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Roles of Estrogen Receptor-α Gene Expression in Reproduction-Related Behaviors in Female Mice*

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ABSTRACT

The role of gene expression of the estrogen receptor-α form (ERα) in the regulation of female reproductive behavior was investigated in estrogen receptor knockout (ERKO) mice, deficient specifically for the ERα, but not the ERβ, gene. Estrogen- or estrogen- plus progesterone-treated gonadectomized ERKO mice did not show any lordosis response. Detailed behavioral analysis revealed that ERKO females were also deficient in sexual behavioral interactions preceding the lordosis response. They were extremely rejective toward attempted mounts by stud male mice, which could not show any intromissions. During resident-intruder aggression tests, gonadally intact ERKO females were more aggressive toward female intruder mice than wild-type (WT) mice. Gonadectomy did not influence the levels of aggressive behavior, and their genotype differences when mice were tested both before and after gonadectomy. However, when mice were tested after gonadectomy for the first time, very few ERKO mice showed aggression. In contrast to aggression, male-type sexual behavior shown by resident mice toward female intruder mice during aggression tests was not different between ERKO and WT mice and was completely abolished after gonadectomy of the resident mice. Finally, it was also found that ERKO females showed greatly reduced levels of parental behavior toward newborn pups placed in their home cage. These changes in parental behavior were not influenced by gonadectomy. ERKO females retrieved significantly fewer numbers of pups with longer latencies compared with wild-type (WT) or heterozygous (HZ) littermates when they were tested as gonadally intact or 20–65 days after gonadectomy. In addition, during parental behavior tests, a significantly higher percentage of ERKO mice exhibited infanticide compared with WT and HZ mice, which rarely showed infanticide. Taken together, these findings suggest that ERα gene expression plays a key role in female mice, not only for sexual behavior but also for other interrelated behaviors, such as parental and aggressive behaviors. In addition, persistence of genotype differences in parental and aggressive behavior after gonadectomy indicates that ERα activation during neural developmental processes may also be involved in the regulation of these behaviors. (*Endocrinology* **139:** 5070–5081, 1998)

IT IS WELL established that the ovarian steroid, estrogen, regulates female reproduction and lordosis behavior by binding to intracellular estrogen receptors (ER) in the brain. This genomic action of estrogen is assumed to be mediated not only through the classical form of ER (now termed ERα), but also possibly through the second form of ER, ERβ, which was recently cloned (1, 2). Recent studies have shown that various brain tissues contain ERβ messenger RNA (mRNA)/protein (3–5) and that 17β-estradiol binds to both forms of ER with a similar affinity (6, 7). The exact roles of the two forms of ER in the regulation of female reproductive behavior are not yet defined.

A direct way to study the specific behavioral role of each ER form is to selectively block gene expression by gene knockout methods. Previously, we examined the behavioral characteristics of ERα gene-deficient (ERKO) mice (8, 9), which lack functional ERα, but not ERβ (3, 10), genes. Using gonadally intact (11) as well as gonadectomized and androgen-replaced (12) male ERKO mice, we found that three types of testosterone-dependent male behaviors, *i.e.* sexual, aggressive, and parental behaviors, were differently affected by the lack of ERα gene expression. Among them, aggressive behavior was most profoundly affected, by an almost complete suppression of offensive attacks, whereas sexual and parental behaviors were only partially reduced in ERKO mice compared with those in wild-type (WT) and heterozygous (HZ) male mice.

Using gonadally intact female ERKO mice, we found that ERα gene disruption greatly modified female sexual behavior (13). When ERKO females were tested with stud male mice, not only did they show a lack of lordosis behavior, but also they were vigorously attacked by the males, which showed frequent attempted mounts to diestrous WT females. As gonadally intact ERKO females are known to have 10-fold elevated levels of estradiol compared with WT females (14), the absence of lordosis suggests that ERα gene expression may be critically important for the induction of lordosis. In the present study we further tested this idea in gonadectomized and estrogen-replaced ERKO female mice. In addition, progesterone is known to facilitate female sexual behavior through its binding to intracellular progesterone receptors that are induced by estrogen in gonadectomized

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females. A recent study in ERKO mice has shown that estrogen can induce progesterone receptor mRNA in a number of hypothalamic areas (15), probably through ERβ, although the exact mechanisms are not known. Effects of progesterone administration on female sexual behavior in estrogen-primed mice, therefore, were also examined in the present study. Elicitation of offensive attacks in stud male mice by gonadally intact ERKO female mice, on the other hand, may be due to indirect peripheral effects of ERα gene disruption. Thus, elevated levels of testosterone found in ERKO females (16, 17) may affect pheromone production, which is known to be regulated almost exclusively through the ER-independent action of testosterone (18). This possibility was also tested in the present study by analyzing the details of behavioral interactions during sexual behavior tests, including male approach and aggressive behavior, in addition to sexual behavior.

Our previous study also revealed that gonadally intact ERKO females vigorously attacked gonadectomized and steroid-primed female intruder mice, although no quantitative analysis was performed in this study (13). In contrast, WT female mice, which were tested on the day of diestrus, were virtually never aggressive. In addition, ERKO females showed greatly reduced levels of pup-retrieving behavior and elevated levels of infanticide toward newborn pups placed in their home cage. These behavioral characteristics of ERKO female mice may simply be due to the elevated levels of testosterone at the time of testing in adulthood. If this is the case, any behavioral changes found in gonadally intact ERKO mice should disappear after gonadectomy. In the present study we compared the levels of aggressive behavior and parental behavior both before and after gonadectomy.

Finally, we have also examined the effects of ERα gene disruption on nonsocial behavior, *i.e.* levels of general activity and anxiety in light/dark transition tests. It was shown, using open-field tests, that ERKO male mice were more active and less fearful than either WT or HZ male mice (11). We tested whether similar effects of ERα gene disruption were detected in female ERKO mice. To assess the contribution of elevated levels of estradiol and testosterone on behavioral characteristics of gonadally intact ERKO female mice, gonadectomized females were also tested.

Materials and Methods

Female ERKO mice and their WT and HZ littermates from a mixed background of C57BL/6J and 129 (8, 9) were used. They were obtained from two separate breeding colonies, maintained at the NIEHS and at the University of Missouri-Columbia. The latter breeding colony originated from the same population as the former and 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. Upon arrival at the Rockefeller University (as young adults), mice were individually housed in plastic cages (30 × 20 × 13 cm) throughout the extent of the studies and 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. In addition, for the behaviors that were predicted to be partially influenced by repeated tests with the same animals, we analyzed and presented the data separately from those tested both before and after gonadectomy and from those tested only after gonadectomy. All tests were videotaped and analyzed by observers who were blind to the genotype of the mice. Females were either tested when gonadally intact (day of the estrous cycle was checked for WT and HZ mice), after gonadectomy, or after various steroid hormone treatments.

Exp 1

Gonadectomized WT (n = 6), ERKO (n = 12), and HZ (n = 6) mice (obtained from the University of Missouri) were tested for sexual behavior for 30 min. They were tested twice, at a 1- to 2-week interval, after the sc injection of estradiol benzoate (EB; two daily injections of 10 μg in 0.1 ml sesame oil). On the day after the second test, they were injected with EB once more, and on the following day they were tested for sexual behavior 5 h after injection with progesterone (500 μg in 0.1 ml sesame oil). To minimize the influence of behavioral differences in male mice, each ERKO female mouse was paired with either a WT or a HZ female mouse, and each pair of mice was tested against the same male mouse on the same day. All behavioral tests were performed in the stud male's home cage.

Three weeks after the last test, mice were injected sc with either EB (5 μg in 0.05 ml sesame oil) or sesame oil (0.05 ml) for 3 consecutive days. They were then perfused 1 h after the last injection, and brain tissues were processed for immunocytochemical detection of ERα.

Exp 2A

WT and ERKO mice obtained from the University of Missouri were divided into three different groups and tested for aggressive behavior. The first two groups of mice were tested twice (2 consecutive days, either metestrus or diestrus for WT mice) when gonadally intact and then once after gonadectomy, on either the 14th day (group A; n = 5/genotype) or the 37th day (group B; n = 7/genotype). The third group of mice was tested twice (with a 1-day interval) starting 24 days after gonadectomy (GDX 24; group C; n = 7 for WT and n = 11 for ERKO). All mice were tested against a female intruder mouse, which was gonadectomized and treated with 10 μg EB and 500 μg progesterone. When they were tested twice when they were intact or twice after gonadectomy, mean data from the two tests were used for the analysis.

Exp 2B

WT and ERKO mice obtained from the NIEHS were tested for aggressive behavior toward a gonadectomized female intruder mouse. They were tested longitudinally, including both before and after gonadectomy, on days 14, 33, and 51 (group A; n = 7/genotype) or only after gonadectomy on days 15 and 32 (group B; n = 8/genotype). At each time, they were tested twice, and the mean data from the two tests were used for the analysis.

Exp 2C

WT and ERKO mice used in Exp 2B (group A) were tested for aggressive behavior toward an olfactory bulbectomized male intruder mouse. They were tested twice as gonadally intact and once on GDX 37.

Exp 2D

Gonadectomized WT and ERKO mice used in Exp 2B (group A) were tested for aggressive behavior against a gonadectomized female intruder mouse. They were tested once on GDX 63. After completion of the behavioral tests, daily injections of EB (10 μg in 0.1 ml sesame oil) were started. On the day after the second and third estrogen injections, mice were tested for aggression.

Exp 3

WT, ERKO, and HZ mice obtained from either the NIEHS (froup 1) or the University of Missouri (group 2, A–D) were tested for parental behavior. Group 1 mice were tested when intact and then after gonadectomy (day 20). Females in group 2 were further divided into four groups, A–D. They were either both before and after gonadectomy or only after gonadectomy (days 20–65).

TABLE 1. Experimental series (flow chart, for each mouse, *top* to *bottom*)

Mice from the NIEHS									
	Series I			Series II			Series III		
Intact	Parental	Exp 3	Group 1				Parental	Exp 3	Group 1
	Agg. to FI	Exp 2B	Group A				LDT		
	Agg. to MI	Exp 2C							
GDX	Gonadectomy			Gonadectomy			Gonadectomy		
14–15	Agg. to FI	Exp 2B	Group A	Agg. to FI	Exp 2B	Group B			
20	Parental	Exp 3	Group 1				Parental	Exp 3	Group 1
32–33	Agg. to FI	Exp 2B	Group A	Agg. to FI	Exp 2B	Group B			
37	Agg. to FI	Exp 2B	Group A						
51	Agg. to FI	Exp 2B	Group A						
63	Agg. to FI	Exp 2D							
	Agg. w/EB to FI	Exp 2D							
	Agg. w/EB to FI	Exp 2D							

Mice from the University of Missouri									
	Series IV			Series V			Series VI		
Intact	LDT	Exp 4							
	Parental	Exp 3	Group 2A						
	Agg. to FI	Exp 2A	Group A	Agg. to FI	Exp 2A	Group B			
GDX	Gonadectomy			Gonadectomy			Gonadectomy		
14	Agg. to FI	Exp 2A	Group A						
20	Parental	Exp 3	Group 2A	Parental	Exp 3	Group 2B	Parental	Exp 3	Group 2C
24							Agg. to FI	Exp 2A	Group C
28							LDT	Exp 4	
37				Agg. to FI	Exp 2A	Group B			
45				Parental	Exp 3	Group 2B			
65							Parental	Exp 3	Group 2D
							Sexual w/EB	Exp 1	
							Sexual w/EB	Exp 1	
							Sexual w/EB + P	Exp 1	
							ICC	Exp 1	

Flow chart describing the order of multiple behavioral tests in each mouse. The series represents a group of mice that were tested at the same time. Numbers in the *left column* indicate the days after gonadectomy. Parental, Parental behavior test; Agg. to FI, aggression test toward female intruder mice; Agg. w/EB to FI, aggression test with estradiol benzoate treatment to female intruder mice; Agg. to MI, aggression test toward male intruder mice; LDT, light/dark transition test; Sexual w/EB, sexual behavior test with estradiol benzoate treatment; Sexual w/EB + P, sexual behavior test with estradiol benzoate and progesterone treatment; ICC, immunocytochemical study for estrogen receptors.

Exp 4

WT and ERKO females were tested for 2 consecutive days either when gonadally intact (n = 13 for WT and n = 16 for ERKO) or on GDX 28 (n = 9 for WT and n = 14 for ERKO) for the light/dark chamber transition behavior. Separate groups of mice were used for each gonadal condition.

Sexual behavior test

Sexual behaviors were tested using singly housed stud Swiss-Webster ((SW)fBR; purchased from Taconic Farms, Germantown, NY) male mice, in the males' home cages, during the dark phase (4–8 h after lights off) of the light cycle under red light. As most ERKO mice did not show regular female sexual behavior, each female was tested for 30 min, and all the behavioral acts displayed by male and female were recorded (*i.e.* instead of simply calculating the lordosis quotient). These behaviors included male and female approaches, social investigation by males (*i.e.* genital sniffing, touching the female's back with forepaws, chasing the female), male sexual behavior (*i.e.* attempted mount, mount, intromission, ejaculation), male aggressive behavior (*i.e.* biting, attacks), female rejective behavior (*i.e.* flee, kick, upright posture), and female receptive behavior (*i.e.* receptive still posture without lordosis, lordosis). Occurrences of these behaviors were recorded sequentially as bouts, which were separated from each other once the distance between the male and the female exceeded more than half of the body length. Each behavioral bout was classified, based on the male behavior, as investigation only, sexual, or aggressive, and the number of bouts of each was counted for each test. Sexual behavioral bouts were further classified based on male and female behaviors. For male behavior, the number of bouts with attempted mount, mount, or intromission/ejaculation were counted. Female responses during sexual behavioral bouts were judged as rejective, receptive without lordosis, or receptive with lordosis. The percentage of each among the total number of sexual behavior bouts was calculated for each test.

Aggressive behavior test

Aggressive behaviors were tested in a resident-intruder paradigm for 15 min during the dark phase (4–8 h after lights off) under red light. Group-housed male or female Swiss-Webster mice were used as intruders. 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 more than 3 sec elapsed between two behavioral aggressive acts, the two behavioral acts were scored as two separate aggressive bouts. Aggressive behavior acts consisted of tail rattling, chasing, boxing, biting, lunge, offensive attack (often accompanied by biting), and wrestling. The number of animals that showed any aggressive behaviors, the latency to the first aggressive act, the cumulative duration of any aggressive behavior bouts, and the number of bouts with offensive attacks were all recorded. In addition, the number of bouts and cumulative duration of male-type sexual behavior by resident female mice, such as attempted mounts and intromission patterns toward the intruder female mice, were also recorded.

Parental behavior test

Females 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, three Swiss-Webster pups (days 3–7 of age) were gently placed in the female's home cage at the farthest end from the nest. Some females were tested for 2 consecutive days with the same three newborn pups (pups were identified by marks on their skin at the end of the first test). The number of pups retrieved to the nest and the latency to retrieval to the nest of the first and all three pups were recorded. Retrieving of pups was scored only if the female 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 females were excluded both from subsequent tests and analysis of pup-retrieving behavior data. Although WT and HZ mice were tested randomly during the estrous cycle, a *post-hoc* analysis revealed that the performance of parental behavior was not affected.

Light and dark chamber transition test

Animals were tested for 10 min on 2 consecutive days for the light/dark chamber transition behavior. The apparatus consisted of one light chamber ($14.5 \times 30 \times 20.5$ cm), which was illuminated from the top with a white light, and one dark chamber ($27 \times 30 \times 20.5$ cm), which was separated from the former by a black partition with a small opening (6×6 cm). At the beginning of the test, a mouse was gently placed in the center of the light chamber with her head facing the dark chamber. The latency to enter the dark chamber, the latency to exit the light chamber (time after the first entry to the dark chamber), the cumulative time spent in the light chamber (time spent before the first entry to the dark chamber was excluded), the number of transitions between two chambers (counted as one if all four paws crossed the partition), and the number of leanings and rearings in the light chamber were measured.

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) anti-ERα antiserum, ER21 or H222 (gift of Dr. G. Greene), in 50 mM Tris-buffered saline (TBS), pH 7.2, containing 0.5% Triton X-100 and 4% normal goat serum (Vector) for 48 h at 4 C; 2) a 1:200 dilution of the biotinylated goat antirabbit secondary antibody (Vector Laboratories, Burlingame, CA) 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 antibody with antigen protein or omitting the primary antibody from the staining procedure.

Statistics

Behavioral data were analyzed by a three-way ANOVA for repeated measurements for the main effects of genotype, gonadal state, and test day and their interactions; a two-way ANOVA for repeated measurements for the main effects of genotype and test day and their interaction; a two-way factorial ANOVA for the main effects of genotype and gonadal state; or one-way ANOVAs for genotype differences or test day differences followed by *post-hoc* pairwise comparisons (Tukey's test), if applicable. Some behavioral data (in which variances were not homogeneous between genotype groups) were analyzed by nonparametric Kruskal-Wallis one-way ANOVA and Mann-Whitney U tests. Differences in the percentage of animals showing certain behaviors were tested with either the χ^2 test or Fisher's exact probability test.

Results

Exp 1: female sexual behavior in gonadectomized and steroid-treated female mice

There were no genotype differences, regardless of the steroid conditions, in the total number of social contact behaviors by male mice (Fig. 1, A-1). That is, male mice sniffed, chased, and tried to touch the backs of the females of all three genotypes. During the tests with ERKO female mice, however, male social approaching behavior was not allowed to develop into male sexual behavior, such as attempted mounts, mounts, and intromissions, due to vigorous rejection by the females. Thus, the total number of sexual behavior responses was lower in ERKO mice than in both WT and HZ mice, although statistically, ERKO mice were only different from HZ mice (Fig. 1, A-2). Only a few females injected with EB alone, but not with EB and progesterone, were attacked by males, and there were no genotype differences (Fig. 1, A-3). During the tests with ERKO mice, male mice showed attempted mounts (demonstrated as the percentage of total sexual behavior, Fig. 1, B-1) and mounts (Fig. 1, B-2), but were never able to achieve intromissions (Fig. 1, B-3). Comparisons in Fig. 1, B-1, also illustrate that most of the sexual behavior shown by males toward ERKO females in all three tests was attempted mounts, whereas the percentage of attempted mounts decreased by the second (EB) and third (EB plus progesterone) tests with the WT and HZ females. Regardless of the steroid conditions, about 80% of the time, ERKO females showed rejections (mainly kick or flee) toward male sexual behavior (Fig. 1, C-1). ERKO females showed prereceptive posture (Fig. 1, C-2), but virtually never displayed lordosis behavior (Fig. 1, C-3). In contrast, both WT and HZ female mice rejected the male (to the same extent as ERKO females) in the first test (Fig. 1, C-1), but they showed increased levels of lordosis behavior in the second (EB) and the third (EB plus progesterone) tests (Fig. 1, C-3).

ERα-immunoreactive cells, stained with either ER21 or H222 antibodies, were found in gonadectomized WT mouse brains but not in ERKO mouse brains. ERα immunoreactivity was greatly reduced in EB-treated WT mouse brains.

Exp 2: aggressive behavior in intact and gonadectomized mice

Exp 2A. Gonadally intact ERKO females (obtained from the University of Missouri) exhibited significantly higher levels of aggression toward gonadectomized and steroid-primed female intruder mice compared with WT mice in both groups A and B (Table 2). Gonadectomy did not abolish aggressive behavior, neither the cumulative duration nor the number of aggressive bouts with offensive attacks, in ERKO mice tested 14 or 37 days after gonadectomy. ERKO mice tested only after gonadectomy, however, were not aggressive (group C).

Exp 2B. ERKO female mice (obtained from the NIEHS) were significantly more aggressive toward gonadectomized (non-steroid-primed) female intruder mice compared with WT females throughout the tests in group A (Fig. 2, A and B). As found in Exp 2A, gonadectomy did not affect the aggressive behavior in mice tested before gonadectomy, whereas those tested only after gonadectomy showed very few aggressive

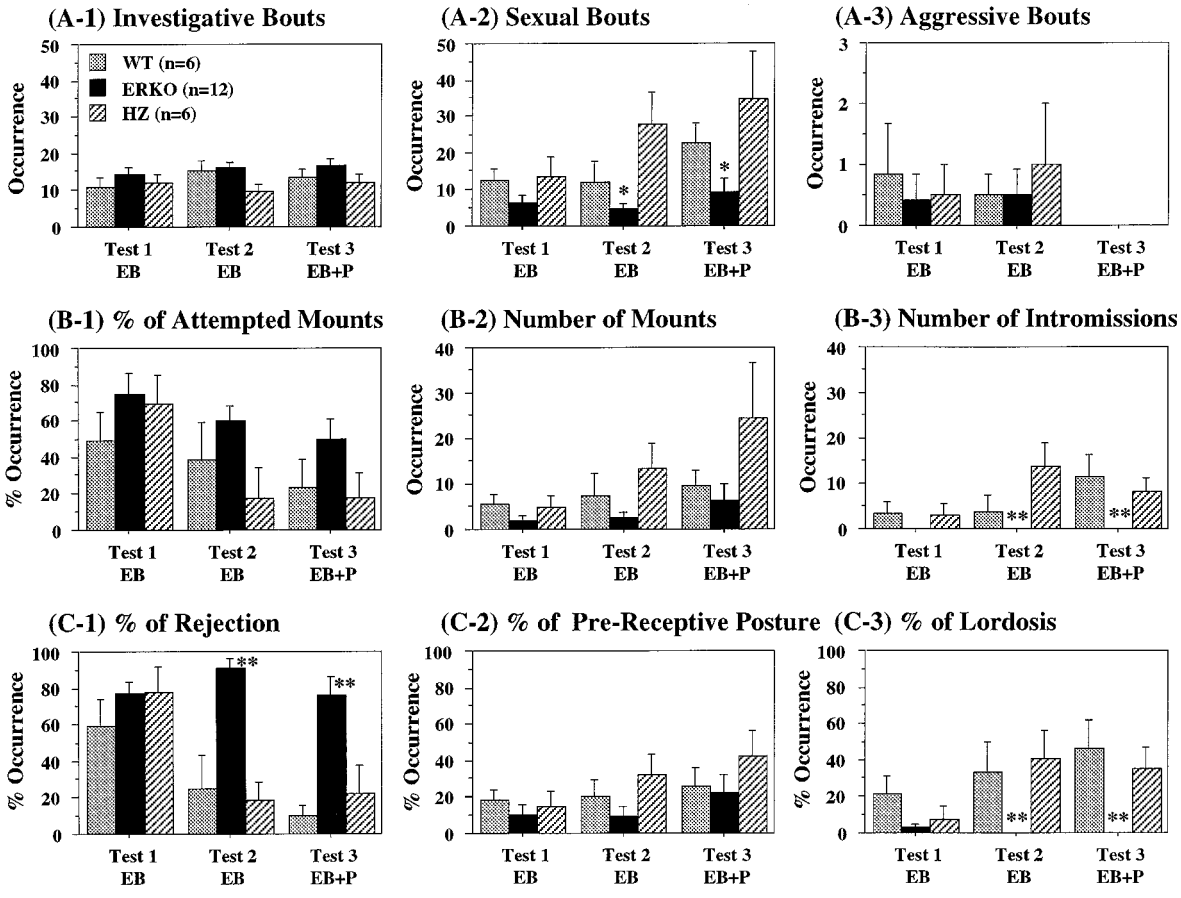

FIG. 1. Effects of ERα gene disruption on female sexual behavior in gonadectomized mice. Behavioral interactions were classified as social investigative (A-1), sexual (A-2), or aggressive (A-3). Male mice investigated females of all three genotypes, but they showed only a small number of sexual behaviors toward ERKO females, especially in tests 2 and 3. Moreover, more than 50% of the sexual behavior shown toward ERKO females was attempted mounts (B-1). Males showed very few mounts (B-2) and no intromissions (B-3) toward ERKO females. Most of the time, ERKO females showed rejection of male sexual behavior (C-1) and very few prereceptive postures (C-2) and never showed lordosis (C-3). **, $P < 0.05$ *vs.* WT and HZ; *, $P < 0.05$ *vs.* HZ.

responses (Table 3). During aggression tests, resident females often showed male-type sexual behavior (mounts and intromission patterns) toward the intruder female mouse. In contrast to aggression, there were no genotype differences in the levels of male-type sexual behavior by the females between WT and ERKO mice before gonadectomy (Fig. 2C). Furthermore, this behavior was greatly reduced by gonadectomy in both genotypes.

Exp 2C. The same female mice used in Exp 2B (group A) were tested against olfactory bulbectomized male intruder mice. None of the WT and ERKO mice exhibited any aggressive behaviors toward males either when intact or on GDX 37 (data not shown), whereas they were aggressive toward female intruder mice (see above, Exp 2B, Fig. 2). Moreover, olfactory bulbectomized male intruder mice (in contrast to our previous findings in intact stud male mice) never showed any aggressive behavior toward either ERKO or WT female mice regardless of the gonadal states.

Exp 2D. Females used in Exp 2B (group A) and 2C were also tested toward gonadectomized (nonsteroid primed) female intruder mice when gonadectomized (day 63) and after EB injections. Before EB treatment, ERKO mice were still more aggressive than WT female mice, as they had been throughout in Exp 2B (see Fig. 2). The mean duration of aggressive behavior (Fig. 3A) and the percentage of mice that showed aggressive behavior (Fig. 3B), however, were greatly reduced by EB treatment in ERKO mice. By the second test after EB treatment, there were no genotype differences in either measurement.

Exp 3: parental behavior in intact and gonadectomized female mice

Proportion of infanticidal mice. A substantial number of gonadally intact ERKO mice showed infanticide during parental behavior tests, whereas very few WT mice showed infanticide (Table 4; groups 1 and 2A). These infanticidal females displayed biting of pups when they were retested on GDX 20. The persistence of infanticide after gonadectomy in ERKO mice was not due to the infanticidal experience before gonadectomy. That is, 40% of ERKO mice showed infanticide in groups tested both before and after gonadectomy (group 2A, 2 of 5) and those tested only after gonadectomy (groups 2B and 2C combined, 6 of 15). In the latter groups of mice, the percentage of infanticidal ERKO mice did not change

TABLE 2. Results of aggressive behavior tests in Exp 2A

	Intact		GDX 14 days		GDX 24–37 days	
	Wild type	ERKO	Wild type	ERKO	Wild type	ERKO
Cumulative duration of aggressive behavior						
Group A	0.5 ± 0.5[a]	9.3 ± 7.0	0.0 ± 0.0	31.0 ± 15.4[b]		
	0–2.5[c]	0–36.5		0–83		
	1/5[d]	3/5	0/5	4/5[b]		
Group B	0.0 ± 0.0	35.2 ± 17.1[e]			0.0 ± 0.0	41.3 ± 20.4[e]
		0–102				0–114
	0/7	3/7			0/7	3/7
Group C					0.0 ± 0.0	0.5 ± 0.4
						0–1.5
					0/7	2/11
No. of bouts with offensive attacks						
Group A	0.0 ± 0.0	1.1 ± 1.1	0.0 ± 0.0	2.2 ± 1.5[e]		
		0–5.5		0–8		
	0/5	1/5	0/5	3/5		
Group B	0.0 ± 0.0	3.0 ± 0.8[e]			0.0 ± 0.0	3.7 ± 2.0[e]
		0–7.5				0–13
	0/7	3/7			0/7	3/7
Group C					0.0 ± 0.0	0.0 ± 0.0
					0/7	0/11

Genotype differences and effects of gonadectomy on cumulative duration of aggressive behavior (*top*) and number of bouts with offensive attacks (*bottom*) toward a steroid-primed (EB, 10 μg; progesterone, 500 μg) gonadectomized female intruder mouse during resident-intruder tests (Exp 2A). Three groups (A–C) of female mice obtained from the University of Missouri were tested before and/or after gonadectomy. Genotype differences in mean duration or number at each test were analyzed using the Mann-Whitney U test, and differences in the percentage of animals showing certain behaviors were tested with the Fisher exact probability test.

[a] Mean ± SEM.
[b] $P < 0.05$ *vs.* wild type.
[c] Range of data.
[d] Number of mice showing behavior/total number of mice tested.
[e] $P < 0.07$ *vs.* wild type.

when they were retested on GDX 45 (group 2B), but was greatly reduced on GDX 65 (group 2C), similar to those tested on GDX 65 for the first time (group 2D). It was found that overall, the ERKO mice obtained from the NIEHS were more infanticidal than those obtained from the University of Missouri. For instance, there were significant differences in the percentage of infanticidal ERKO mice on GDX 20 between the two populations, *i.e.* 8 of 10 mice in group 1 from the NIEHS *vs.* 8 of 20 mice in group 2 from the University of Missouri (see Table 4; $\chi^2(1) = 4.286$; $P < 0.05$), although ERKO mice were still more infanticidal than WT mice in both populations (group 1: $P = 0.023$, by Fisher's test; group 2: $\chi^2(1) = 8.229$; $P < 0.001$). Finally, none of the HZ female mice demonstrated infanticide.

Pup retrieving behavior in noninfanticidal females. Retrieving behavior of noninfanticidal mice in group 2, B–D (mice tested only after gonadectomy), was further analyzed (Fig. 4). On both GDX 20–21 (a) and GDX 65 (b), ERKO females displayed reduced levels of retrieving behavior compared with those of WT and HZ female mice, *i.e.* retrieved a significantly lower number of pups (Fig. 4A) with longer latencies (Fig. 4, B and C). It should be noted that on GDX 65, most of the ERKO females no longer showed infanticide (Table 4), but their retrieving behaviors were still markedly different from those of WT and HZ mice.

Behavioral correlation between infanticide and aggressive behavior. Some mice were tested for both parental and aggressive behaviors. It was found that except for one ERKO female, all females that showed infanticide also exhibited aggressive behavior toward a female intruder mouse (Table 5), regardless of the gonadal status. In contrast, there were some ERKO females that only showed aggression but not infanticide. All the WT females that showed infanticide also exhibited aggression.

Exp 4: genotype differences in light/dark chamber transition test and effects of gonadectomy

Data were first analyzed by three-way ANOVAs for repeated measurements for the main effects of genotype, gonadal state, and test day and their interactions. These tests revealed significant differences between the two tests in all measurements except the latency to exit the light chamber. The number of transitions, number of rearings and leanings, and cumulative duration spent in the light chamber decreased in the second test compared with those in the first test. However, no interaction with genotype was detected in any of these measurements (although the degree of decrease was larger in the gonadectomized groups compared with the intact groups in some measurements). Therefore, genotype differences and effects of gonadal states were analyzed and demonstrated using mean values of two tests calculated for each mouse (Fig. 5). Both the latency to enter the dark chamber and the latency to exit to the light chamber (after entry to the dark chamber) were not different between ERKO and WT mice regardless of the gonadal states, although both latencies were longer in gonadectomized mice of both genotypes (Fig. 5, A and B). ERKO mice, however, tended to exit to the light chamber fewer times [Fig. 5C; $F(1,48) = 8.81$; $P = 0.06$] and spent significantly less time in the light chamber [Fig. 5D; $F(1,48) = 5.54$; $P < 0.05$] compared with WT

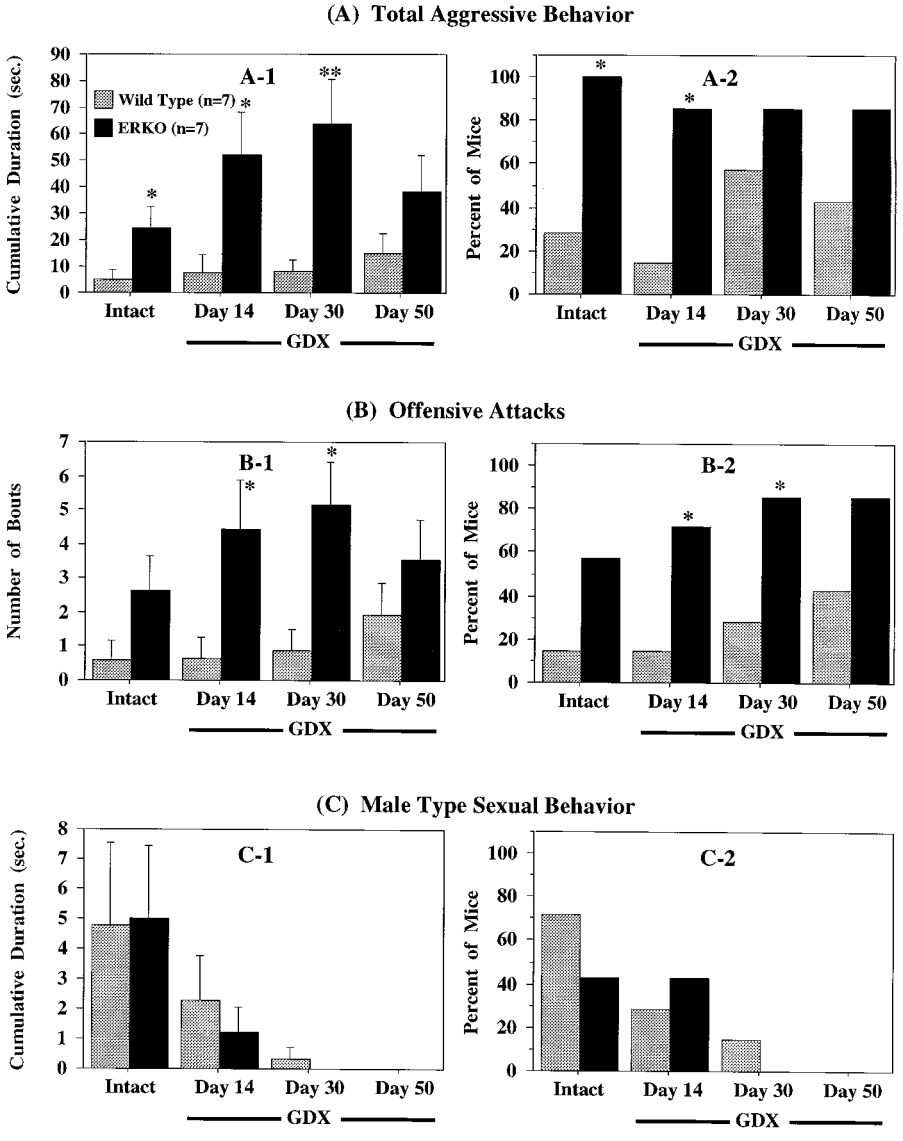

FIG. 2. Genotype differences and effects of gonadectomy on aggressive and male-type sexual behavior toward a gonadectomized female intruder mouse during resident-intruder tests. ERKO females showed higher levels of aggression, measured as cumulative durations of total aggressive behavior bouts (A-1), number of bouts with offensive attacks (B-1), and the percentage of animals showing any aggression (A-2) or offensive attacks (B-2). Gonadectomy did not abolish aggressive behavior. In contrast, ERKO and WT females showed similar levels of male-type sexual behavior toward female intruder mice (C-1 for cumulative duration, and C-2 for the percentage of mice), which was markedly reduced by gonadectomy. Two-way ANOVA for repeated measurements revealed that there were overall significant genotype differences in the mean duration of aggression [A-1; $F(1,36) = 6.84$; $P < 0.05$] and the mean number of offensive attacks [B-1; $F(1,36) = 4.99$; $P < 0.05$], but not in the mean duration of male-type sexual behavior (C-1). As interactions between genotype and test days were also significant, genotype differences were analyzed in each test (*, $P < 0.05$ *vs.* WT). Data for the percentage of mice showing aggression (A-2), offensive attacks (B-2), and male-type sexual behavior (C-2) were analyzed for genotype differences in each test using Fisher's exact probability test (*, $P < 0.05$ *vs.* WT).

mice. In both genotypes, the intact mice exited to the light chamber more often and spent a longer period of time there compared with the gonadectomized mice. The numbers of leanings and rearings in the light chamber were also significantly lower in ERKO compared with WT mice [Fig. 5E; $F(1,48) = 5.72$; $P < 0.05$]. Finally, a few gonadectomized HZ mice (n = 7) were tested and found to be similar to WT mice (data not shown).

Discussion

In the present study we extensively described the behavioral characteristics of gonadally intact and gonadectomized female ERKO mice obtained from two separate breeding colonies. Although there were some small, but significant, differences, *e.g.* the percentage of infanticidal ERKO mice, *etc.*, between the two groups of mice, we found very consistent behavioral changes due to ERα gene disruption despite the fact that they were maintained separately for a few generations. These findings suggest that ERKO mice serve as a powerful tool to assess behavioral effects of ERα-specific gene manipulation. It should also be noted, however, that effects of gene manipulation in ERKO mice, as in any other knockout mice, are permanent throughout the life of the animal and global across the entire body of the animal. Therefore, we could not completely differentiate effects at the time of testing in adulthood and those during developmental processes. Furthermore, as discussed below, ERα gene disruption may have secondary effects, such as elevated levels of estrogen and testosterone, which may also affect brain functions through developmental processes. It is also possible that some of the real effects of specific gene disruption may be concealed by compensating mechanisms. It is necessary to elucidate underlying mechanisms of behavioral modification in ERKO mice in further studies.

Sexual behavior

Detailed behavioral analysis in the present study revealed that ERKO females were deficient not only in the ability to show lordosis responses, but also in prelordotic behavioral interactions with male mice. During the sexual behavior

TABLE 3. Results of aggressive behavior tests in Exp 2B

	GDX 15 days		GDX 32 days	
	Wild type	ERKO	Wild type	ERKO
Total aggressive behavior				
Mean duration ± SEM	0	3.75 ± 3.61	0	8.63 ± 8.63
Range		0–29		0–69
No. of mice[a]	0/8	2/8	0/8	1/8
Offensive attacks				
Mean no. ± SEM	0	0.19 ± 0.19	0	1.00 ± 1.00
Range		0–1.5		0–8
No. of mice	0/8	1/8	0/8	1/8
Male-type sexual behavior				
Mean duration ± SEM	0.63 ± 0.63	0	0.06 ± 0.06	0
Range	0–5		0–0.5	
No. of mice	1/8	0/8	1/8	0/8

Genotype differences in total aggressive behavior (*top*), number of bouts with offensive attacks (*middle*), and male-type sexual behavior (*bottom*) toward a gonadectomized female intruder mouse during resident-intruder tests (group B mice in Exp 2B). Female mice obtained from the NIEHS were tested only after gonadectomy on days 15 and 32. Unlike those obtained from NIEHS and tested before and after gonadectomy (group A mice in Exp 2B; see Fig. 2), very few ERKO female mice in this group were aggressive. Genotype differences in the mean duration or number at each test were analyzed using the Mann-Whitney U test, and differences in the percentage of animals showing certain behaviors were tested with Fisher's exact probability test.

[a] Number of mice that showed behavior/total number of mice tested.

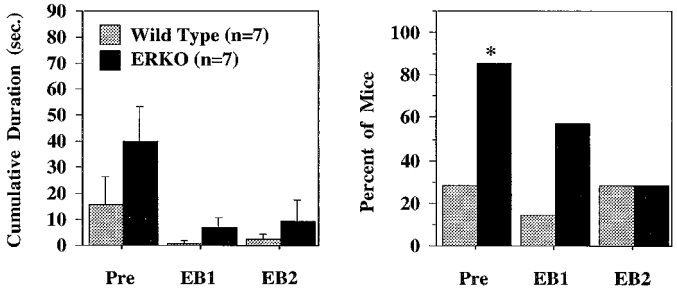

FIG. 3. Effects of estrogen treatment on cumulative duration of aggressive behavior and the percentage of mice showing aggression. Genotype differences were analyzed in each behavioral test using *t* test or Fisher's exact probability test (*, $P < 0.05$ *vs.* WT).

tests, stud male mice approached and explored females of all three genotypes. However, male mice could not show full mounting behavior toward ERKO female mice, which rarely showed lordosis. These results are consistent with previous findings (19) and suggest that ERα activation by estrogen is critically important for the expression of normal sexual behavior in female mice. The presence of ERβ, which is known to bind to estradiol with a similar affinity as ERα (6, 7), did not alter the observed phenotype or lead to the induction of sexual behavior in ERKO female mice.

Progesterone treatment failed to induce any lordosis behavior in ERKO females, although it completely suppressed male aggressive behavior toward ERKO females and slightly increased the percentage of prereceptive posture. A recent study has shown that estrogen induces progesterone receptor mRNA in the hypothalamus of ERKO female mice (15), probably through ERβ-mediated mechanisms. These findings together suggest that ERα gene expression is still necessary for synergistic regulation by estrogen and progesterone for the induction of lordosis behavior. On the other hand, some other aspects of sexual behavioral interaction may be partially regulated by progesterone through ERα-independent genomic mechanisms or nongenomic mechanisms (20).

In our previous studies it was found that gonadally intact ERKO female mice were vigorously attacked by stud male mice without any sign of normal sexual behavioral interactions (13). It was assumed that ERKO females had male-type characteristics, such as pheromone production. In contrast, in the present study estrogen-treated gonadectomized ERKO females were not attacked by stud males. These findings suggest that certain male-type characteristics were produced in gonadally intact ERKO females by elevated levels of testosterone through androgen receptor (AR)-dependent mechanisms consistent with previous studies (18). Indeed, we found that preputial (clitoral) glands are highly stimulated in intact ERKO females compared with those in WT females due to an excess of serum androgen (17). In addition, these peripheral characteristics of ERKO females decreased within 2–3 weeks after gonadectomy (Lindzey, J., and K. S. Korach, unpublished observation) when the serum testosterone levels were not detectable in ERKO female mice (Ogawa, S., K. S. Korach, and D. W. Pfaff, unpublished data).

Aggressive behavior

It was found that aggressive behavior greatly increased in ERKO female mice compared with that in WT mice. They showed offensive attacks toward female intruder mice. In marked contrast, induction of offensive attacks was almost completely suppressed in male ERKO mice (11, 12). Therefore, these findings together suggest that ERα gene expression is critically important for the induction of aggressive behavior in male mice but not in female mice. This is consistent with previous findings in a series of studies by Simon's group. These studies have shown that both testosterone and estrogen restore aggressive behavior in gonadectomized male mice (21), whereas only testosterone, but not estrogen, is effective to induce aggression in female mice (22, 23). Higher levels of aggressive behavior are found in gonadally intact ERKO female mice, which may be due to the elevated levels of testosterone. In addition, it is possible that ERKO females might have elevated levels of testosterone during the neonatal period. This may further sensitize ERKO females for aggression-promoting effects of testosterone

TABLE 4. Proportion of infanticidal mice

	Intact			GDX day 20			GDX day 45			GDX day 65		
	WT	KO	HZ	WT	KO	HZ	WT	KO	HZ	WT	KO	HZ
Group 1	1/9 (11)[a]	8/10 (80)[b]		2/9 (22)	8/10 (80)[c,d]							
Group 2	0/4 (0)	2/5 (40)		0/16 (0)	8/20 (40)[b]	0/7 (0)	0/7 (0)	2/7 (29)		0/8 (0)	1/14 (7)	0/7 (0)
2A	0/4	2/5		0/4	2/5							
2B				0/7	2/7		0/7	2/7				
2C				0/5	4/8	0/7				0/5	0/8	0/7
2D										0/3	1/6	

Genotype differences and effects of gonadectomy on the proportion of infanticidal mice (Exp 3). Female mice obtained from the NIEHS (group 1) or the University of Missouri (group 2) were tested before and/or after gonadectomy. Genotype differences in the percentage of animals showing infanticide (in *parentheses*) were tested with either the χ^2 test or Fisher's exact probability test. There were also significant differences in the percentage of infanticidal ERKO mice on GDX day 20 between the two populations.

[a] Number of mice that showed infanticide/total number of mice tested.

[b] $P < 0.01$ *vs.* wild type.

[c] $P < 0.05$ *vs.* wild type.

[d] $P < 0.05$ *vs.* group 2.

through ERα-independent mechanisms. Previous studies have shown that adult females neonatally treated with testosterone exhibit higher levels of aggression in response to testosterone as an adult (21, 24, 25). Furthermore, it is shown that neonatal ER stimulation may not be necessary for the ER-independent aggression-promoting effects of testosterone in female mice, as neonatal androgenization with AR-specific agonists effectively induces aggressive behavior in response to testosterone in adult female mice (22, 26). Therefore, it is assumed that a lack of ERα gene expression during the neonatal period may not impair the development of neural substrates for aggression in female ERKO mice, in marked contrast to the case in male ERKO mice. Rather, it is conceivable that AR-dependent mechanisms for development of neural substrates for aggression may not only be intact, but may also be accentuated in female ERKO mice. Our recent studies in neonatal mice revealed that there was a great increase in AR-immunoreactive cells in female ERKO mouse brains compared with those in WT and HZ female brains starting around postnatal day 12, whereas there were no obvious genotype differences in male brains (27). Further studies about the effects of neonatal androgenization on the development of aggressive behavior in ERKO female mice may elucidate the specific role of neonatal activation of AR, ERα, and/or ERβ in sex-specific controlling mechanisms of aggressive behavior.

In two different populations of ERKO female mice (*i.e.* NIEHS- and University of Missouri-derived mice), we consistently found that gonadectomy might affect the first induction of aggressive behavior, but not the maintenance of aggressive behavior. Thus, ERKO females showing aggressive behavior before gonadectomy remained aggressive at least 63 days after gonadectomy. On the other hand, ERKO females tested only after gonadectomy were rarely aggressive. It is unlikely that this was simply due to the repetition of aggressive behavior tests, because most intact ERKO mice showed aggressive behavior during the first aggression tests. It is also unlikely that gonadectomy failed to change the hormonal conditions in ERKO females, as male-type sexual behavior shown by resident ERKO female mice against female intruder mice was abolished by gonadectomy. Indeed, it was found that serum testosterone levels were not detectable 3 weeks after gonadectomy in ERKO female mice (Ogawa, S., K. S. Korach, and D. W. Pfaff, unpublished data). Taken together, these findings suggest that the maintenance of aggressive behavior in female mice may not be influenced by the hormonal environment at the time of the testing. The persistence of already induced aggression after gonadectomy in females is repeatedly reported in both rats (28) and mice, as found in WT female mice derived from the NIEHS population in the present study. Aggressive behavior of ERKO females was not an exception in this sense. This is markedly contrasted with the rapid decrease and complete abolishment of aggressive behavior after gonadectomy in male mice tested similarly in this laboratory (12, 29). Moreover, in ERKO mice with aggressive experience before gonadectomy, there was even a trend toward an increase in levels of aggression after gonadectomy. This was also the case, to some extent, in WT female mice. It is generally assumed that levels of already induced aggression in female mice are further increased, even after gonadectomy, by repetition of tests and/or prolonged periods of social isolation.

Aggressive behavior in gonadectomized ERKO females was unexpectedly reduced by estrogen administration. This was probably not due simply to daily repetition of aggression tests. When they were tested on 2 consecutive days throughout the experiment (Exp 2B), there were no apparent trends toward a decrease in aggression in the second tests. Estrogen is indeed known to inhibit spontaneously induced aggressive behavior in postpartum female mice (30). However, as both aggression and estradiol levels were elevated in gonadally intact ERKO females, it was predicted that estrogen might inhibit aggression, possibly through ERα, only in gonadectomized WT, not in ERKO, mice. Findings in the present study suggest that acute injection of estrogen may work to inhibit aggression through either ERβ-mediated or nongenomic mechanisms.

Parental behavior

It was found in the present study that parental behavior was greatly reduced in ERKO females. ERKO mice not only showed poor pup-retrieving behavior, as indicated by the number of pups retrieved and the latency to retrieve the pups, but also overall more than 50% of ERKO females ex-

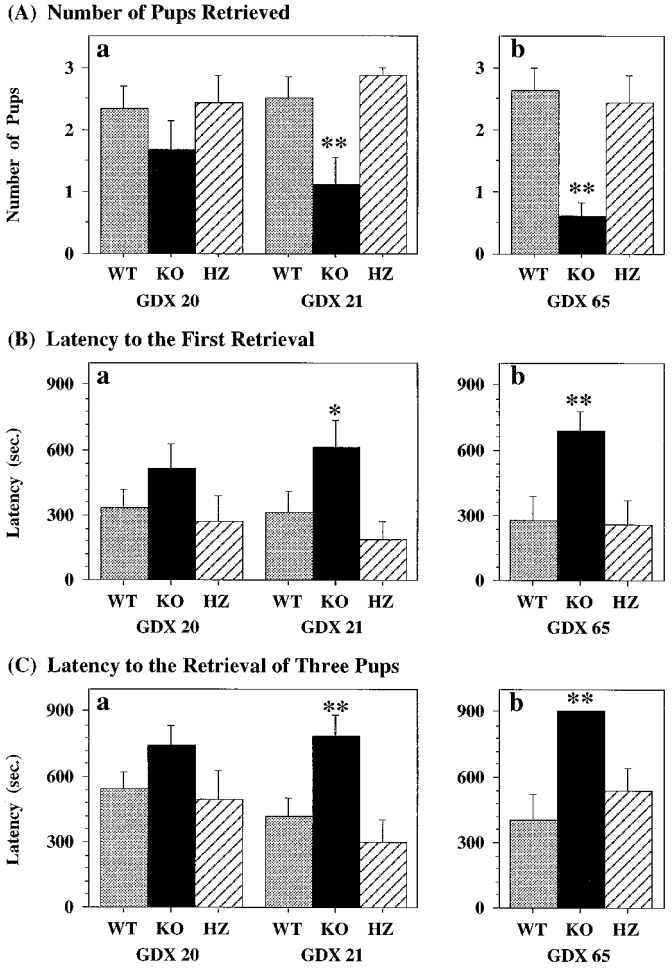

FIG. 4. Genotype differences in parental behavior in gonadectomized females (group 2, B–D) tested 20–21 days (a) and/or 65 days (b) after gonadectomy. Animals that showed infanticide were excluded (see text). ERKO females showed reduced levels of pup-retrieving behavior, measured as the number of pups retrieved (A), the latency to the retrieval of the first pup (B), and the latency to the retrieval of all three pups (C), compared with WT and HZ mice. GDX 20–21: n = 12 for WT, n = 9 for ERKO, and n = 7 for HZ mice; GDX 65: n = 8 for WT, n = 13 for ERKO, and n = 7 for HZ mice. **, $P < 0.05$ *vs.* WT and HZ; *, $P < 0.05$ *vs.* HZ.

hibited infanticide when they were tested 20 days after gonadectomy. These behavioral profiles in gonadectomized females were not different from those in gonadally intact females in the present as well as the previously reported study (13). On the other hand, when tested 65 days after gonadectomy, ERKO female mice still retrieved fewer pups with longer latency compared with females of the other two genotypes, but they no longer showed high levels of infanticide.

The higher incidence of infanticide in gonadally intact ERKO females may be partially due to the elevated levels of testosterone at the time of testing in adulthood. It is known that plasma levels of testosterone in gonadally intact ERKO females are 2 (in University of Missouri-derived mice) (16) to 10 (in NIEHS-derived mice) (17) times higher than those in WT female mice. These elevated levels of testosterone may stimulate infanticide in ERKO females through ARs, as previous studies have shown that dihydrotestosterone is as effective as testosterone in inducing infanticide in gonadectomized female Rockland Swiss albino mice (31). However, a number of studies also showed that estrogen is effective in inducing infanticide in gonadectomized female mice (31, 32). Estradiol levels are also known to be 10 times higher in gonadally intact ERKO females compared with WT female mice (14). Therefore, we cannot rule out the possibility that ERβ also plays a role in the induction of infanticide in ERKO females, although the exact mechanisms and brain regions of its action are not known at this point.

TABLE 5. Behavioral correlation between aggression and infanticide

Intact females			GDX females		
WT	Aggression		WT	Aggression	
	Y	N		Y	N
Infanticide			Infanticide		
Y	0	0	Y	2	0
N	0	19	N	2	17
ERKO	Aggression		ERKO	Aggression	
	Y	N		Y	N
Infanticide			Infanticide		
Y	9	1	Y	9	1
N	4	5	N	4	5

Genotype differences and effects of gonadectomy on behavioral correlations between aggressive behavior toward gonadectomized (either steroid-primed or nonprimed) female intruder mice and infanticide. Y, Number of mice that showed behavior; N, number of mice that did not show behavior.

There is much evidence suggesting that increased levels of infanticide in ERKO females may not be due simply to the steroid hormone environment at the time of testing. The lack of ERα stimulation during the perinatal period may play a role in the behavioral modification found in ERKO females. It is known that neonatal androgenization after gonadectomy in female mice has a suppressive effect on testosterone-inducible infanticide in adulthood (33). Furthermore, this study has shown that the effects of neonatal testosterone are partially blocked by concurrent injection of the antiestrogen MER-25. Therefore, it is possible that ERKO females might have elevated levels of testosterone during the neonatal period, but due to the lack of ERα stimulation, they might develop greater responsiveness to testosterone in adulthood (which is known to be elevated), thereby inducing infanticide. In addition to the neonatal period, the fetal (34, 35) and peripubertal (32) hormonal environments may need to be considered to elucidate the exact mechanism of behavioral modification in ERKO females.

Finally, it is also conceivable that the increases in infanticide as well as aggression toward female intruder mice in ERKO mice might be due to the alteration in the processing of chemosensory information. Particularly, chemosensory cues from pups are known to be important inhibitors of infanticide in both sexes. Therefore, ERKO females, like males, might show greatly increased levels of infanticide because they failed to properly process chemosensory cues from pups. To date, it is known only that ERKO mice are

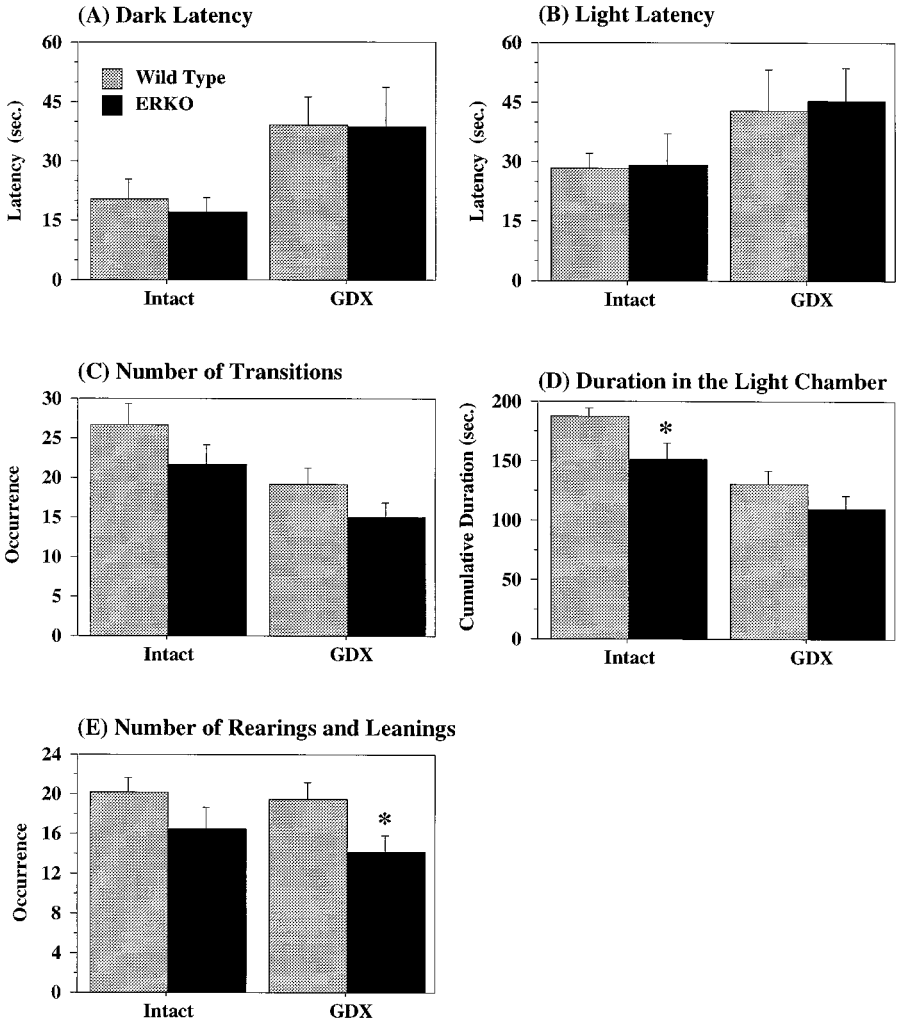

FIG. 5. Effects of ERα gene disruption and gonadectomy on the dark latency (A), light latency (B), number of transitions (C), cumulative time spent in the light chamber (D), and number of rearings and leanings in the light chamber (E). ERKO females tended to be less active than WT females regardless of gonadal state. ERKO females also spent less time in the light chamber compared with WT females, although the latency to enter the dark chamber or exit to the light chamber for the first time was not different between the two genotypes. Separate groups of mice were used for each gonadal condition. Intact: n = 13 for WT and n = 16 for ERKO mice; GDX 28: n = 9 for WT and n = 14 for ERKO mice. *, $P < 0.05$ *vs.* WT.

capable of smelling certain strong odors that are most likely processed by the main olfactory system. It is not known whether their accessory olfactory system and processing of pheromonal cues remain intact.

Anxiety and general activity

Gonadally intact ERKO females spent less time in the light compartment during the dark/light transition tests than WT females. In the open-field tests, ERKO females were less active and also spent less time in the center area (Ogawa, S., K. S. Korach, and D. W. Pfaff, unpublished data). Although the difference was relatively small in both cases, these results consistently suggest that anxiety and fear levels might be increased in ERKO female mice compared with those in WT females. These findings are dramatically contrasted to those found in gonadally intact male ERKO mice, which showed increased levels of activity and spent more time in the center area during the open-field tests compared with both WT and HZ male mice (11). The present study also revealed that genotype differences in the dark/light transition tests were not abolished by gonadectomy in female mice. Our recent findings in the open-field tests also confirmed that gonadectomized male ERKO mice were more active and spent more time in the center field than male WT mice, whereas gonadectomized female ERKO mice were less active and spent less time in the center field compared with female WT mice (Ogawa, S., K. S. Korach, and D. W. Pfaff, unpublished data). Taken together, these findings suggest that ERα gene disruption affects nonsexual and nonsocial behaviors in a sexually dimorphic manner.

The findings in the present study taken together demonstrate that ERα gene expression plays a key role in the regulation of female reproductive behavior, which not only includes the lordosis response but also a number of interrelated behaviors, such as parental and aggressive behaviors. Furthermore, they suggest that behavioral effects of ERα gene disruption may not be simply due to the lack of ERα activation at the time of the testing in adults, but may also derive from the lack of ERα gene expression during developmental processes.

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