 2009 August ; 56(2): 254–263. doi:10.1016/j.yhbeh.2009.05.007.

Androgen- and estrogen-independent regulation of copulatory behavior following castration in male B6D2F1 mice

Jin Ho Park^{*}, Paul Bonthuis, Alice Ding, Salehin Rais, and Emilie F. Rissman

Department of Biochemistry and Molecular Genetics and Program in Neuroscience, University of Virginia Medical School, Charlottesville, VA 22908, USA

Abstract

Male reproductive behavior is highly dependent upon gonadal steroids. However, between individuals and across species, the role of gonadal steroids in male reproductive behavior is highly variable. In male B6D2F1 hybrid mice, a large proportion (about 30%) of animals demonstrate the persistence of the ejaculatory reflex long after castration. This provides a model to investigate the basis of gonadal steroid-independent male sexual behavior. Here we assessed whether non-gonadal steroids promote mating behavior in castrated mice. Castrated B6D2F1 hybrids that persisted in copulating (persistent copulators) were treated with the androgen receptor blocker, flutamide, and the aromatase enzyme inhibitor, letrozole, for 8 weeks. Other animals were treated with the estrogen receptor blocker, ICI 182,780, via continual intraventricular infusion for 2 weeks. None of these treatments eliminated persistent copulation. A motivational aspect of male sexual behavior, the preference for a receptive female over another male, was also assessed. This preference persisted after long-term castration in persistent copulators, and administration of ICI 182,780 did not influence partner preference. To assess the possibility of elevated sensitivity to sex steroids in brains of persistent copulators, we measured mRNA levels for genes that code for the estrogen receptor- α , androgen receptor, and aromatase enzyme in the medial preoptic area and bed nucleus of the stria terminalis. No differences in mRNA of these genes were noted in brains of persistent versus non-persistent copulators. Taken together our results suggest that non-gonadal androgens and estrogens do not maintain copulatory behavior in B6D2F1 mice which display copulatory behavior after castration.

Keywords

Male copulatory behavior; Sexual behavior; Partner preference; B6D2F1 hybrid mice; Libido; Androgen receptors; Aromatase; Estrogen receptors

Introduction

A variety of male social behaviors that contribute to successful reproduction and survival is modulated by gonadal steroids. Much evidence shows that these behaviors decline after castration and are restored following hormone replacement (reviewed in Hart (1974), Hull (2006)). Although testicular steroids are essential and castration eliminates male sexual behavior (MSB) in most well studied rodent models, there are a few notable exceptions. These exceptions allow us to delineate the underpinnings of gonadal steroid-independent MSB. Long-term retention of the ejaculatory reflex after castration has been reported in Siberian hamsters (Park et al., 2004) and in the hybrid B6D2F1 mouse (Manning and Thompson, 1976; McGill

^{*}Corresponding author. Department of Biochemistry and Molecular Genetics, PO Box 800733, University of Virginia, Charlottesville, VA 22908, USA. Fax: +1 434 243 8433. jinhopark@virginia.edu (J.H. Park).

and Manning, 1976); ~30% of the males in both rodent species retained the ejaculatory reflex in mating tests conducted 25 weeks after castration (Clemens et al., 1988; Park et al., 2004).

One possible contributing factor to the persistence of MSB may be non-gonadal hormones (e.g., neurosteroids), although adrenal hormones do not play a role in persistent copulation as adrenalectomy does not eliminate copulation in castrated B6D2F1 mice that persist in mating activities (Thompson et al., 1976). In the B6D2F1 hybrid male mouse, one hypothesis is that MSB is maintained after long-term castration by non-gonadal sources of estrogens (Sinchak and Clemens, 1989a; Sinchak et al., 1996). However, hormonal characterization of the hybrid mouse has failed to reveal anything exceptional (Quadagno et al., 1979; Sinchak et al., 1996). Plasma testosterone (T), estradiol (E2), and dihydrotestosterone concentrations and hypothalamic nuclear estrogen receptors (ER) were reduced to the same extent in castrated hybrid mice that continued or ceased to copulate (herein after referred to as persistent copulators and non-persistent copulators, respectively; Clemens et al., 1988; Sinchak et al., 1996). Also, activity of the aromatase enzyme, which converts T to E2, and total ER binding was similar between persistent and non-persistent copulators (Sinchak et al., 1996). In sum, there are no data in support of the hypothesis that persistent copulators have quantifiable differences in any aspect of E2 production or utilization, nor are there any data suggesting that MSB in the B6D2F1 hybrid mouse is maintained by direct actions of steroids in the brain. Alternatively, the neural mechanisms that mediate MSB may be sensitive enough to respond to low concentrations of androgens or estrogens present after castration. To determine whether the low remaining concentrations of non-gonadal steroids mediate persistent copulation, an androgen receptor antagonist, flutamide, an aromatase inhibitor, letrozole, or an estrogen receptor antagonist, ICI 182,780, was administered to castrated B6D2F1 mice which were then tested for male sexual behavior. In addition, we measured mRNA levels for genes that code for the estrogen receptor- α (Esr1), androgen receptor (AR), and aromatase enzyme (Cyp19) in the medial preoptic area (mPOA) and bed nucleus of the stria terminalis (BNST), two integral neural sites known to mediate male sexual behavior.

Social preference behavior is dependent on a combination of chemosensory cues and hormones (Wood and Coolen, 1997; Wood and Newman, 1995). Sexually experienced male rats (Stern, 1970; Paredes et al., 1998) and Syrian hamsters (Fiber and Swann, 1996; Swann, 1997) prefer the odors of estrous females over those of non-estrous females or males. This preference disappears a few weeks after castration, and is restored by T treatment (e.g., Stern (1970) for rats; Gregory et al. (1975) for Syrian hamsters). The loss of this olfactory preference may contribute to the males' reduced mating activity after castration. However, Siberian hamsters that demonstrated persistent copulation were still responsive to sexually salient odors 4 months after castration while this responsiveness was absent in those that were non-persistent copulators (Costantini et al., 2007). To test whether B6D2F1 hybrid male mice demonstrating persistent copulation also exhibit a partner preference of estrous females over males, we observed partner preferences in persistent and non-persistent copulators. In addition, to test whether the persistence of partner preference after castration was mediated by estrogens, we administered ICI 182,780 to B6D2F1 hybrid persistent copulators.

Materials and methods

Animals

Mice used in these studies were weaned at 20–21 days of age, single-sex group-housed until the beginning of each of the experiments (between 50 and 80 days of age), and individually housed afterward for the rest of the experiments. All animals were maintained on a 12:12 light:dark cycle (lights off at 1200 h EST). All of the mice received food (Harlan Diet 8604; Harlan Teklad, Madison, WI) and water *ad libitum* in the University of Virginia Animal Care Facility.

Male B6D2F1 hybrid mice (*Mus musculus*) were derived from crossing C57BL/6J females with DBA/2J males for all experiments other than Experiment 2, in which the mice were all C57BL/6J ($n=18$). One cohort of male hybrid mice ($n=43$) was used for Experiments 1 and 5 and another cohort ($n=50$) for Experiments 3 and 4.

C57BL/6J mice were used as stimulus females for behavioral testing. Females were ovariectomized and injected s.c. with 0.5 μg estradiol benzoate (dissolved in sesame oil) 48 h prior to testing. Three to five hours prior to testing, stimulus females were injected s.c. with 0.83 μg progesterone. The females were group-housed in the same colony room as the experimental males.

All animal procedures were conducted in accordance with our animal protocol, approved by the University of Virginia Committee on Animal Care and Use.

Behavioral testing

MSB was tested under dim red lights during the dark phase of a light/dark cycle (Wersinger et al., 1997). All males were habituated 1 h prior to the introduction of the stimulus female in an 18 $\times$ 30 $\times$ 14 cm clear Plexiglas testing cage containing the male's home cage bedding. The home cages were only changed after the completion of each behavioral test; thus, the Plexiglas testing cage contained either one or two week-old bedding at the time of behavioral testing.

MSB tests began with the introduction of a hormone-treated stimulus female. Once the male mounted, the test continued to a criterion of a successful ejaculatory reflex or for 120 min, whichever occurred first. If the stimulus female became unreceptive during testing she was replaced with another stimulus female.

All tests were recorded and scored at a later time by an observer blind to the classification of the individuals. During each behavioral test, the behavioral components recorded were: mount frequency (MT, the number of mounts not accompanied by an intromission that preceded the ejaculation or the termination of the test), intromission frequency (INT, the number of intromissions that preceded the first ejaculation), mount latency (ML; time from the introduction of a receptive female to the first mount), intromission latency (IL; time from the introduction of a receptive female to the first intromission), and ejaculation latency (EL; interval between the first intromission and ejaculation).

Experiment 1. Effects of long-term exposure to letrozole and flutamide on MSB before and after castration in B6D2F1 hybrid mice

Prior to castration, male B6D2F1 hybrid mice were provided sexual experience by pairing each with a stimulus female weekly for 3 tests. Those that ejaculated on at least two of the three tests were used in this study ($n=43$), and behavioral testing continued on a bi-weekly schedule.

Phase 1—Gonad-intact male hybrid mice were randomly assigned to either the treatment group, which received a 90-day constant release subcutaneous (s.c.) flutamide pellet (Innovative Research, Sarasota, FL; 25 mg/pellet; $n=20$), or the control group, which received a blank silastic capsule similar in size to the flutamide pellet ($n=23$). Male mice were tested three times, bi-weekly, for sexual behavior. The original flutamide pellets were replaced with a new pellet at the time of castration.

Phase 2—After 6 weeks of behavioral testing, males treated with flutamide received either daily s.c. injections of letrozole (10.0 μg /animal in 0.1 ml of 0.3% hydroxyl propyl cellulose in phosphate buffered saline; $n=13$), a non-steroidal aromatase inhibitor that prevents the conversion of T to E2 in brain and adipose tissues without binding to either AR or ER, or

vehicle ($n=7$). In addition, mice with the blank silastic capsules were also treated with either letrozole ($n=11$) or vehicle ($n=12$). This dosage of letrozole is sufficient to significantly reduce circulating E2 levels in mice (Mandava et al., 2001; Tekmal et al., 1999). Pretreatment with letrozole significantly inhibited testosterone-induced exacerbation of seizures in male Swiss–Webster mice, demonstrating letrozole effectively acting in the brain (Reddy, 2004). The effects of letrozole have also been shown to be extremely effective when administered s.c. at doses as low as 5–10 μg per day (Lu et al., 1998, 1999). Tests for sex behavior continued bi-weekly for an additional 3 tests over 6 weeks with letrozole or saline injections 15 min prior to the introduction of the female.

Phase 3—After 12 weeks of behavioral testing through Phases 1 and 2, all the males treated with flutamide and/or letrozole were castrated while control mice treated with the blank capsule and vehicle injections were either castrated ($n=7$) or sham-castrated ($n=5$). Treatments during Phase 2 continued for all mice during Phase 3. Male mice were tested bi-weekly for 8 weeks post-operatively. Males that continued to copulate after castration were considered to be persistent copulators if they demonstrated the ejaculation reflex on at least three out of the four tests.

Experiment 2. Effect of flutamide on MSB and peripheral reproductive tissue in C57BL/6J mice

Sexually naive C57BL/6J male mice underwent gonadectomy between 50 and 60 days of age. Eight weeks later, they received either a 90-day constant release flutamide pellet (identical to those used in Experiment 1; $n=9$) or a blank capsule ($n=9$). All males were implanted with silastic capsules (Dow Corning, Midland, MI) filled with crystalline T (1 cm length, 1.02 mm inner diameter, 2.16 mm outer diameter) one week later. Two weeks after T implantation, behavioral tests for MSB were conducted once a week for a total of 4 tests. The day after the last behavioral test, animals were sacrificed and body weight, seminal vesicle, preputial gland, and penis weights were recorded.

Experiment 3. Effect of ICI 182,780 on persistent MSB in B6D2F1 hybrid male mice after castration

Prior to castration, B6D2F1 hybrid male mice were provided sexual experience in four weekly tests. Males that ejaculated on at least 3 of the 4 tests were used in this study ($n=48$). Males were either castrated ($n=38$) or sham-castrated ($n=10$), and tested for male sexual behavior every two weeks beginning one week after castration. Males were considered to be persistent copulators if they demonstrated the ejaculation reflex on at least two of the three tests, including the last test, conducted on weeks 11–15 post-castration.

Chronic intracerebroventricular cannula implantation—Four months after castration, persistent copulators ($n=11$) were anesthetized with a ketamine–xylazine mixture (100 mg/kg ketamine and 10 mg/kg xylazine injected i.p.), and an intracerebroventricular cannula with osmotic pump (ALZET Brain Infusion Kits, Alzet) was implanted into the right lateral ventricle (coordinates: 0.3 mm caudal, 1.0 mm lateral to bregma, and 3.0 mm below the skull surface) for chronic infusions of either ICI 182,780 ($1.5 \mu\text{g}/\text{kg}^{-1}/\text{day}^{-1}$; TOCRIS, Ellisville, MO; dissolved in 0.6% DMSO in CSF; $n=6$) or vehicle (CSF; Harvard; $n=5$). Several studies have shown that ICI 182,780 binds with ERs with a high affinity and is completely devoid of estrogenic activity *in vivo* (Wakeling and Bowler, 1992; Wakeling et al., 1991). The amount of ICI 182,780 we used, constantly infused into the lateral ventricle, has been demonstrated to significantly block estrogen action on ER *in vivo* (Aguado-Llera et al., 2007; Xue et al., 2007). Persistent copulators treated with ICI 182,780, or vehicle, were tested for male sex behavior twice at an interval of one week, 17–19 weeks post-castration. At the

end of the experiment, the animals were euthanized and their brains were rapidly frozen and stored at -80°C . The accuracy of the lateral ventricle cannula implantation was verified.

Experiment 4. mRNA levels of *Esr1*, *AR*, and *Cyp19* of persistent versus non-persistent copulators

Quantitative real-time PCR (qRT-PCR) was used to assess differences in the amount of mRNA in the mPOA and BNST between persistent ($n=3$) and non-persistent copulators ($n=4$) from the Castrate control group from Experiment 1.

Brain tissue preparation—Persistent and non-persistent copulators and age-matched sham-castrated B6D2F1 hybrid mice were sacrificed. Their brains were rapidly frozen and stored at -80°C . Brains were cut into 120 μm thick coronal sections with a Bright cryostat. The brain areas containing the mPOA and BNST were dissected bilaterally from ~8 single sections. Samples were homogenized at room temperature in QIAzol Lysis Reagent (Qiagen) and stored at -80°C until ready to be processed for RNA isolation.

RNA isolation for quantitative RT-PCR—Total RNA was isolated from mouse brain tissues using RNeasy[®] Lipid Tissue Mini kit (Qiagen) as described by the manufacturer's protocol. The quantity (A260) and quality (A260/A280) of RNA were determined with a Bio-Rad SmartSpec Plus spectrophotometer.

qRT-PCR—The cDNA templates were prepared from the total RNA using SuperScript[™] Reverse Transcriptase (RT) (Invitrogen, Carlsbad, CA). The reverse transcription reaction consisting of 1 μg total RNA, 500 ng oligo (dT)_{12–18}, 500 μM each dNTP, 10 mM DTT, 40 U RNaseOUT[™] RNase inhibitor, and 200 U SuperScript[™] RT was incubated at 37°C for 1 h and then heat inactivated at 70°C for 15 min.

Real time-PCR was performed using ABI Prism[®] 7300 Real Time PCR System with Sequence Detection Software version 1.2.3 (Applied Biosystems, Foster City, CA). In a 25- μl PCR reaction volume, 1 ng cDNA was mixed with iTaq[™] SYBR[®] Green Supermix with ROX (Bio-Rad) and 500 nM primers. Separate β -actin endogenous control reactions were used to normalize RNA input. Oligonucleotide primers were designed using consensus sequence from the NCBI genomic alignment database and Primer Express version 2.0 and synthesised by Invitrogen (Carlsbad, CA; Table 1). The real-time PCR conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. After the last PCR cycle, a dissociation melting curve stage was run according to software protocol. Target and endogenous control genes were measured in triplicate for each cDNA sample during each real-time run to avoid inter-sample variance. For each RNA sample a no-reverse transcriptase reaction was run in parallel to cDNA synthesis, and measured by qRT-PCR to control for contamination and genomic amplification. Each qRT-PCR reaction was verified for a single PCR product of expected size with the dissociation melting curve stage, and some samples were checked with gel electrophoresis.

Normalization and quantifications of the genes of interest, and β -actin mRNA were performed with the comparative cycle thresholds (C_T) method as described in the ABIPRISM7700 sequence detection system user bulletin (#2). Validation experiments were conducted to test for equally efficient target and endogenous control gene amplification as described in the user bulletin. All of the primers were at between 90 and 110% efficient for all amplifications.

Experiment 5. Partner preferences of persistent versus non-persistent copulators

Sexually experienced male B6D2F1 hybrid mice were tested for partner preferences one week prior to castration ($n=24$ randomly selected males of the 48 males from Experiment 3). The

stimulus animals were placed under inverted wire pencil holders (Spectrum Diversified Designs, Inc.) at opposite ends of the testing arena (18×30×14 cm clear Plexiglas). All tests were performed during the dark phase of the light–dark cycle under red light. The subjects were placed in the testing arena with empty wire pencil holders at the beginning of each test and allowed to habituate for 10 min. The stimulus mice for the partner preference tests were a gonad-intact B6D2F1 hybrid adult male and an ovariectomized female (C57BL/6J) that was hormonally primed (as described above). When the subject was in physical contact with either the subject or the wire pencil holder, time spent investigating was recorded. The arena and pencil holders were carefully cleaned with a 10% EtOH solution between tests. Intact males ($n=9$), persistent ($n=8$) and non-persistent ($n=6$) copulators were tested again for partner preferences 7 weeks after castration. Mice were considered persistent copulators if they displayed the ejaculatory reflex on all 3 tests prior to partner preference testing.

Persistent copulators from Experiment 3 that were treated with ICI 182,720, were also tested similarly for partner preference after two weeks of treatment with the ER antagonist ~24 h after the last behavioral test for MSB that was described in Experiment 3.

Statistical analysis—Chi-square tests were used to compare differences in the proportion of males displaying copulatory behavior between groups. Repeated ANOVAs were used to analyze the number of mounts and intromissions, as well as mount, intromission and ejaculation latencies of all mice. One-way ANOVAs were used to analyze time spent investigating a female versus a male in the partner preference tests and to analyze mRNA levels of *Esr1*, *AR* and *Cyp19* between persistent and non-persistent copulators. Post-hoc comparisons were conducted using the Fisher Protected Least Significant Difference test where appropriate. Observed differences were considered significant if $p<0.05$. Statistical tests were run using the Statview program (Statview 5; SAS Institute, Cary, NC, USA).

Results

Experiment 1. Long-term exposure to letrozole combined with flutamide fails to block mating behavior before or after castration in B6D2F1 hybrid mice

After randomly assigning males to the 5 different groups in Experiment 1, statistical analysis of MSB during the last behavior test prior to drug treatments revealed no significant differences of mount, intromission, and ejaculation latencies and number of mounts and intromissions among the five groups (data not shown).

Phase 1—Treatment with flutamide for 6 weeks in gonadally-intact B6D2F1 hybrid males did not affect the percentage of males ejaculating when compared with control males ($X^2(1)=0.01$, $p=0.92$; Fig. 1). Of the males that ejaculated, there were no statistical differences in mount, intromission, and ejaculation latencies and number of mounts and intromissions between males were treated with flutamide for 6 weeks and controls (Table 2).

Phase 2—There were no differences in the proportion of gonad-intact hybrid males displaying ejaculations among the groups ($X^2(3)=3.0$, $p=0.39$; Fig. 1). In addition, there were no differences in mount latencies or number of mounts among the males that ejaculated in each treatment group (Table 2). In general, there were very few differences in the latencies to intromit or ejaculate or number of intromissions among the males that ejaculated in the three groups. However, males that ejaculated that were treated with flutamide (for 8 weeks) and both flutamide and letrozole (for 2 weeks) had intromission latencies that were longer than control males ($p<0.05$ for both comparisons) and males treated with letrozole for 2 weeks only ($p<0.05$ for both comparisons); these differences disappeared at the next two time points. In addition, control males had longer ejaculation latencies than males treated with letrozole alone for 6

weeks ($p<0.05$) and more intromissions than the other three groups ($p<0.05$ for all comparisons; Table 2).

Phase 3—Treatment with either flutamide or letrozole separately or treatment of both simultaneously did not effect the proportion of persistent copulators displaying the ejaculatory reflex in tests conducted over the 8 weeks following castration ($X^2(4)=6.6$, $p=0.16$; Fig. 1). The numbers of mounts and intromissions were significantly higher in the sham-castrated males and the untreated castrated males when compared with the three other groups 4 weeks after castration (Table 2). Castrated males and flutamide-treated castrated males displayed longer intromission latencies when compared to sham-castrated males ($p<0.05$ for both comparisons). Castrated males treated with both flutamide and letrozole ($p<0.05$ for both comparisons) 6 weeks after surgery also took longer to ejaculate when compared with the other three groups 8 weeks post-surgery ($p<0.05$; Table 2). A significantly smaller number of mounts was observed in untreated castrated males when compared with letrozole treated castrated males ($p<0.05$) and castrated males treated with both flutamide and letrozole ($p<0.05$) 8 weeks post-surgery (Table 2).

Experiment 2. Administration of flutamide affected male sexual behavior and resulted in diminished peripheral reproductive tissue in castrated C57BL/6J mice implanted with a T-filled silastic capsule

Five weeks after flutamide treatment, the proportion of castrated, T-implanted C57BL/6J demonstrating MSB was reduced in comparison to untreated males, although the statistical analysis did not reach significance (Fig. 2A). After 6 weeks, MSB was recovered in 67% of flutamide-treated males, (Fig. 2A). Of the males that ejaculated, there were no differences in mount, intromission, and ejaculation latencies and number of mounts and intromissions between C57BL/6J males treated with flutamide for 6 weeks and controls (Table 3).

Seminal vesicle and preputial gland weights were significantly lighter in males with a testosterone-filled silastic capsule that were treated with flutamide for 6 weeks ($F_{(1,16)}=11.185$, $p<0.05$ and $F_{(1,16)}=94.337$ $p<0.001$, respectively; Figs. 2B, C). Penile weight, when corrected for body mass, was not effected by the flutamide treatment ($F_{(1,16)}=1.264$, $p>0.05$; Fig. 2D).

Experiment 3. Administration of ICI 182,780 did not affect persistent copulation in B6D2F1 hybrid male mice

Continuous infusion of ICI 182,780 into the right lateral ventricle of B6D2F1 hybrid males that displayed persistent copulation did not affect behavior. The percentage of ICI 182,780 versus vehicle infused males did not differ ($X^2(1)=0.78$, $p=0.38$; Fig. 3). There were no differences in mount, intromission, and ejaculation latencies and number of mounts and intromissions between persistent copulators that received ICI 182,780 and those that received vehicle (Table 4).

Experiment 4. Similar mRNA levels of androgen receptor, estrogen receptor- α , and aromatase enzyme in brains of persistent and non-persistent copulators

The efficiencies of the target amplification and the reference amplification were approximately equal by demonstrating that the absolute value of the slope of log input amount versus ΔC_T for all genes was <0.1 (data not shown). In each of the no-reverse transcriptase reaction, there was no amplification, indicating that there was no genomic contamination in our mRNA isolation.

Persistent copulators were randomly selected from each group; because there were no statistical differences in mRNA levels or components of MSB in the persistent copulators among all of the groups, the groups were collapsed into one group. Levels of AR, Esr1, and Cyp19 mRNA

in the mPOA and BNST did not differ between persistent and non-persistent copulators ($F_{(1,5)}=0.777, p > 0.05$, $F_{(1,5)}=2.483, p > 0.05$, and $F_{(1,5)}=0.033, p > 0.05$, respectively; Fig. 4).

Experiment 5. Partner preference for a receptive female was retained in persistent copulators

Prior to castration, B6D2F1 hybrid males interact with receptive females more than males (Fig. 5A). The preference for investigating females rather than males, which was evident in the sham-castrated males, was retained in persistent copulators after castration and was reversed in non-persistent copulators ($F_{(2,20)}=7.538, p < 0.01$; Fig. 5A). In addition, treatment with ICI 182,780 for two weeks did not change the preference for investigating females over males when ICI-treated and vehicle-treated persistent copulators were compared ($F_{(1,8)}=1.359, p > 0.05$; Fig. 5B).

Discussion

Here we show that persistent copulation after castration is not dependent upon the low concentrations of circulating androgens or estrogens in castrated B6D2F1 hybrid male mice. A high proportion of castrated hybrid mice treated with flutamide, displayed the ejaculatory reflex after eight weeks of treatment. In addition, 40% of castrated hybrid mice treated with flutamide and letrozole displayed the ejaculatory reflex when tested for MSB 8 weeks after castration (Fig. 1). A few components of MSB in a few of the groups in Experiment 1 did reveal a significant difference between groups (Table 2). However, the differences disappeared by the next behavioral test and there were no general trends to indicate that either of the drug treatments impacted MSB. Furthermore, 2 weeks of continuous icv infusions of ICI 182,780 did not block persistent copulation (Fig. 3). We also show that levels of mRNA for AR, Esr1, and Cyp19 in the mPOA and BNST are not different between persistent and non-persistent copulators (Fig. 4). These results support an earlier study in which total ER binding was similar between persistent and non-persistent copulators. In addition, aromatase enzyme activity was uniformly low in the mPOA of castrates, regardless of their ability to mate (Sinchak et al., 1996). In conjunction with the behavioral data, these results suggest that persistent copulation in castrated B6D2F1 males is not dependent upon the remaining low concentrations of circulating non-gonadal androgens and estrogens.

The expression of persistent copulation in castrated B6D2F1 mice has been attributed to non-gonadal sources of estrogens (Sinchak et al., 1996), mostly based on data presented in abstract form (Sinchak and Clemens, 1989a,b). The abstracts described a significant, but not complete, reduction in MSB in persistent copulators implanted with silastic capsules containing an aromatase inhibitor, 1,4,6-androstatrien-3,17-dione (ATD) (Sinchak and Clemens, 1989a,b). However, because there are few details, we cannot speculate on potential variables which may have differed between those experiments and our current study. Perhaps the use of letrozole, rather than ATD, is the cause of the conflicting results. Letrozole, in addition to anastrozole and exemestane, is one of the third-generation aromatase inhibitors that has been used clinically to treat breast cancer and that has been shown to be more potent and more selective at inhibiting aromatase activity than previous aromatase inhibitors (reviewed in Eisen et al. (2008), Miller (2006)). Other methodological differences (i.e. dose, treatment duration, testing conditions, etc.) may explain these discrepancies.

Administration of flutamide and letrozole was not sufficient to block MSB in gonad-intact B6D2F1 hybrid male mice (Fig. 1); however, this does not mean that the doses used were ineffective in blocking male sexual behavior in castrated hybrid males. The concentration of endogenous ligand remaining in circulation is minimal to undetectable in these castrated hybrid mice, regardless of whether they are persistent or non-persistent copulators (Clemens et al., 1988; Sinchak et al., 1996). Moreover, the doses of flutamide, letrozole, and ICI 182,780 used,

which were all based on dosages that have been shown to be highly effective in prior studies, should be more than sufficient to out-compete the very small residual amounts of steroids that would have remained after castration. In addition, the dose of flutamide used in this study was sufficient to block the effects of testosterone on maintaining the weights of peripheral androgen-dependent reproductive tissues in castrated C57BL/6J males implanted with a T-filled silastic capsule (Figs. 2B, C), increasing our confidence in the effectiveness of the dosage of flutamide. There is also evidence demonstrating that although antagonists may have difficulty competing with the endogenous ligands in gonad-intact males, the same dosage becomes effective when given with exogenous ligand in castrates. For example, flutamide treatment was not sufficient to block male sexual behavior in testes-intact rats (Sodersten et al., 1975). But in another study in which castrates had received a similar flutamide treatment along with T implants, the restoration of MSB was blocked (Gladue and Clemens, 1980). Lastly, the dose of letrozole used was 10 fold greater than the highest concentration used in previous studies that demonstrated that letrozole was very effective in preventing the aromatization of testosterone to E2 *in vivo* (Mandava et al., 2001; Tekmal et al., 1999). Pre-treatment with letrozole (100 mg/kg, i.p.) significantly inhibited testosterone-induced seizures in male Swiss-Webster mice (20–30 g), demonstrating letrozole action in the brain (Reddy, 2004). Letrozole have also been shown to be extremely effective when administered s.c. at doses as low as 5–10 µg per day (Lu et al., 1999) or 10 µg per day (Lu et al., 1998).

Five weeks of flutamide treatment in castrated C57BL/6J males implanted with a T-filled silastic capsule resulted in a lower proportion of males demonstrating MSB compared to controls (Fig. 2A). However, this difference disappeared on the following test, raising the possibility that the 90 day flutamide capsule may have started to secrete lower amounts of flutamide after 5 weeks in some of the males. In Experiment 1, males were treated with the flutamide pellets for 7 weeks; however, there were no differences in MSB between the persistent copulators treated with flutamide for 5 weeks and those treated for 7 weeks (Fig. 1; Table 2).

The major mechanism of action for gonadal steroids is by acting on specific receptors which bind DNA and regulate transcription (Mani and O'Malley, 2002; McKenna et al., 1999; Tsai and O'Malley, 1994). In addition, estrogen and androgen receptors can exhibit ligand-independent activation under appropriate conditions (reviewed in Weigel and Zhang (1998)). Also, other factors (i.e. neurotransmitters, growth factors, etc.) may activate steroid hormone receptors through membrane receptors or second messenger pathways even in the absence of steroid hormones (reviewed in Cenni and Picard (1999), Culig et al. (1997)). *In vitro* cells which were once responsive to anti-hormone treatment may develop resistance resulting in an endocrine insensitive phenotype (reviewed in Hiscox et al. (2006)). Although unlikely, it is possible that non-steroidal ligands may bind and activate androgen receptors despite the presence of flutamide. A potential role for any of these factors acting in a ligand-independent manner in the regulation of persistent copulation has yet to be investigated. It is also unlikely that ERs are activated in the presence of ICI 182,720. Due to its steroidal structure and long side-chain, ICI 182,720 induces a conformational change with the estrogen receptor that prevents ER dimerization and leads to the rapid degradation of the ICI-ER complex, ultimately producing loss of cellular ER (Dauvois et al., 1993). Moreover, this is not the case for dopamine activation of progesterin receptors (PR) since PR antagonists and antisense oligonucleotides block cross-talk between PR and dopamine-initiated signaling pathway (Mani et al., 1994).

In addition to MSB, persistent copulators maintained a preference for female odor cues. Our results are in accordance with a recent study in Siberian hamsters. In hamsters, persistent copulators preferred the soiled bedding of estrous females over male soiled bedding or non-soiled bedding, but such preferences were absent in non-persistent copulators (Costantini et al., 2007). Male rat preferences for odors of estrous females disappear three weeks after

castration and are restored by testosterone treatment (Stern, 1970). Thus, considering that tests were conducted 7 weeks after castration, the robust preference of B6D2F1 castrates for the odors of sexually receptive females is notable. It remains to be determined whether the neural response of the olfactory mating circuit to salient female odors differs between persistent and non-persistent copulators. Because administration of ICI 182,720 did not eliminate the preference for female odor cues, it is unlikely that the neural mechanisms that mediate whole-body odor preferences are more sensitive to low concentrations of estrogens from non-gonadal sources of persistent copulators than those of non-persistent copulators. However, because of the small group numbers, the possibility that statistical difference was due to the fact that the experiment may have been underpowered cannot be ruled out. The androgen receptor (AR) may organize normal responses to olfactory cues in mice (Bodo and Rissman, 2007, 2008). Mice carrying the testicular feminization (Tfm) mutation of the AR (Bodo and Rissman, 2007) and female mice treated on the day of birth with dihydrotestosterone, a non-aromatizable androgen, resulted in the demasculinization and masculinization, respectively, of olfactory preferences (Bodo and Rissman, 2008). Thus, it would be of interest to determine the role, if any, of the AR in the persistence of the salience of female odor cues in castrated B6D2F1 that continue to copulate.

An alternative mechanism that may be responsible for continued copulation is dopamine (DA) stimulation. The relationship between mating behavior and DA has been well studied in male rats (reviewed in Hull et al. (2006)). Increases in DA secretion in the mPOA occur when adult male rats and hamsters are exposed to estrous females (Hull et al. 1995, 1997), and pharmacological blockade of DA receptor activation reduces expression of MSB in sexually inexperienced adults (Lumley and Hull, 1999). In sexually experienced castrated rats, administration of the DA agonist apomorphine partially restored MSB (Malmnas, 1976; Scaletta and Hull, 1990). ER- α knockout mice, which usually show little MSB, copulated normally after receiving systemic injections of apomorphine, a classic non-selective DA agonist (Wersinger and Rissman, 2000). Testosterone may mediate the facilitation of MSB by increased DA release in the mPOA (reviewed in Dominguez and Hull (2005)). We are currently investigating whether increased DA release in the mPOA facilitates MSB in long-term castrated B6D2F1 hybrid males that demonstrate persistent copulation.

First generation hybrid mice created by crossing two inbred strains display a high degree of individual genetic variation. Individual differences in sexual responsiveness of B6D2F1 castrates may reflect these genetic differences as well as the influence of environmental variables. For example, intrauterine position is an important environmental factor in determining sexual behavior in adulthood and in responsiveness to gonadal hormones in a number of rodent species (Meisel and Ward, 1981; vom Saal and Bronson, 1978; vom Saal et al., 1983). Another environmental variable that has received a good deal of attention of late is maternal behavior. Maternal care can alter the neuroendocrinology underlying female sexual behavior as well as maternal behavior, and these maternal effects are mediated by epigenetic modifications (Cameron et al., 2008; Champagne et al., 2003). Variation in maternal care may influence the development of the neural circuitry underlying male sexual behavior (Akbari et al., 2008), and thus male sexual behavior in adulthood (Moore, 1984), and studies on epigenetic and genetic contributions to the display of persistent copulation after castration are currently underway.

Acknowledgments

We thank Aileen Wills for her technical assistance. We are also grateful for the comments and suggestions made by the anonymous reviewers in the revision of this manuscript. This work was supported by NIH grant R01MH057759 and a grant from the Mellon Prostate Cancer Center at the University of Virginia (EFR). JHP was supported by the Center for Cellular and Molecular Studies in Research Training grant (T32HD07382) and NIH grant K99HD056041-1.

References

- Aguado-Llera D, Arilla-Ferreiro E, Chowen JA, Argente J, Puebla-Jimenez L, Frago LM, Barrios V. 17Beta-estradiol protects depletion of rat temporal cortex somatostatinergic system by beta-amyloid. *Neurobiol Aging* 2007;28 (9):1396–1409. [PubMed: 16843571]
- Akbari EM, Budin R, Parada M, Fleming AS. The effects of early isolation on sexual behavior and *c-fos* expression in naive male Long–Evans rats. *Dev Psychobiol* 2008;50 (3):298–306. [PubMed: 18335501]
- Bodo C, Rissman EF. Androgen receptor is essential for sexual differentiation of responses to olfactory cues in mice. *Eur J Neurosci* 2007;25 (7):2182–2190. [PubMed: 17419752]
- Bodo C, Rissman EF. The androgen receptor is selectively involved in organization of sexually dimorphic social behaviors in mice. *Endocrinology* 2008;149 (8):4142–4150. [PubMed: 18467440]
- Cameron NM, Shahrokh D, Del Corpo A, Dhir SK, Szyf M, Champagne FA, Meaney MJ. Epigenetic programming of phenotypic variations in reproductive strategies in the rat through maternal care. *J Neuroendocrinol* 2008;20 (6):795–801. [PubMed: 18513204]
- Cenni B, Picard D. Ligand-independent activation of steroid receptors: new roles for old players. *Trends Endocrinol Metab* 1999;10 (2):41–46. [PubMed: 10322393]
- Champagne FA, Francis DD, Mar A, Meaney MJ. Variations in maternal care in the rat as a mediating influence for the effects of environment on development. *Physiol Behav* 2003;79 (3):359–371. [PubMed: 12954431]
- Clemens LG, Wee BE, Weaver DR, Roy EJ, Goldman BD, Rakerd B. Retention of masculine sexual behavior following castration in male B6D2F1 mice. *Physiol Behav* 1988;42 (1):69–76. [PubMed: 3387479]
- Costantini R, Park J, Beery A, Paul M, Ko J, Zucker I. Post-castration retention of reproductive behavior and olfactory preferences in male Siberian hamsters: role of prior experience. 2007;51:149–155.
- Culig Z, Hobisch A, Hittmair A, Peterziel H, Radmayr C, Bartsch G, Cato AC, Klocker H. Hyperactive androgen receptor in prostate cancer: what does it mean for new therapy concepts? *Histol Histopathol* 1997;12 (3):781–786. [PubMed: 9225161]
- Dauvois S, White R, Parker MG. The antiestrogen ICI 182780 disrupts estrogen receptor nucleocytoplasmic shuttling. *J Cell Sci* 1993;106 (Pt 4):1377–1388. [PubMed: 8126115]
- Dominguez J, Hull E. Dopamine, the medial preoptic area, and male sexual behavior. *Physiol Behav* 2005;86:356–368. [PubMed: 16135375]
- Eisen A, Trudeau M, Shelley W, Messersmith H, Pritchard KI. Aromatase inhibitors in adjuvant therapy for hormone receptor positive breast cancer: a systematic review. *Cancer Treat Rev* 2008;34 (2):157–174. [PubMed: 18164821]
- Fiber JM, Swann JM. Testosterone differentially influences sex-specific pheromone-stimulated Fos expression in limbic regions of Syrian hamsters. *Horm Behav* 1996;30 (4):455–473. [PubMed: 9047271]
- Gladue BA, Clemens LG. Flutamide inhibits testosterone-induced masculine sexual behavior in male and female rats. *Endocrinology* 1980;106 (6):1917–1922. [PubMed: 7371596]
- Gregory E, Engel K, Pfaff D. Male hamster preference for odors of female hamster vaginal discharges: studies of experiential and hormonal determinants. *J Comp Physiol Psychol* 1975;89 (5):442–446. [PubMed: 1238437]
- Hart BL. Gonadal androgen and sociosexual behavior of male mammals: a comparative analysis. *Psychol Bull* 1974;81:383–400. [PubMed: 4370053]
- Hiscox S, Morgan L, Green T, Nicholson RI. Src as a therapeutic target in anti-hormone/anti-growth factor-resistant breast cancer. *Endocr Relat Cancer* 2006;13 (Suppl 1):S53–S59. [PubMed: 17259559]
- Hull EM, Du J, Lorrain DS, Matuszewich L. Extracellular dopamine in the medial preoptic area: implications for sexual motivation and hormonal control of copulation. *J Neurosci* 1995;15 (11):7465–7471. [PubMed: 7472498]
- Hull EM, Du J, Lorrain DS, Matuszewich L. Testosterone, preoptic dopamine, and copulation in male rats. *Brain Res Bull* 1997;44 (4):327–333. [PubMed: 9370196]

- Hull, E.; Wood, R.I.; McKenna, K.E. Neurobiology of male sexual behavior. In: Neill, J.D., et al., editors. *Knobil and Neill's Physiology of Reproduction*. 3. New York: 2006. p. 1729-1824.
- Lu Q, Yue W, Wang J, Liu Y, Long B, Brodie A. The effects of aromatase inhibitors and antiestrogens in the nude mouse model. *Breast Cancer Res Treat* 1998;50 (1):63–71. [PubMed: 9802621]
- Lu Q, Liu Y, Long BJ, Grigoryev D, Gimbel M, Brodie A. The effect of combining aromatase inhibitors with antiestrogens on tumor growth in a nude mouse model for breast cancer. *Breast Cancer Res Treat* 1999;57 (2):183–192. [PubMed: 10598045]
- Lumley LA, Hull EM. Effects of a D1 antagonist and of sexual experience on copulation-induced Fos-like immunoreactivity in the medial preoptic nucleus. *Brain Res* 1999;829 (1–2):55–68. [PubMed: 10350530]
- Malmnas CO. The significance of dopamine, versus other catecholamines, for L-dopa induced facilitation of sexual behavior in the castrated male rat. *Pharmacol Biochem Behav* 1976;4 (5):521–526. [PubMed: 951430]
- Mandava U, Kirma N, Tekmal RR. Aromatase overexpression transgenic mice model: cell type specific expression and use of letrozole to abrogate mammary hyperplasia without affecting normal physiology. *J Steroid Biochem Mol Biol* 2001;79 (1–5):27–34. [PubMed: 11850204]
- Mani, S.; O'Malley, B. Mechanism of progesterone receptor action in the brain. In: Pfaff, AADW.; Etgen, AM.; Fahrbach, SE.; Rubin, RT., editors. *Hormones, Brain and Behavior*. Vol. 3. Academic Press; Amsterdam: 2002. p. 643-682.
- Mani SK, Allen JM, Clark JH, Blaustein JD, O'Malley BW. Convergent pathways for steroid hormone- and neurotransmitter-induced rat sexual behavior. *Science* 1994;265 (5176):1246–1249. [PubMed: 7915049]
- Manning A, Thompson ML. Postcastration retention of sexual behaviour in the male BDF1 mouse: the role of experience. *Anim Behav* 1976;24 (3):523–533. [PubMed: 962192]
- McGill TE, Manning A. Genotype and retention of the ejaculatory reflex in castrated male mice. *Anim Behav* 1976;24 (3):507–518. [PubMed: 962190]
- McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev* 1999;20 (3):321–344. [PubMed: 10368774]
- Meisel RL, Ward IL. Fetal female rats are masculinized by male littermates located caudally in the uterus. *Science* 1981;213 (4504):239–242. [PubMed: 7244634]
- Miller WR. Aromatase inhibitors and breast cancer. *Minerva Endocrinol* 2006;31 (1):27–46. [PubMed: 16498362]
- Moore CL. Maternal contributions to the development of masculine sexual behavior in laboratory rats. *Dev Psychobiol* 1984;17 (4):347–356. [PubMed: 6745497]
- Paredes RG, Tzschenke T, Nakach N. Lesions of the medial preoptic area/anterior hypothalamus (MPOA/AH) modify partner preference in male rats. *Brain Res* 1998;813 (1):1–8. [PubMed: 9824656]
- Park JH, Takasu N, Alvarez MI, Clark K, Aimaq R, Zucker I. Long-term persistence of male copulatory behavior in castrated and photo-inhibited Siberian hamsters. *Horm Behav* 2004;45 (3):214–221. [PubMed: 15047017]
- Quadagno DM, McGill TE, Yellon SM, Goldman BD. Neither non-contact exposure nor mating affect serum LH and FSH in male B6D2F1 house mice. *Physiol Behav* 1979;22 (1):191–192. [PubMed: 451033]
- Reddy DS. Testosterone modulation of seizure susceptibility is mediated by neurosteroids 3alpha-androstanediol and 17beta-estradiol. *Neuroscience* 2004;129 (1):195–207. [PubMed: 15489042]
- Scaletta LL, Hull EM. Systemic or intracranial apomorphine increases copulation in long-term castrated male rats. *Pharmacol Biochem Behav* 1990;37 (3):471–475. [PubMed: 2087489]
- Sinchak, K.; Clemens, LG. Expression of ejaculatory reflex in castrated male B6D2F1 hybrid mouse (*Mus musculus*) is reduced by the aromatase inhibitor ATD. *Conference on Reproductive Behavior*; 1989a.
- Sinchak K, Clemens LG. Expression of ejaculatory reflex in castrated male B6D2F1 hybrid mouse (*Mus musculus*) is reduced by the aromatase inhibitor ATD. *Society for Neuroscience*. 1989b

- Sinchak K, Roselli CE, Clemens LG. Levels of serum steroids, aromatase activity, and estrogen receptors in preoptic area, hypothalamus, and amygdala of B6D2F1 male house mice that differ in the display of copulatory behavior after castration. *Behav Neurosci* 1996;110 (3):593–602. [PubMed: 8889004]
- Sodersten P, Gray G, Damassa DA, Smith ER, Davidson JM. Effects of a non-steroidal antiandrogen on sexual behavior and pituitary–gonadal function in the male rat. *Endocrinology* 1975;97 (6):1468–1475. [PubMed: 1204574]
- Stern JJ. Responses of male rats to sex odors. *Physiol Behav* 1970;5 (4):519–524. [PubMed: 5535505]
- Swann JM. Gonadal steroids regulate behavioral responses to pheromones by actions on a subdivision of the medial preoptic nucleus. *Brain Res* 1997;750 (1–2):189–194. [PubMed: 9098544]
- Tekmal RR, Kirma N, Gill K, Fowler K. Aromatase overexpression and breast hyperplasia, an in vivo model—continued overexpression of aromatase is sufficient to maintain hyperplasia without circulating estrogens, and aromatase inhibitors abrogate these preneoplastic changes in mammary glands. *Endocr Relat Cancer* 1999;6 (2):307–314. [PubMed: 10731124]
- Thompson WL, Abeles FB, Beall FA, Dinterman RE, Wannemacher RW Jr. Influence of the adrenal glucocorticoids on the stimulation of synthesis of hepatic ribonucleic acid and plasma acute-phase globulins by leucocytic endogenous mediator. *Biochem J* 1976;156 (1):25–32. [PubMed: 60101]
- Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* 1994;63:451–486. [PubMed: 7979245]
- vom Saal FS, Bronson FH. In utero proximity of female mouse fetuses to males: effect on reproductive performance during later life. *Biol Reprod* 1978;19 (4):842–853. [PubMed: 743525]
- vom Saal FS, Grant WM, McMullen CW, Laves KS. High fetal estrogen concentrations: correlation with increased adult sexual activity and decreased aggression in male mice. *Science* 1983;220 (4603):1306–1309. [PubMed: 6857252]
- Wakeling AE, Bowler J. ICI 182,780, a new antioestrogen with clinical potential. *J Steroid Biochem Mol Biol* 1992;43 (1–3):173–177. [PubMed: 1525058]
- Wakeling AE, Dukes M, Bowler J. A potent specific pure antiestrogen with clinical potential. *Cancer Res* 1991;51 (15):3867–3873. [PubMed: 1855205]
- Weigel NL, Zhang Y. Ligand-independent activation of steroid hormone receptors. *J Mol Med* 1998;76 (7):469–479. [PubMed: 9660165]
- Wersinger SR, Rissman EF. Dopamine activates masculine sexual behavior independent of the estrogen receptor alpha. *J Neurosci* 2000;20 (11):4248–4254. [PubMed: 10818161]
- Wersinger SR, Sannen K, Villalba C, Lubahn DB, Rissman EF, De Vries GJ. Masculine sexual behavior is disrupted in male and female mice lacking a functional estrogen receptor alpha gene. *Horm Behav* 1997;32 (3):176–183. [PubMed: 9454668]
- Wood RI, Newman SW. Integration of chemosensory and hormonal cues is essential for mating in the male Syrian hamster. *The Journal of Neuroscience* 1995;15:7261–7269. [PubMed: 7472480]
- Wood RI, Coolen LM. Integration of chemosensory and hormonal cues is essential for sexual behaviour in the male Syrian hamster: role of the medial amygdaloid nucleus. *Neuroscience* 1997;78 (4):1027–1035. [PubMed: 9174071]
- Xue B, Pamidimukkala J, Lubahn DB, Hay M. Estrogen receptor-alpha mediates estrogen protection from angiotensin II-induced hypertension in conscious female mice. *Am J Physiol Heart Circ Physiol* 2007;292 (4):H1770–H1776. [PubMed: 17142339]

Fig. 1.

Percentages of hybrid mice treated with flutamide, letrozole, or both that displayed ejaculatory behaviors during pre- and post-operative testing. After providing sexual experience, gonadally-intact B6D2F1 hybrid males were treated with either a 90-day constant release flutamide pellet or a blank silastic capsule for 6 weeks during Phase 1. Males that were treated with flutamide during Phase 1 either continued treatment with flutamide or were treated with both flutamide and letrozole in Phase 2. Control males were placed into either a group that received letrozole or a group that did not receive any treatments for 6 weeks during Phase 2. During Phase 3, control males were either sham-castrated or castrated. The males that were receiving flutamide, letrozole or both during Phase 2 were castrated and tested for male sexual behavior during Phase 3. *Significantly higher than all other groups.

Fig. 2.

(A) Percentage of castrated C57BL/6J treated with testosterone and a 90-day constant release flutamide pellet or a blank capsule displaying ejaculatory behaviors. Mean+SE (B) preputial gland mass (C) seminal vesicle mass, and (D) penis/body mass ratio of castrated C57BL/6J treated with testosterone and a 90-day constant release flutamide pellet or a blank capsule after 6 weeks of treatment. *Significantly lower than controls.

Fig. 3. Percentage of persistent copulators (those that ejaculated on at least 2 of 3 tests from weeks 11 to 15 post-castration) that displayed ejaculatory behavior after constant icv infusions with either the anti-estrogen, ICI 182,780, or vehicle between weeks 17 and 19 post-castration.

Fig. 4. mRNA levels in the medial preoptic area and bed nucleus of the stria terminalis of persistent ($n=3$) and non-persistent ($n=4$) copulators of (A) Esr1, (B) AR, and (C) Cyp19. Samples were taken within 24 h after the last male sexual behavioral test 8 weeks after castration.

Fig. 5.

(A) Mean+SE time spent investigating a receptive female minus time spent sniffing a gonad-intact B6D2F1 hybrid male. Prior to castration, 24 randomly selected B6D2F1 hybrid males were tested for partner preference. Groups were composed of sham-castrated males ($n=9$), persistent ($n=8$) and non-persistent ($n=6$) copulators that were tested for partner preference 7 weeks after surgery. *Significantly different than all other groups. (B) Mean+SE time spent investigating a receptive female minus time spent sniffing a gonad-intact B6D2F1 hybrid male. Groups were composed of persistent copulators that were tested for partner preference 19 weeks after surgery after treatment with either ICI 182,780 or vehicle for 2 weeks.

Table 1Forward and reverse nucleotide sequences of *Esr1*, *AR*, *Cyp19* and β -actin primers.

Gene	Accession #		Nucleotide sequence (start location)
<i>Esr1</i>	NM_007956	Forward	5'-AATTCTGACAATCGACGCCAG (650)
		Reverse	5'-GTGCTTCAACATTCTCCCTCCTC (994)
<i>AR</i>	NM_013476	Forward	5'-GGAGAACTCTTCAGAGCAAG (1553)
		Reverse	5'-AGCTGAGTCATCCTGATCTG (446)
<i>Cyp19</i>	NM_007810	Forward	5'-ACCTCGGGCTACGTGGATGTG (582)
		Reverse	5'-GATGTTTGGTTTGATGAGGAGAGC (740)
β -actin	NM_007393	Forward	5'-CCAGATCATGTTTGAGACCTTCAA (439)
		Reverse	5'-CCAGAGGCGTACAGGGATAGC (519)

Table 2

Mean $\pm$ SE of mount latencies (ML), intromission latencies (IL), and ejaculation latencies (EL) and number of mounts (MT) and intromissions (INT) of B6D2F1 hybrid male mice that ejaculated from Experiment 1 (n =sample number).

	<i>n</i>	ML	$\pm$ SE	IL	$\pm$ SE	EL	$\pm$ SE	MT	$\pm$ SE	INT	$\pm$ SE
PRE1	32	358.8	74.3	450.0	120.3	1196.3	162.4	24.1	2.5	221.8	22.1
PRE2	44	243.8	61.3	129.0	68.5	626.5	105.6	11.7	1.4	155.8	18.7
PRE3	43	108.6	5.8	22.5	5.8	203.4	29.5	6.1	0.7	91.2	9.9
2	20	55.5	4.2	47.3	8.0	347.0	105.1	8.1	2.1	108.0	26.8
	24	88.4	21.8	63.7	28.7	437.7	117.1	6.7	1.7	78.0	19.1
4	17	152.9	32.8	102.9	47.4	142.5	24.1	5.7	1.1	61.8	4.8
	24	198.1	46.6	95.4	40.8	220.3	48.6	6.5	1.3	75.8	10.5
6	19	49.7	5.1	45.8	17.8	417.4	130.1	14.8	2.6	96.7	15.1
	22	53.1	6.1	65.0	15.3	606.0	116.7	21.8	3.6	83.2	14.5
8	10	163.3	29.3	76.2 ^a	28.2	393.3	110.5	15.4	4.0	135.3	33.3
	12	97.9	16.0	37.1 ^a	13.2	616.3	236.5	10.4	2.1	66.4	4.9
	12	122.5	28.7	149.5	57.6	405.7	133.9	12.8	2.9	94.5	18.5
	7	139.4	43.2	564.6	424.1	453.9	152.8	19.7	4.2	170.7	51.9
10	11	129.6	71.2	41.4	21.0	738.7	279.6	18.2	3.1	134.5	20.4
	13	78.2	13.0	56.7	18.7	498.9	142.6	19.6	3.6	84.9	13.6
	11	61.3	10.4	26.1	9.1	730.5	209.7	23.9	5.9	134.5	27.5
	5	159.0	55.4	91.2	41.2	699.8	213.7	28.2	5.3	134.6	39.6
	11	44.5	6.6	90.6	44.9	1195.3	307.7	32.3	8.2	240.6	77.6
12	11	40.2	4.9	163.8	111.9	452.4 ^b	140.7	22.3	3.9	96.5 ^b	27.3
	13	33.1	3.3	92.1	40.1	664.6	198.7	20.9	5.0	97.3 ^b	25.5
	6	43.2	4.4	258.5	147.8	454.5	197.4	21.8	3.9	43.0 ^b	6.8
14	6	78.5	18.6	1195.7	1108.3	974.7	446.2	16.5	3.9	57.5	11.6
	5	60.0	1.8	709.4	316.4	433.2	150.2	17.8	3.3	75.4	29.3
	4	82.5	27.3	97.5	29.5	377.8	208.5	13.8	6.8	100.3	35.2
	9	47.8	5.0	1042.7	533.9	747.2	331.3	23.4	9.4	84.0	32.0
	4	85.5	9.6	1456.5	437.9	144.3	114.9	15.8	3.1	28.8	5.1
16	7	85.1	19.5	893.6	411.6	964.3	295.7	11.7 ^c	1.5	64.7 ^c	10.5

		<i>n</i>	ML	±SE	IL	±SE	EL	±SE	MT	±SE	INT	±SE
18	Castrate control	4	151.5	69.4	1114.8	455.8	847.3	456.3	19.5	6.7	143.0	58.1
	Sham control	5	75.6	9.3	75.4	31.1	1000.8	307.2	29.8	8.4	173.0	65.7
	Flutamide and letrozole castrate	8	62.0	11.3	1152.1	540.1	1182.6	230.3	13.5 ^c	2.4	61.8 ^c	19.4
	Flutamide castrate	4	85.5	15.4	446.0	234.6	1819.5	600.8	13.5 ^c	1.0	35.3 ^c	6.4
	Letrozole castrate	5	72.0	17.3	445.0	252.8	1483.4	559.5	16.8	5.4	58.0	9.3
20	Castrate control	4	55.0	10.6	1562.8	785.9	1061.3	394.9	20.0	3.0	108.3	62.4
	Sham control	5	78.0	5.4	31.4 ^d	9.9	491.4	247.0	13.4	4.3	119.0	34.3
	Flutamide and letrozole castrate	6	62.3	17.4	281.3 ^d	112.6	913.8	379.9	16.7	3.6	51.3	5.2
	Flutamide castrate	4	53.8	4.1	1258.8	532.4	993.5	445.8	15.0	2.7	57.8	6.3
	Letrozole castrate	5	61.6	19.1	388.8	301.5	968.2 ^e	159.2	15.6 ^f	2.3	75.8	18.6
	Castrate control	2	59.5	37.5	27.5	15.5	4128.5	88.5	32.0	11.0	151.5	94.5
	Sham control	5	100.4	24.6	125.6	50.8	676.4 ^e	202.7	22.6	4.4	113.0	32.7
	Flutamide and letrozole castrate	7	79.7	11.4	314.7	302.4	2668.4	544.0	17.3 ^f	3.4	67.6	7.9
	Flutamide castrate	3	68.7	8.4	595.3	276.8	929.7 ^e	56.6	17.3	3.5	70.3	16.5

Numbers in the first column indicate weeks of treatment (PRE1 indicates first behavioral test prior to treatments). Groups included control 1 (gonad-intact males treated with a blank silastic capsule) and flutamide (gonad-intact males treated with a flutamide pellet), letrozole (gonad-intact males treated with daily injections of letrozole), control 2 (gonad-intact males treated with a blank silastic capsule and daily injections of saline), flutamide and letrozole (gonad-intact males treated with a flutamide pellet and daily injections of letrozole), letrozole castrate (castrated males treated with daily injections of letrozole), castrate control (castrated males treated with a blank silastic capsule and daily injections of saline), sham control (gonad-intact males treated with a blank silastic capsule and daily injections of saline), flutamide and letrozole castrate (castrated males treated with a flutamide pellet and daily injections of letrozole), and flutamide castrate (castrated males treated with a flutamide pellet).

^aSignificantly lower than flutamide group.

^bSignificantly lower than letrozole group.

^cSignificantly lower than sham control group.

^dSignificantly lower than castrate control and flutamide castrate groups.

^eSignificantly lower than castrate control and flutamide and letrozole castrate groups.

^fSignificantly lower than castrate control group.

Mean±SE of mount (ML), intromission (IL), and ejaculation (EL) latencies and number of mounts (MT) and intromissions (INT) of C57/B6j male mice that ejaculated that were treated either with a flutamide pellet (flutamide) or a blank silastic capsule (control) in Experiment 2.

Table 3

	N	ML	±SE	IL	±SE	EL	±SE	MT	±SE	INT	±SE
Week 3	3	245.0	63.6	285.0	66.7	806.3	287.3	18.3	9.6	263.3	86.0
Flutamide	3	251.7	83.0	637.3	338.1	1350.0	499.9	16.3	3.9	180.3	61.0
Week 4	5	171.2	46.2	79.0	21.9	844.2	174.5	25.4	5.4	211.6	46.9
Flutamide	3	594.3	376.3	151.7	85.8	554.0	124.4	14.3	3.0	155.3	30.9
Week 5	5	260.2	80.2	262.8	234.3	1064.2	327.9	16.2	5.1	202.8	31.5
Flutamide	2	375.0	169.0	155.0	76.0	715.5	319.5	19.5	11.5	83.0	38.0
Week 6	5	53.8	13.9	19.2	10.7	1230.4	435.0	31.4	9.2	211.8	39.1
Flutamide	6	506.8	300.2	32.7	14.0	688.0	93.2	22.8	3.6	153.2	14.6

Table 4

Mean $\pm$ SE of mount (ML), intromission (IL), and ejaculation latencies (EL) and number of mounts (MT) and intromissions (INT) of B6D2F1 hybrid male mice that ejaculated that were infused icv with either CSF (control) or ICI 182,720 for one and two weeks from Experiment 3.

7 days icv infusions					14 days icv infusions							
Control			ICI 182,720			Control			ICI 182,720			
<i>n</i>	Mean	±SE	<i>n</i>	Mean	±SE	<i>n</i>	Mean	±SE	<i>n</i>	Mean	±SE	
ML (s)	5	383.4	316.1	6	62.3	22.2	4	180.5	46.5	5	182.4	80.2
IL (s)	4	787.5	746.7	5	519.0	252.8	3	1934.3	1900.4	4	557.5	463.6
EL (s)	4	1590.8	354.3	5	2240.0	459.8	2	539.5	0.5	4	1387.8	326.9
MT	5	23.0	4.5	6	28.8	4.3	5	9.6	3.2	6	9.7	2.5
INT	5	163.0	77.0	6	149.0	69.0	5	39.8	21.1	6	38.5	16.2