0013-7227/98/$03.00/0
Endocrinology

Vol. 139, No. 12
Printed in U.S.A.

Modifications of Testosterone-Dependent Behaviors by Estrogen Receptor-α Gene Disruption in Male Mice*

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ABSTRACT

The role of the α form of estrogen receptor (ERα) gene expression in the regulation of testosterone-dependent male reproductive behaviors was investigated using ER knockout mice (ERKO), which are specifically deficient in functional ERα, but not ERβ, gene expression. Previous studies in gonadally intact ERKO mice revealed that male aggressive behavior was greatly reduced by the lack of a functional ERα gene. In the present study the almost complete suppression of male-typical offensive attacks was further confirmed in ERKO mice that had been singly housed since weaning. Regarding aggression, it was also found that ERα gene disruption virtually abolished the propensity to initiate offensive attacks, even though ERKO mice could elicit attacks from resident C57BL/6J mice as wild-type (WT) and heterozygous littermates. Daily injection of testosterone propionate (TP) was completely ineffective in inducing aggressive behavior in gonadectomized ERKO mice, whereas it successfully restored aggression in WT mice. In contrast, male sexual behaviors, mounts and intromissions, were induced by daily injection of TP in both gonadectomized ERKO and WT mice. In addition to TP, dihydrotestosterone propionate (DHTP) was also effective in restoring mounts in ERKO mice, although DHTP was much more potent in WT mice than in ERKO mice. Neither TP nor DHTP, however, ever induced ejaculation in ERKO mice. These results together with previous findings in gonadally intact ERKO mice suggest that ERα may be responsible for the regulation by testosterone of consummatory, but not motivational, aspects of male sexual behavior. Finally, ERKO male mice retrieved newborn pups placed in their home cage with similar latencies to males of the two other genotypes. During parental behavior tests, however, a higher percentage of ERKO mice (70%) showed infanticide compared with WT mice (35%). The latter result was interpreted as showing that ERα activation by testosterone during the perinatal period may exert a suppressive effect on testosterone-inducible infanticide in adulthood. With respect to three major testosterone-dependent behavioral systems reflecting masculinization, these findings demonstrate three different types of effects due to ERα gene disruption. (*Endocrinology* **139:** 5058–5069, 1998)

IT IS WELL known that testosterone regulates various behavioral and neuroendocrine functions in male mice. In the brain, as in many peripheral tissues, testosterone not only acts through androgen receptors (AR) in its original form or as the 5α-reduced form (dihydrotestosterone), but also partly through estrogen receptors (ER), after being aromatized to estrogen. The relative importance of these two mechanisms in specific reproductive behaviors, *e.g.* sexual, aggressive, and parental, has been studied extensively during the last 3 decades. These studies included the comparisons of potency of testosterone with its 5α-reduced (*i.e.* dihydrotestosterone) or aromatized metabolites (*i.e.* estradiol), testosterone treatment in conjunction with aromatization inhibitors, or specific receptor antagonists for androgen or estrogen receptors.

Recently, however, the second form of ER, ERβ, was cloned (1, 2), and subsequently its messenger RNA (mRNA)/protein was localized in various brain regions (3–5). Although its exact functions and mechanisms of action are not yet determined, it is known to bind to 17β-estradiol with a similar affinity to that of the classical ER (6, 7), now termed ERα. Therefore, our knowledge about ER-dependent actions of testosterone needs to be reevaluated. One direct way is to actively manipulate the gene expression of specific ER through the use of antisense DNA (8) or gene-targeting methods. Especially useful for the latter approach are ERα gene-deficient (ERKO) mice (9, 10), which lack functional ERα, but have ERβ (5, 11) genes. They provide us with a great opportunity to study the role of ERα in a number of different behaviors and in a number of different endocrine conditions. Previously, we have characterized behavioral modifications induced by the lack of functional ERα in gonadally intact male (12) and female (13) ERKO mice. These studies revealed that ERKO male mice almost completely lacked ejaculatory behavior as well as male-typical offensive attacks, whereas some components of male sexual behavior remained intact. In the present study we have examined the effects of ERα gene disruption on male reproductive behaviors by controlling their previous social experience as well as their gonadal conditions, *i.e.* tested after gonadectomy and androgen replacement.

It is well demonstrated that aggressive behavior in male mice can be affected by a number of experiential factors, such as social isolation, previous social experiences, and type of opponents (14). Also, offensive and defensive components of

Received June 16, 1998.
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* This work was supported by the Harry Frank Guggenheim Foundation (to S.O.), the University of Missouri molecular biology program (to D.B.L.), and NIH Grant HD-05751 (to D.W.P.).

male aggressive behavior in mice may have different genetic bases (15, 16). Therefore, previous findings of greatly reduced levels of aggressive behavior in ERKO male mice need to be confirmed in behavioral paradigms and conditions that are sensitive to detect the possible contribution of these factors to behavioral changes induced by ERα gene disruption. In the present study, male mice singly housed since the time of weaning were tested in three different aggressive behavioral paradigms, which enabled us to measure both offensive and defensive components of male aggressive behavior. In addition, we have examined the effects of gonadectomy and subsequent testosterone replacement on aggressive behavior to further confirm insensitivity of ERKO male mice to estrogen as an aromatization product of testosterone.

In contrast to aggressive behavior, gonadally intact ERKO male mice were not completely deficient in their sexual behavior, *i.e.* although they almost never ejaculated (even during extended hour behavioral tests with highly receptive female mice) and showed greatly reduced levels of intromissions, some ERKO mice maintained high levels of mounts (12). These behavioral findings together with the fact that ERKO mice have either slightly (17) or significantly (17a) higher testosterone levels than wild-type (WT) or heterozygous (HZ) mice suggest that mounting behavior may be preserved by AR-dependent and/or ERβ-dependent actions of testosterone in ERKO mice. To determine the contribution of AR in residual mounting behavior in ERKO male mice, the effects of dihydrotestosterone treatment on sexual behavior were tested in the present study.

Parental behavior in mice is also known to be regulated by gonadal steroids in both sexes. Some of the naive male mice show parental behavior (retrieving of newborn pups), whereas a high percentage of mice either ignore or show infanticide. In a number of different strains of mice, it has been demonstrated that infanticide is a sexually dimorphic behavior, in that more males show infanticide than females. Several lines of evidence suggest that these sex differences in infanticide may be regulated by testosterone stimulation not only during adulthood but also during perinatal periods (18, 19). Previously, we have reported that ERα gene disruption greatly modified parental behavior in female mice (13). Parental behavior in gonadally intact ERKO female mice, which was measured as retrieval of newborn pups to the nest, was found to be greatly reduced. In addition, about one third of ERKO female mice showed infanticide, whereas none of the WT females did. The effects of ERα gene disruption on male parental behavior have not been investigated to this date. In the present study, therefore, we aimed to determine the role of ERα in testosterone regulation of parental behavior in male mice. In the first experiment, male mice singly housed since weaning were used, because previous housing conditions may affect parental behavior (20). In addition, as previous copulatory behavior experience (ejaculation) is known to time dependently affect subsequent parental behavior in male mice (21–23), a second set of animals without any sexual experience was tested.

Materials and Methods

Male ERKO mice and their WT and HZ littermates from mixed background of C57BL/6J and 129 (9, 10) were used. They were obtained from two separate breeding colonies, maintained at NIEHS and at the University of Missouri-Columbia. The latter breeding colony originated from the same population as the former, but was maintained separately thereafter. Due to small differences in the maintenance procedures of each breeding colony, genetic backgrounds were not exactly identical between the two groups of mice when they were used for the study. Some mice from the NIEHS were individually housed starting at weaning age (21 days old), whereas others were group housed. Upon arrival at the Rockefeller University (as young adults), all mice were individually housed in plastic cages (30 × 20 × 13 cm) throughout the extent of the studies and were maintained on a 12-h light, 12-h dark cycle at constant temperature (22 C). Food and water were available *ad libitum*. The same mice were used for a number of different behavioral tests. We designed the order of tests, which is illustrated in Table 1, to minimize potential confounds of multiple tests as much as possible. All tests were videotaped and analyzed by observers who were blind to the genotype of the females. Males were tested as gonadally intact, after gonadectomy, or after sc injection of either testosterone or dihydrotestosterone.

A special opportunity derived from obtaining ERKO mice from two separate sources was that we could isolate behavioral changes due to ERα gene loss from other small incidental differences in genetic background, handling during early development, feeding, *etc.* Once the mice arrived at the Rockefeller University, they were all treated identically in all respects described above.

Exp 1

WT (n = 12), ERKO (n = 15), and HZ (n = 17) mice (series 1a and 1b; see Table 1) were singly housed since weaning age at the NIEHS and were transferred to the Rockefeller University as young adults. They were then tested for aggressive behavior in three different paradigms: once in a resident-intruder paradigm toward an olfactory bulbectomized (OBX) Swiss-Webster ((SW)fBR purchased from Taconic Farms, Germantown, NY) male intruder mouse, twice (on consecutive days) in a homogeneous set test paradigm (one ERKO mouse and one HZ mouse were not tested), and once as an intruder against a C57BL/6J male resident mouse (one WT, three ERKO, and one HZ mice were excluded from the analysis because C57BL/6J resident mice were socially inactive during these tests).

Exp 2

WT and ERKO (n = 10/genotype) mice (series 2 mice obtained from the University of Missouri; see Table 1) were tested for aggressive behavior in the resident-intruder paradigm against an OBX Swiss-Webster male mouse three times before gonadectomy and three more times after gonadectomy [days 20, 41, and 63 (GDX 20, 41, and 63)]. After aggressive behavior and sexual behavior were abolished by gonadectomy, mice from each genotype were assigned one of two treatment groups, daily injection (sc) of either testosterone propionate (TP; 250 μg in 25 μl sesame oil) or vehicle (25 μl sesame oil). They were then tested three more times for aggressive behavior on days 3, 7, and 11. To compare the effect of TP treatment on aggressive behavior with that on sexual behavior, mice were also tested for sexual behavior toward sexually receptive female mice, once 45 days after gonadectomy and once after the final aggression test (either the 13th or 14th day of TP or oil treatment).

Exp 3

WT and ERKO (n = 10/genotype) mice (series 3 mice obtained from both the NIEHS and the University of Missouri; see Table 1) were tested for sexual behavior against a steroid-primed female Swiss-Webster mouse before and after gonadectomy (GDX 8, 15, and 22) as well as after daily dihydrotestosterone propionate (DHTP; 200 μg/day; days 6, 13, 22, and 29) treatment. Some of the WT males showed complete recovery of sexual behavior (*i.e.* showed ejaculation) by the second or third tests after DHTP treatment and were not tested thereafter.

Exp 4A

Gonadally intact WT, ERKO, and HZ mice (n = 10/genotype; series 1b mice used in Exp 1, all obtained from the NIEHS; see Table 1) housed

TABLE 1. Experimental design

Series 1 (NIEHS)		
1a: n = 2 WT, n = 5 ERKO, and n = 7 HZ		
1b: n = 10/genotype		
	Standard opponent test in resident-intruder paradigm	Exp 1
	Sexual behavior test	Group 1b only; data not shown
	Homogeneous set tests	Exp 1
	Aggression test against C57BL/6J resident mouse	Exp 1
	Parental behavior test	Exp 4A (group 1b only)
Series 2 (University of Missouri)		
Day 1–4	Parental behavior test	Exp 4B
Day 16–17	Standard opponent aggression test	Exp 2
Day 41–42	Standard opponent aggression test	Exp 2
Day 50–52	Standard opponent aggression test	Exp 2
Gonadectomy		
GDX Day 20	Standard opponent aggression test	Exp 2
GDX Day 30–32	Parental behavior test	Exp 4B
GDX Day 41	Standard opponent aggression test	Exp 2
GDX Day 45	Sexual behavior test	Exp 2
GDX Day 63	Standard opponent aggression test	Exp 2
Daily injection of TP or OIL		
Day 3	Standard opponent aggression test	Exp 2
Day 7	Standard opponent aggression test	Exp 2
Day 11	Standard opponent aggression test	Exp 2
Day 13–14	Sexual behavior test	Exp 2
Series 3 (NIEHS and University of Missouri)		
	Sexual behavior test	Exp 3
Gonadectomy		
Day 8	Sexual behavior test	Exp 3
Day 15	Sexual behavior test	Exp 3
Day 22	Sexual behavior test	Exp 3
Daily injection of DHTP		
Day 6	Sexual behavior test	Exp 3
Day 13	Sexual behavior test	Exp 3
Day 22	Sexual behavior test	Exp 3
Day 29	Sexual behavior test	Exp 3

individually starting at weaning age were tested once for their parental behavior (with two pups).

Exp 4B

Ten WT and 10 ERKO male mice (series 2 mice used in Exp 2, all obtained from the University of Missouri; see Table 1) were tested for parental behavior (with three pups), once before gonadectomy and once 30–32 days after gonadectomy. They were sexually naive during both tests.

Exp 5

WT and ERKO male mice were injected sc with TP (100 μg/day for 21 days), DHTP (200 μg/day for 28 days), or vehicle (sesame oil). They were then perfused, and the brain tissues were processed for immunocytochemical detection of ERα and AR.

Aggressive behaviors

All tests were performed during the dark phase (4–8 h after lights off) of the light-dark cycle. An aggressive bout was defined as a continuous series of behavioral interactions including at least one aggressive behavioral act (see below). Three seconds was the maximum amount of time that could elapse between aggressive behavioral acts to be considered part of the same aggressive bout; if intervals between the occurrences of two aggressive behavioral acts exceeded 3 sec, the two behavioral acts were scored as two separate aggressive bouts. Chasing, boxing, tail rattling, biting, and offensive attack (often accompanied by biting and wrestling), previously shown to be typical for intermale (male *vs.* male) aggression (15, 24), were defined as aggressive behavior acts.

Standard opponent test in resident-intruder paradigm. Each male was tested in his home cage (as a resident) against a group-housed (four or five mice per cage) OBX male Swiss-Webster intruder mouse for 15 min. Expression of aggression in mice is mainly regulated by olfactory cues, and therefore, OBX intruders rarely show aggression. However, as their gonads are intact, they can elicit aggressive behaviors from resident mice (24, 25). By testing against olfactory bulbectomized intruder mice, therefore, aggressive behaviors of resident animals, which were not influenced by any experience of defeat, were measured. For each experimental male, cumulative duration of aggressive bouts, latency to the first aggressive act (900 sec were given to mice that did not show any aggression), number of aggressive bouts with offensive attacks, as well as cumulative duration of sexual behavior by resident mice toward intruder mice (chasing with attempted mounts) were recorded.

Homogeneous set test in neutral cage. Pairs of body weight-matched (±3 g) males from the same genotype were tested in a clean neutral cage (30 × 20 × 13 cm) on 2 consecutive days. They were first placed on either side of the test cage, which was divided in the center by transparent acrylic board. After a 5-min adaptation period, the divider was removed, and males were tested for aggression for 15 min. For each pair, cumulative duration of aggressive bouts, latency to the first aggressive act (900 sec were given to mice that did not show any aggression), and number of aggressive bouts with offensive attacks were scored.

Aggression test against C57BL/6J resident mouse. Each male was introduced into the home cage of an individually housed, body weight-matched (±3 g) C57BL/6J (13–15 weeks old; The Jackson Laboratory, Bar Harbor, ME) male mouse for 15 min. Each aggressive bout was classified as either an offensive (experimental mice were dominant) or a defensive (experi-

mental animals were submissive) aggressive bout, and the cumulative duration of each type of aggression was calculated.

Sexual behaviors

Male sexual behaviors were measured during a 30-min behavioral test with a Swiss-Webster female mouse in the male's home cage during the dark phase (4–8 h after lights off) of the light-dark cycle. All females were ovariectomized and sc injected with 10 μg estradiol benzoate (48 h before the tests) and 500 μg progesterone (4–7 h before the tests) to ensure high sexual receptivity. For each male, the numbers and latencies of mounts, intromissions, and ejaculations were recorded.

Parental behaviors

Males were tested in their home cages for 15 min for the retrieving of pups to their nests during the light phase of the light-dark cycle. On the day before the tests, they were given 1.5 g cotton on their cage top and allowed to make nests. At the beginning of the tests, two or three newborn Swiss-Webster pups (3–7 days of age) were gently placed in the male's home cage at the end farthest from the nest. The number of pups retrieved to the nest and latency to retrieval to the nest of the first and all three pups were recorded. Retrieving of pups was scored only if the male carried the pups inside the nest. If infanticidal behavior (biting of pups) was observed, the behavioral tests were terminated immediately after biting started, and these males were excluded from both subsequent tests and analysis of pup-retrieving behavior data.

Immunocytochemistry for steroid receptors

Mice were deeply anesthetized and perfused transcardially with 1) 100 mM PBS containing 0.1% heparin, pH 7.2; and 2) 4% paraformaldehyde in 100 mM phosphate buffer (PB), pH 7.2. The brains were removed, postfixed in 4% paraformaldehyde in PB, and stored for 24 h at 4 C in PB containing 30% sucrose. Brain tissues were cut at 30 μm on a freezing microtome. Free floating sections were incubated in 1) either anti-ERα (ER21: gift of Dr. G. Greene) or anti-AR (Affinity BioReagent, Golden, CO) antiserum in 50 mM Tris-buffered saline (TBS), pH 7.2, containing 0.5% Triton X-100 and 4% normal goat serum (Vector Laboratories, Burlingame, CA) for 48 h at 4 C; 2) a 1:200 dilution of the biotinylated goat antirabbit secondary antibody (Vector) in TBS containing 0.5% Triton X-100 and 4% of normal goat serum for 120 min at room temperature; and 3) the avidin-biotin complex (Vectastain ABC Elite kit, Vector Laboratories) in TBS containing 0.5% Triton X-100 for 60 min at room temperature. Sections were treated with 0.05% diaminobenzidine and 0.03% hydrogen peroxide in TBS, pH 7.8. Control conditions involved either preadsorption of antiserum with antigen protein or omitting the primary antiserum from the staining procedure.

Statistics

Behavioral data were analyzed by either a two-way ANOVA for repeated measurements for the main effects of genotype, test day, and their interaction or by one-way ANOVAs for genotype differences or test day differences, followed, if applicable, by *post-hoc* pairwise comparisons. Some behavioral data (in which variances were not homogeneous between genotype groups) were analyzed by nonparametric tests (Kruskal-Wallis one-way ANOVA and Mann-Whitney U test for independent samples, or Friedman's ANOVA and the Wilcoxon matched pairs signed ranks test for repeated measurements). Differences in the percentage of animals showing certain behaviors were tested with the χ^2 test, Fisher's exact probability test, or binomial test for related samples with small expected values.

Results

Exp 1: comparisons of aggressive behavior in three different paradigms in gonadally intact males

Aggression toward OBX intruders. Both WT (6 of 12) and HZ (8 of 17) showed aggressive behavior, but none of the ERKO mice did ($\chi^2(2) = 10.65$; $P < 0.01$). Some of the WT (4 of 12) and HZ (7 of 17) mice also showed offensive attacks, but none of the ERKO mice did [$\chi^2(2) = 7.82$; $P < 0.05$]. The mean duration of aggressive behavior [Fig. 1A; H(2) = 9.98; $P < 0.01$], mean number of bouts with offensive attacks [Fig. 1B; H(2) = 7.75; $P < 0.05$], and mean latency to the first aggressive act [Fig. 1C; H(2) = 9.96; $P < 0.01$] were different among the three genotypes. ERKO mice were significantly less aggressive than WT and HZ mice ($P < 0.05$), which were not different from each other. Some mice showed attempted mounts toward intruder mice, but there were no genotype differences in the mean cumulative duration in this behavior [Fig. 1D; F(2,41) = 0.17; P = NS].

Homogeneous set tests. In the first test, only one ERKO pair showed aggression, whereas more than half of the WT pairs (four of six) and HZ pairs (six of eight) showed aggression [$\chi^2(2) = 6.21$; $P < 0.05$]. There were genotype differences in mean cumulative duration of aggressive bouts [Fig. 2A; H(2) = 6.40; $P < 0.05$]. ERKO mice were significantly less aggressive than either WT or HZ mice ($P < 0.05$), which were not different from each other. In the second test, more than half of the ERKO pairs (four of seven) showed aggressive behavior. They were not significantly different from the WT (five of six) and HZ (seven of eight) pairs. The mean duration of aggression of ERKO mice also increased compared with that during the first test ($P < 0.05$), but they were still significantly less aggressive compared with the other two genotypes [Fig. 2A; H(2) = 8.55; $P < 0.05$]. None of the ERKO pairs, however, showed offensive attacks in either test, whereas most of the aggressive pairs of WT (four and five pairs in test 1 and test 2, respectively) and HZ (four and seven pairs in test 1 and test 2, respectively) showed offensive attacks [test 1: $\chi^2(2) = 6.87$; $P < 0.05$; test 2: $\chi^2(2) = 14.02$; $P < 0.001$]. There were also genotype differences in the mean number of attacks [Fig. 2B; test 1: H(2) = 5.85; $P = 0.054$; test 2: H(2) = 12.27; $P < 0.01$]. ERKO mice were distinguishable from the other genotypes. WT and HZ mice showed equal number of attacks in the first test, but HZ mice showed more attacks in the second test than in the first test ($P < 0.05$). No such increase was found in WT mice. Finally, a two-way ANOVA for repeated measurements on the latency data (Fig. 2C) revealed that there were no genotype differences in the mean latency [F(2,18) = 3.14; P = NS]. Latencies, however, were significantly shorter in test 1 compared with test 2 [F(1,18) = 10.70; $P < 0.01$], although paired t tests in each genotype revealed that the differences were significant only in WT mice.

Test against C57BL/6J resident mouse. As expected, C57BL/6J resident mice showed vigorous aggressive behavior toward intruder mice. Some of the intruder mice (*i.e.* ERKO, WT, or HZ), however, were dominant in certain aggressive bouts (Fig. 3A). The mean cumulative duration of these offensive bouts was different among the three genotypes [H(2) = 6.40; $P < 0.05$]. ERKO mice were significantly less aggressive than both WT and HZ mice ($P < 0.05$), which were not different from each other. In contrast, there were no significant genotype differences in mean cumulative duration of defensive bouts [Fig. 3B; F(2,37) = 0.20; P = NS], suggesting that ERKO mice were attacked as frequently as WT and HZ mice by resident C57BL/6J mice.

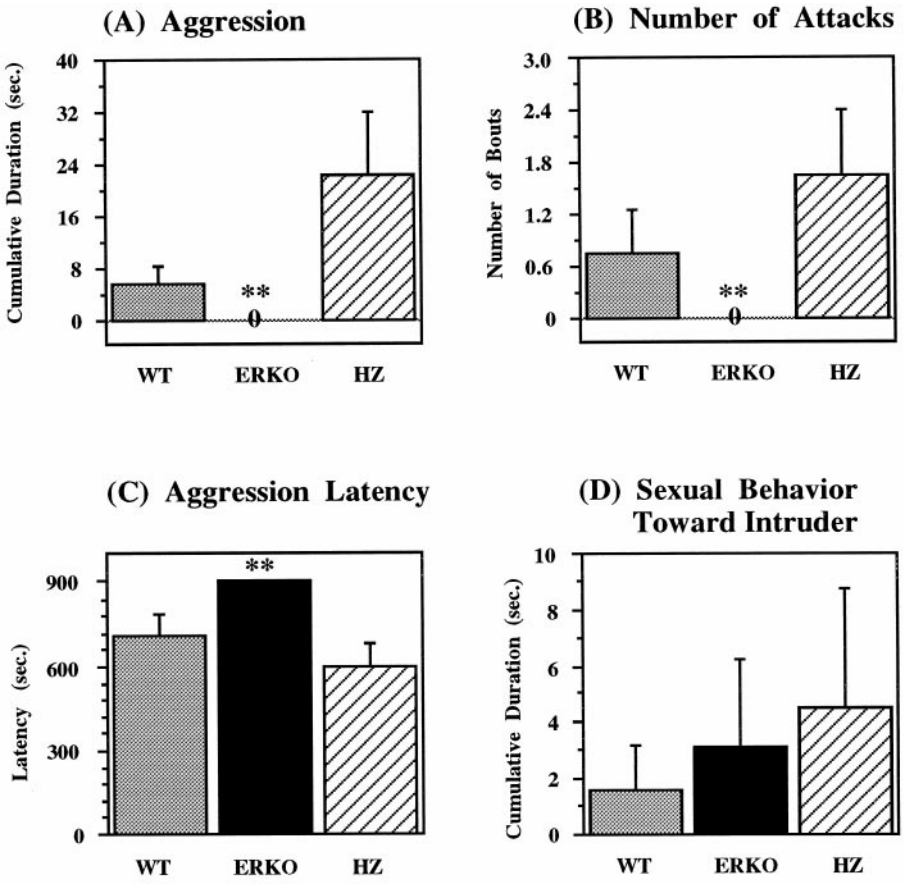

FIG. 1. Genotype differences in the levels of aggressive behavior in the resident-intruder tests. WT (n = 12), ERKO (n = 15), and HZ (n = 17) male mice, which had been singly housed since weaning, were used. The reduction of aggressive behavior by ERα gene disruption was detected in this experiment. ERKO mice were significantly different from both WT and HZ mice (**, $P < 0.05$, by Mann-Whitney U test) in all three measurements of aggressive behavior (A–C), but they showed equivalent levels of sexual behavior toward male intruders.

Exp 2: effects of gonadectomy and replacement dose of testosterone on aggressive behavior

Effects of gonadectomy. WT male mice were much more aggressive than ERKO mice, which rarely showed aggression (Fig. 4A). During three tests performed before gonadectomy, 10%, 30%, and 70% of WT mice showed aggressive behavior, whereas only one ERKO mouse showed aggression (in the third test). Aggressive behavior of WT mice was, as expected, decreased substantially by gonadectomy from the levels seen during intact tests [by Friedman ANOVA, comparison of means of three intact tests, GDX 20, 41, and 63; $\chi^2(3) = 18.80$; $P < 0.01$], and at 63 days, none of the WT mice was aggressive. It was found that ERKO male mice were as socially active during aggressive behavior tests as WT mice. Gonadally intact ERKO and WT mice showed equivalent levels of sexual behavior (attempted mounts) toward intruder male mice (Fig. 4B). This behavior completely disappeared after gonadectomy in both WT [$\chi^2(3) = 13.71$; $P < 0.01$] and ERKO [$\chi^2(3) = 22.77$; $P < 0.01$] mice.

Effects of testosterone. Daily TP injection restored aggressive behavior in WT mice (Fig. 5A), but failed to do so in ERKO mice (Fig. 5B). By 11 days after daily TP injection, WT mice became significantly more aggressive than the levels at GDX 63 ($z = -1.83$; $P < 0.05$, one-tailed). In contrast, sexual behavior, which was abolished after gonadectomy (day 45; data not shown), was induced in TP-treated mice of both genotypes. Thus, compared with the oil-treated group, the TP-treated mice in each genotype tended to show greater number of mounts (Fig. 6A) and intromissions (Fig. 6B). The percentage of mice that showed at least one mount or intromission in the TP-treated groups was 80% in the WT mice and 66% in the ERKO mice, significantly higher than those in the oil-treated groups (Fig. 6C).

Exp 3: effects of gonadectomy and dihydrotestosterone treatment on male sexual behavior

Effects of gonadectomy. The levels of each of three tests after GDX and four tests after DHTP were compared with those of the intact test, using either the two-tailed binomial test for related samples with small expected frequencies (the percentage of mice showing certain behavior) or the two-tailed paired *t* test (mean frequency and latency). Male sexual behaviors, as indicated by the percentage of mice showing mounts, intromissions, or ejaculation (Fig. 7, A–C); the mean frequency of mounts or intromissions (Fig. 7, D and E); and the latency to the first mount (Fig. 7F), were reduced by gonadectomy in both WT and ERKO mice. However, in WT mice, gonadectomy reduced ejaculation and intromissions more quickly and by a larger magnitude, whereas the frequency of mounts was not affected, and 80% of mice still showed mounts at the third test. In ERKO mice, both mounts and intromissions were reduced, and mount latency was increased by gonadectomy, although 20% of mice still showed mounts 22 days after gonadectomy. In a separate group of animals, we tested sexual behavior for a long period of time after gonadectomy (18, 34, and 111 days) and found

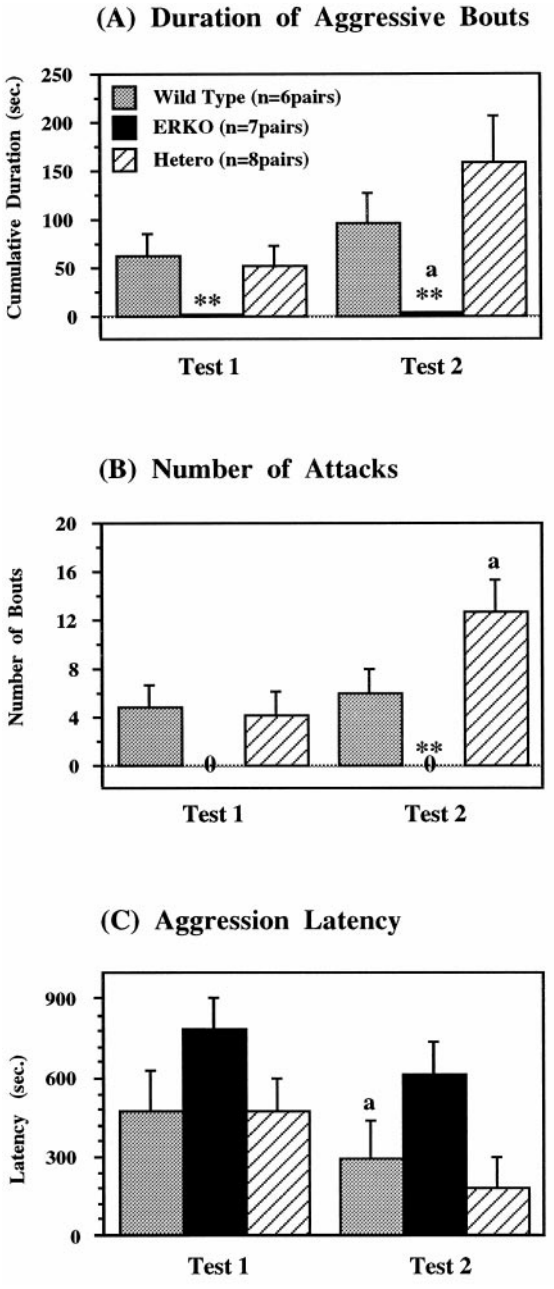

FIG. 2. Genotype differences in the levels of aggressive behavior in the homogeneous set tests. The duration of aggressive bouts (A) and the number of attacks (B) were analyzed using nonparametric tests, as the variances were not homogeneous among the three genotypes. Genotype differences in each test were analyzed by Kruskal-Wallis one-way ANOVA followed by *post-hoc* pairwise comparisons with Mann-Whitney U test (**, $P < 0.05$ *vs.* WT and HZ). Changes between test days were analyzed by paired *t* tests in each genotype (a, $P < 0.05$ *vs.* test 1). Aggression latency data (C) were analyzed by a two-way ANOVA for repeated measurements, followed by paired *t* tests for comparisons between test days in each genotype (a, $P < 0.05$ *vs.* test 1).

that some mice (either HZ or ERKO mice) showed sexual behavior at 34 days, but not 111 days after gonadectomy (data not shown).

Effects of dihydrotestosterone. DHTP restored male sexual behavior in both WT and ERKO mice, although the rate of recovery was much slower in ERKO compared with WT mice. By the time of the second tests (after 2 weeks of daily injection of DHTP), the mean frequency of intromissions and mount latency was completely restored, *i.e.* significantly different from GDX 3 but not from intact levels. Sixty percent of WT mice (*vs.* 40% of intact) ejaculated during 30-min sexual behavior tests. In contrast, the mean mount latency of ERKO mice was still significantly longer than intact levels, and only 1 mouse showed intromissions in the second test. Only after 29 daily injections of DHTP did ERKO mice show recovery of sexual behavior, in terms of the percentage of mice that showed mounts and the mean frequency of intromissions. It should also be noted that none of the ERKO mice ejaculated either intact mice or after DHTP treatment, although some ERKO mice showed high levels of mounts or intromissions.

Exp 4: parental behavior in gonadally intact and gonadectomized male mice

Exp 4A. It was found that 50% of WT, 80% of ERKO, and 60% of HZ mice showed infanticide. Most of the mice that did not show infanticide licked pups frequently but never retrieved pups to the nest, except one WT and one HZ mouse that retrieved all pups given to the nest.

Exp 4B. As found in Exp 4A, a higher percentage of the ERKO mice (60%) showed infanticide compared with the WT mice (20%) before gonadectomy (Fig. 8A). Analysis with combined data (Exp 4, A and B) revealed that ERKO mice (70%) indeed showed higher levels of infanticide than WT mice [35%; $\chi^2(1) = 4.91$; $P < 0.05$]. After gonadectomy, on the other hand, there was no genotype difference. Thus, the percentage of ERKO mice showing infanticide tended to decrease (from 60% to 30%), whereas no such change was found in WT mice. Pup-retrieving behavior was further analyzed by including only the animals that did not show infanticide. These analyses revealed that ERKO males were not significantly different from WT mice in the number of pups retrieved (Fig. 8B), the latency for retrieving the first pup (Fig. 8C), or the latency to retrieve all three pups (data not shown) both before and after gonadectomy.

Exp 5: immunocytochemical studies for steroid receptors in testosterone or dihydrotestosterone-treated mice

Both testosterone and dihydrotestosterone induced AR-immunoreactive cells, which were greatly reduced by gonadectomy, in a number of hypothalamic and limbic areas as well as in midbrain periaquiductal gray in both ERKO and WT mouse brains. As expected, ERα-immunoreactive cells were found in gonadectomized WT mouse brains but not in ERKO mouse brains.

Discussion

In the present study we have described behavioral characteristics of ERKO male mice, which are deficient in functional ERα, but not ERβ, gene expression, to determine the specific role of ERα gene expression in normal development of testosterone-dependent male behaviors. These knockout mice serve as a powerful tool to assess the effects of active

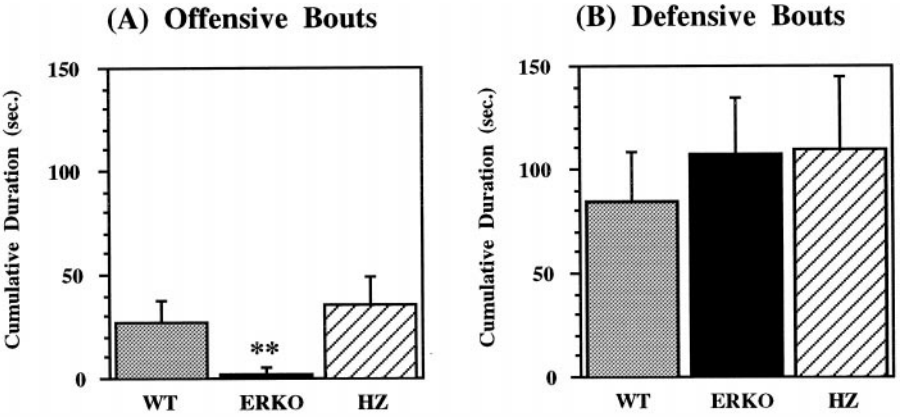

FIG. 3. Results of aggression tests against C57BL/6J resident male mouse. WT (n = 11), ERKO (n = 12), and HZ (n = 15) male mice, which were singly housed since weaning, were used. Genotype differences were detected (by Kruskal-Wallis one-way ANOVA) only in the levels of offensive bouts, but not those of defensive bouts. *Post-hoc* pairwise comparisons with Mann-Whitney U test revealed that ERKO mice were significantly less aggressive than either WT or HZ mice (**, $P < 0.05$).

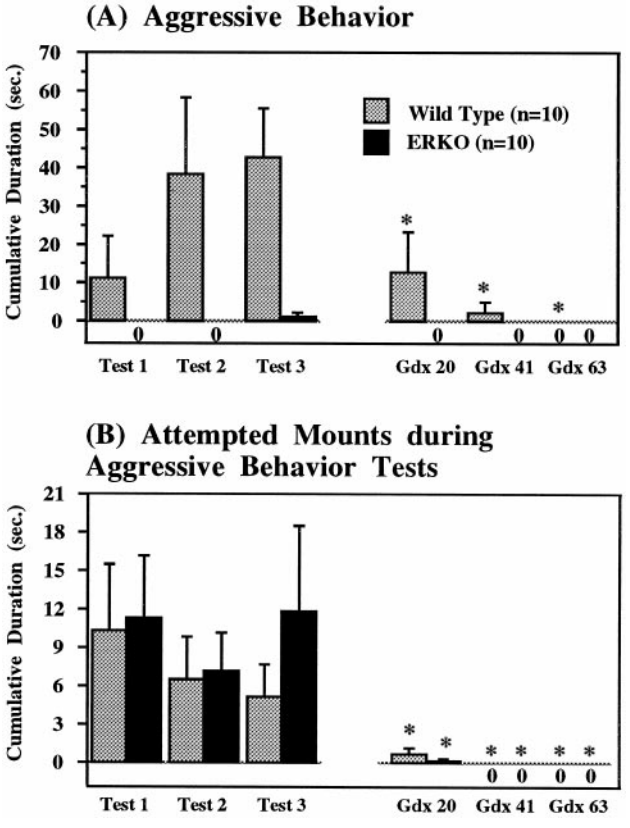

FIG. 4. Effects of gonadectomy on aggressive behavior and attempted mounts toward male intruder during aggression tests. Both behaviors were reduced by gonadectomy. The levels of aggressive behavior and sexual behavior in each of three tests after gonadectomy were compared with those before gonadectomy (average of three tests) using the Wilcoxon matched pairs signed ranks test (two-tailed; *, $P < 0.05$).

manipulation of specific gene expression on a number of different behaviors in a number of different circumstances (*e.g.* endocrine conditions, *etc.*). However, it should also be noted that there are a number of pitfalls for this type of gene manipulation. That is, the effects of gene manipulation by the gene-targeting method are permanent throughout the life of the animal and are global across the entire body of the animal. Furthermore, it is possible that compensating mechanisms may conceal the real effects of specific gene disruption. Findings in the present study may need to be further confirmed in brain site-specific and time-dependent manipulation of gene expression with the use of antisense DNA methods as well as conditional knockout mice.

It should also be noted that the same mice were used for more than one behavioral test in the present study because we had a very limited number of mice during the early stage of behavioral characterization with newly developed knockout mice. As we described above, we carefully designed the order of tests to minimize as much as possible potential confounds of multiple tests. Also, as we tested mice from the three genotypes simultaneously, differential responses, if any, to repeated tests between genotypes can be interpreted as a part of the effect of gene disruption. Nevertheless, it is still possible that some of the behavioral effects of ERα gene disruption could be either over- or underestimated in the present study due to the multiple usage of the same animals. This possibility needs to be tested in future studies.

Aggressive behavior

The present study, which used singly housed mice, has confirmed and extended the previously found effects of ERα gene disruption on male aggressive behavior (12). To all three types of opponents, ERKO male mice rarely showed offensive attacks (the most vigorous form of intermale aggression), whereas both WT and HZ littermates were highly aggressive. Therefore, reduced levels of aggression reported previously in ERKO mice (12) were not due to possible defeat experiences during group housing before aggression tests. In addition, equivalent levels of defensive aggressive bouts found in the present study in ERKO mice, compared with WT and HZ mice, in the tests with resident C57BL/6J male mice suggest that ERKO mice are normal in their ability to elicit aggressive behavior in opponent male mice.

In our previous studies we concluded that reduced levels of aggression in gonadally intact ERKO male mice are not due to differences in plasma levels of testosterone and/or estradiol, which are equivalent (estradiol) or higher (testosterone) in ERKO male mice compared with WT mice (17, 17a, 26). The results from TP-treated gonadectomized mice in the present study further support this idea. Thus, daily injection of TP successfully restored aggressive behavior, suppressed by gonadectomy, in WT mice, but failed to induce any aggression in ERKO mice. In contrast, TP induced sexual behavior in 66% of ERKO mice even though they had very little

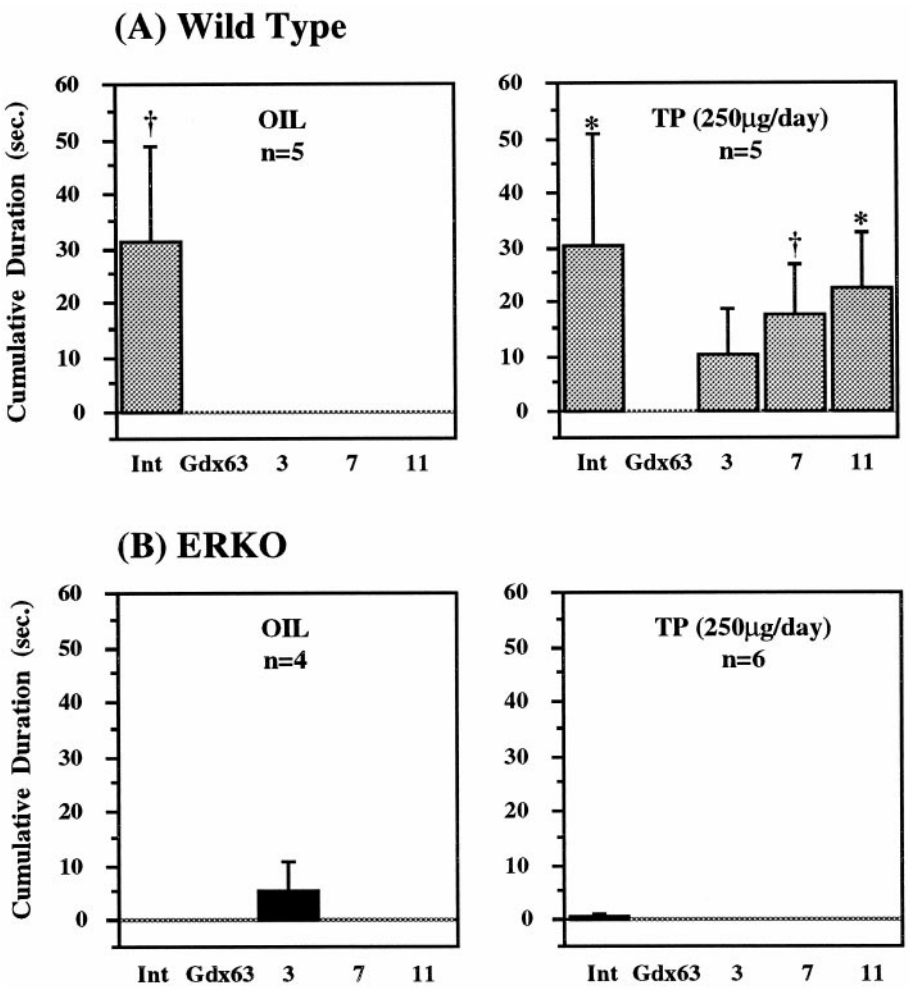

FIG. 5. Effects of TP replacement on aggressive behavior. TP restored aggressive behavior in WT mice, but failed to induce any aggressive behavior in ERKO mice. Levels of aggressive behavior after daily injection of either TP or vehicle (OIL) were compared with those on GDX 63 in each treatment group using the Wilcoxon matched pairs signed ranks test (one-tailed; *, $P < 0.05$; †, $P = 0.054$)

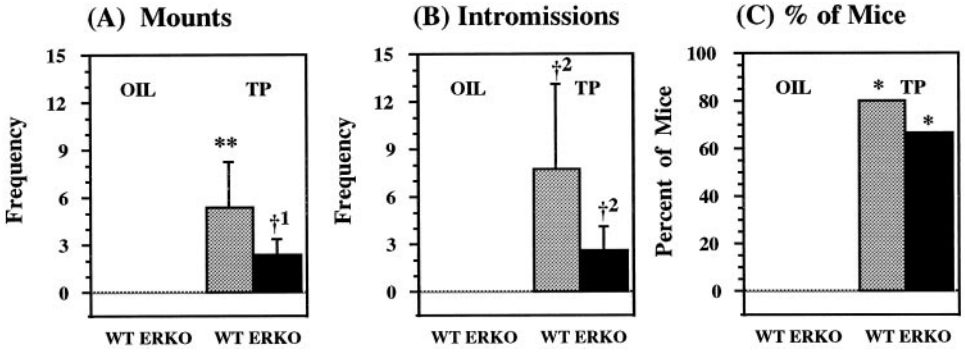

FIG. 6. Effects of TP replacement on sexual behavior. In contrast to its effects on aggressive behavior, TP induced sexual behavior in both WT and ERKO mice. The mean frequency of mounts (A) or intromissions (B) in the TP-treated group were compared with that in the OIL-treated group in each genotype using the Mann-Whitney U test (one-tailed; **, $P < 0.01$; †1, $P = 0.068$; †2, $P = 0.057$). The percentage of mice that showed at least one mount or intromission (C) were tested using Fisher's exact probability test (one-tailed; *, $P < 0.05$ *vs.* oil-treated group). n = 5 for oil-treated and TP-treated WT mouse groups, n = 4 for oil-treated ERKO mouse group, and n = 6 for TP-treated ERKO mouse group.

previous sexual experience. (They were tested only once 45 days after gonadectomy, and none of the ERKO mice showed sexual behavior.) These findings suggest that ERα gene expression may be more crucial for the facilitatory action of testosterone on male aggressive behavior than for other types of reproductive behavior. It is known that both AR and ER may be involved in the induction of aggressive behavior in male mice, and the relative importance of ER- *vs.* AR-dependent mechanisms differs among three lines of outbred mice (27). Therefore, the genetic background of ERKO mice may partially account for the great reduction of aggressive behavior by the lack of functional ERα. However, it is assumed that multiple processes induced by ERα gene disruption may also be involved in the almost complete disap-

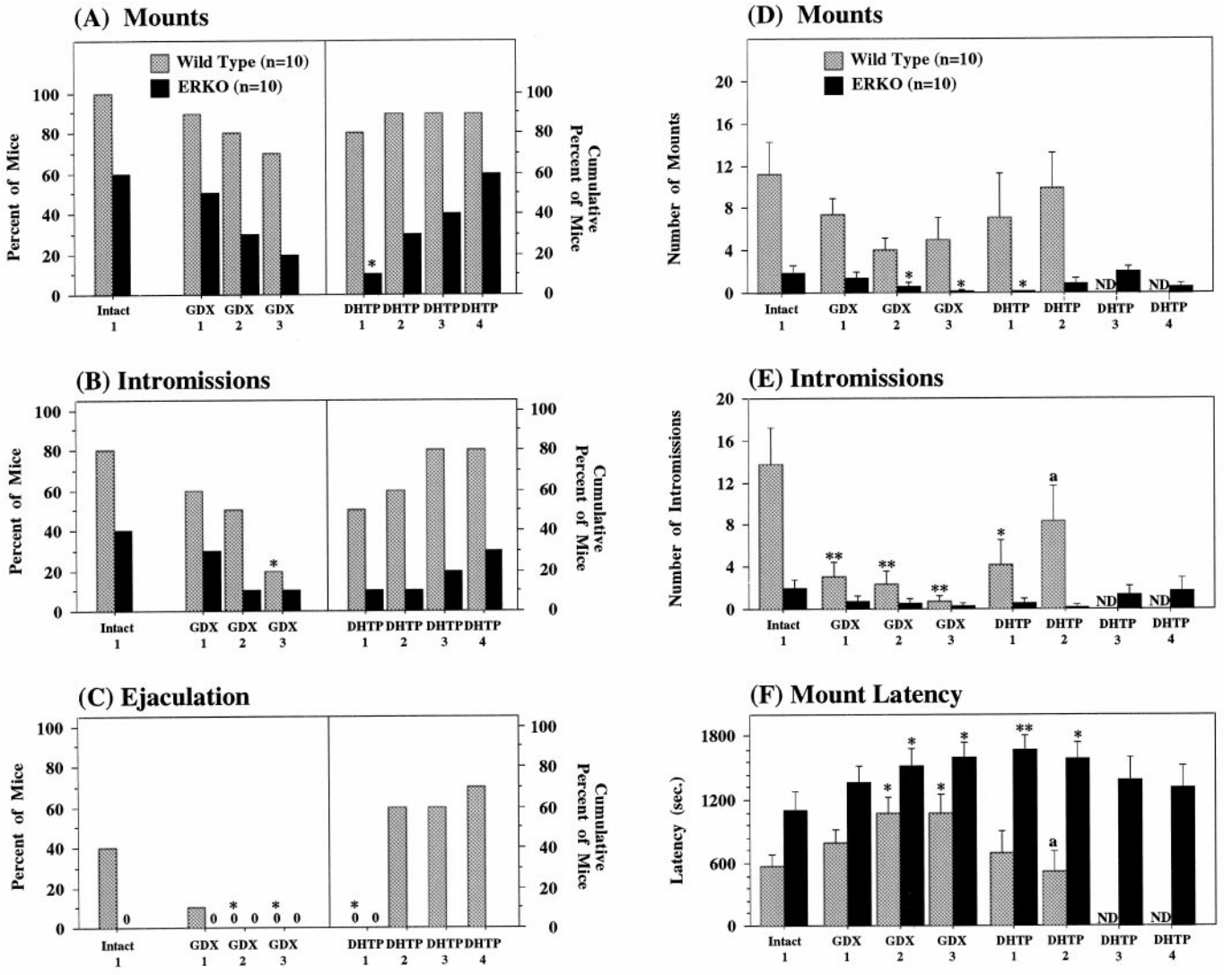

FIG. 7. Effects of gonadectomy (GDX) and DHTP treatment on male sexual behavior. Gonadectomy reduced sexual behavior, and DHTP restored it to the intact levels in both WT and ERKO mice, although ERKO showed slower recovery compared with WT mice. The levels in each of three tests after GDX and four tests after DHTP were compared with those in the intact test using either the binomial test for related samples with small expected frequencies (A–C; two-tailed; *, $P < 0.05$ *vs.* the intact test) or the paired *t* test [D–F; two-tailed; **, $P < 0.01$; *, $P < 0.05$ (*vs.* the intact test)]. The percentage of animals that showed the behavior (A–C) after DHTP treatment are shown as a cumulative number, as some WT mice were not tested at the third and fourth tests.

pearance of male-type offensive attacks in ERKO mice. Thus, lack of ERα activation may have a cascade effect on a number of neural processes involved in the regulation of aggressive behavior in male mice. It is known that proper ER activation by testosterone, after being aromatized to estradiol, during perinatal periods is essential for normal sexually dimorphic development of the central nervous system. Therefore, a lack of such stimulation in ERKO male mice due to a lack of ERα, but not necessarily testosterone itself, may severely affect the development of brain substrates regulating aggression.

It is also possible that an important part of the underlying mechanisms for the behavioral effects of ERα gene disruption depends on altered processing of chemosensory information. ERKO male mice did not attack any male mice that were gonadally intact, *i.e.* olfactory bulbectomized male intruders in the resident-intruder paradigm, opponent mice from the same genotype in homogeneous set tests, or C57BL/6J resident mice. It can be assumed that this was due to a failure in ERKO male mice to recognize them as a proper target of intermale aggression. This possibility needs to be further tested by examining whether ERKO male mice will show aggression toward other types of opponent, *e.g.* gonadectomized male mice. Likewise, it might be possible that chemosensory cues from pups that normally suppress infanticide were not processed properly in ERKO male mice, and therefore, they showed higher levels of infanticide compared with those in WT male mice.

Finally, it is conceivable that ERβ, known to bind to 17β-estradiol with an affinity similar to that of ERα (6, 7), may also play some role in the regulation of aggressive behavior as well as other types of reproductive behavior. Recent studies have shown that ERβ mRNA levels in the hypothalamic

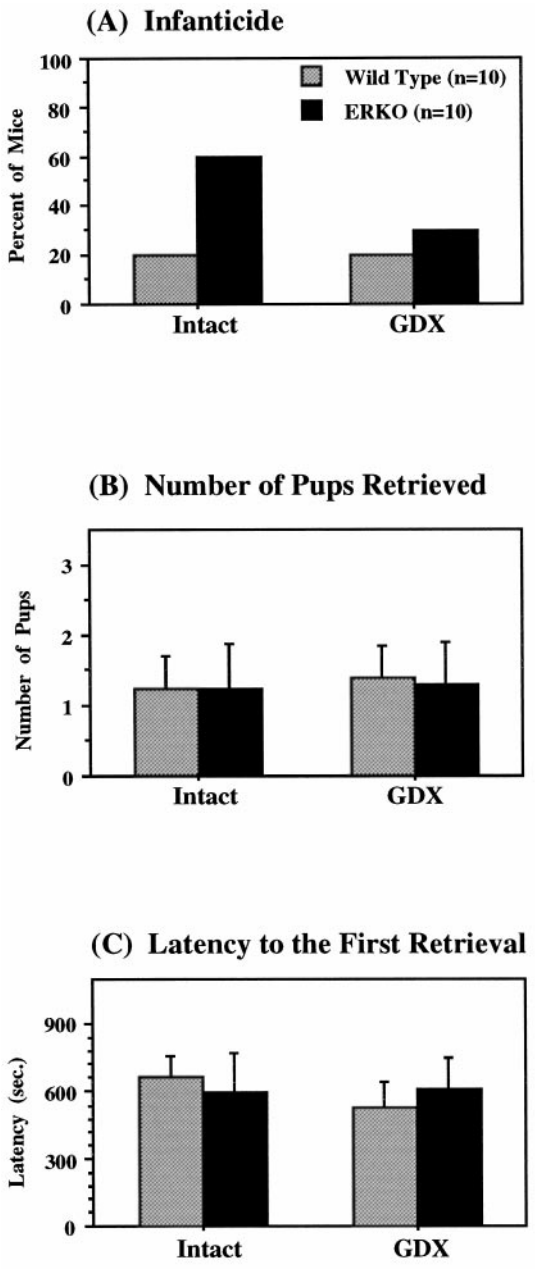

FIG. 8. Effects of ERα gene disruption on male parental behavior. There were genotype differences in the percentage of mice that showed infanticide before, but not after gonadectomy (A; see text). Pup-retrieving behavior (animals that showed infanticide were excluded), measured as the number of pups retrieved (B) and the latency to the retrieval of the first pup (C), was not different between ERKO and WT mice eithr before or after gonadectomy.

tissues in ERKO mice are not different from those of WT mice (5, 11). However, normal functioning of ERβ can be attenuated in ERKO mice as a result of lack of formation of heterodimers with ERα (28) and therefore may affect the induction of aggressive behavior. Furthermore, ERβ may also be involved in organizational effects of testosterone on aggression during the perinatal period. Expression of ERβ mRNA or protein during the neonatal period has recently been demonstrated in the rat (29) and ERKO mouse (30) brains, respectively. However, the exact role of ERβ in contrast to that of ERα in the organizational actions of testosterone on aggressive behavior needs to be determined in further studies.

Sexual behavior

It was found that TP treatment could induce mounts in both WT and ERKO male mice with very little sexual experience (Exp 2; see above). In DHTP-treated groups (Exp 3), again 60% of ERKO mice showed mounts in response to DHTP treatment. In both experiments, however, androgens failed to induce any ejaculation in ERKO mice. These results are consistent with our previous findings in gonadally intact male mice (12) and suggest that ERα may be crucial for the induction of ejaculation, but not for mounting behavior in male mice. Recent studies in male rats have shown that different components of male sexual behavior are regulated by different brain mechanisms. Thus, testosterone-implanted gonadectomized male rats showed a great reduction in ejaculation after 2 weeks of delivery of the aromatase inhibitor, fadrozole (31). In contrast, a smaller reduction of intromission was found only after 4 weeks of fadrozole delivery. In addition, it is shown that motivational aspects of male sexual behavior (preferential discrimination of receptive females) are not affected by fadrozole in testosterone-implanted male rats (32). We also found previously that ERKO male mice showed a similar interest in the odors of receptive female mice as WT mice (33). Furthermore, ultrasonic vocalizations emitted by ERKO mice in response to sexually receptive female mice were not different from those of WT mice (Ogawa, S., R. J. Barfield, K. S. Korach, and D. W. Pfaff, unpublished observation). Taken together, these findings suggest that ERα may be more responsible for the regulation of the consummatory elements than for the motivational aspects of male sexual behaviors by testosterone. These behavioral effects could also be partly mediated by a lack of ERα-dependent action of testosterone (as an aromatization precursor of estradiol) during brain development (as discussed above).

Finally, it should be noted that in the present study the mean frequency of mounts before gonadectomy was much lower in ERKO mice than that in WT mice, although more than half (60%) of ERKO mice showed mounts (Exp 3). This is in contrast to our previous studies in intact male mice (12), in which ERKO mice showed equivalent number of mounts as WT mice. There are several possibilities to explain this difference. One major factor is the difference in generations after gene disruption was first introduced to the foundation population. In our previous study, which used animals obtained from the NIEHS at five different times, we found that the overall levels of sexual behavior varied among these stages of the overall study. In conjunction with this, a relatively small sampling from each generation and a large individual variation in ERKO mice may also contribute to this effect. In our most recent study, some ERKO mice showed more than 100 mounts during 2-h tests without showing any ejaculation (which is atypical of WT or HZ mice), whereas other ERKO mice showed no mounts at all. Moreover, although we treated all animals identically after the arrival to

the laboratory, it is still possible that minor changes in housing conditions over the period of study may be responsible. Finally, in the present study mice were tested repeatedly for a long period of time. It is possible that WT mice became more and more sexually active with copulatory experience, but ERKO mice did not.

Parental behavior

It was found that more ERKO male mice tended to show infanticide while they were gonadally intact compared with WT mice. Although this difference was small, a similar trend was found regardless of the housing condition before the experiment, either singly housed mice obtained from the NIEHS (Exp 4A) or group-housed mice obtained from the University of Missouri (Exp 4B). These findings (maintenance of high levels of infanticide) are in marked contrast to the findings on aggressive behavior (almost complete elimination) and suggest that neural mechanisms affecting these two systems are different. Unlike its effects on aggression, testosterone maintained its behavioral effects on infanticide without functional ERα. These results may be interpreted as showing that testosterone in adulthood facilitates infanticide mainly through AR and/or ERβ. Slightly higher plasma levels of testosterone found in ERKO mice (17) may account for a small, but consistently higher, percentage of infanticide in ERKO mice compared with that in WT mice in the present study. A number of studies, however, revealed that levels of infanticide in male mice are not simply correlated with the circulating testosterone levels at the time of testing (18, 34, 35). Another interpretation of the present results (not mutually exclusive with the first interpretation) is that lack of ERα activation by testosterone (after being aromatized to estradiol) during the perinatal period may also contribute to the maintenance of infanticide in this genotype despite the lack of ERα activation at the time of testing in adulthood. Several studies suggest that prenatal as well as neonatal testosterone exposure may have suppressive effects on testosterone-inducible infanticide in adulthood. Thus, male mice neonatally gonadectomized (before day 10) showed more infanticide than sham-operated mice (gonadectomized in adulthood) when they were tested for infanticide after testosterone treatment in adulthood (36, 37). Studies examining the effects of intrauterine position revealed that 0 M (zero-male; low prenatal testosterone exposure) male mice gonadectomized at birth were not different from 2 M (two-males) male mice (also gonadectomized at birth) in spontaneous infanticide, but showed higher testosterone-inducible infanticide (36). Furthermore, studies in female mice suggest that ER-dependent actions of testosterone may be responsible, as 1) female mice neonatally gonadectomized and treated with TP or EB, but not DHTP, also showed lower levels of infanticide in response to adult testosterone treatment; and 2) this suppressive effect of neonatal androgenization was partially prevented by neonatal treatment with antiestrogen, MER-25 (37). Therefore, if this perinatal action of testosterone is indeed mediated primarily through ERα, it is assumed that in ERKO mice, perinatal testosterone fails to manifest its suppressive effects on testosterone-inducible infanticide in adulthood. This may account for the higher percentage of infanticidal ERKO mice compared to WT mice in intact (*i.e.* testosterone-inducible), but not gonadectomized (*i.e.* spontaneous), conditions. Our preliminary study revealed that testosterone indeed restored infanticide to the pregonadectomy levels in gonadectomized ERKO, but not WT, males. The exact mechanisms of this facilitatory action of testosterone in adult ERKO mice, including the role of ERβ, however, needs to be determined in further studies.

The findings in the present study together demonstrate that three types of testosterone-dependent male behavior reflecting masculinization are affected differently by ERα gene disruption. It should also be noted that two forms of active social approaches to other mice are different from each other with respect to their dependency on ERα. That is, sexual approaches by ERKO male mice to females, coupled with mounting, were almost normal, whereas attacks against other male mice were abolished.

Acknowledgments

The authors are thankful to Dr. Geoffrey Greene for his generous gifts of ER antiserum and peptides. The authors are also indebted to Dr. C. Pavlides for critical reading of the manuscript, to Mr. J. Gordan and Vincent Eng for their technical assistance, and to Ms. Lucy Frank for her editorial assistance.

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