0013-7227/97/$03.00/0
Endocrinology

Vol. 138, No. 1
Printed in U.S.A.

Estrogen Receptors are Essential for Female Sexual Receptivity

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Abstract. One of the most important, robust and evolutionarily conserved functions for neural estrogen receptor (ER) is as a mediator of female sexual behavior. Using homozygotic ER knockout (ERKO) mice we tested the hypothesis that ER controls female receptivity. Females with either two normal copies of the ER gene (wild-types), or an insertational disruption (knockouts) of the ER were ovariectomized. Each female was treated with 17β-estradiol (EB) alone, and with EB in combination with progesterone, prior to tests for behavioral receptivity. Under both hormonal conditions female ERKO mice did not display sexual receptivity whereas wild-type litter-mates were receptive to males. Male behavior indicated that females of both genotypes were equally attractive. Brain tissues were examined with immunocytochemical methods showed that ERKOs had greatly reduced levels of ER immunoreactivity in hypothalamus. In sum, the data show that ER is required for the display of sexual receptivity, but is not essential for female attractivity.

It is well established that estrogens are critical for the expression of female sexual behavior in all vertebrates (1). Research on this topic has employed a variety of techniques and all data collected suggest that hypothalamic estrogen receptor (ER) regulates expression of female sexual behavior (1). Several lines of evidence show that multiple forms of ER may reside in brain. There is evidence that a membrane bound ER exists in brain (2). In addition, several isoforms of ER mRNA have been found in pituitary and brain (3,4). Recently a second genomic ER (ERβ) has been cloned and sequenced (5).

The experimental approaches used previously cannot determine specifically which subtype(s) of ER regulate sexual behavior. To determine whether the ER that was first characterized (6; also referred to as ERα) mediates female sexual behavior we have employed the ERKO mouse. This mouse was generated from an embryonic stem cell containing an introduced Neo sequence into the second exon of the ER gene (7). In the study reported here, standard behavioral testing paradigms were used to assess sexual receptivity and attractivity of female mice lacking ER, as compared with wild-types.

Methods and Materials

Animals

Mice were mixed 129/J and C57BL/6J background. They were housed on a 12:12 light:dark cycle (lights off at 1300h EDT). Each was housed individually at weaning (18 days of age) and received *ad lib* access to food (Purina mouse chow) and water. Subjects were generated by crossing heterozygotic mating pairs carrying a single copy of the disrupted ER gene (7). The resulting offspring were screened by PCR amplification of tail DNA. The methods used were a modification and simplification of those employed previously (7). The stud males (n=10) used were adult heterozygotes.

Surgery and Hormone Treatment

Adult female mice were subjected to bilateral ovariectomy (OVX). All surgery was conducted under general anesthesia (20 mg Ketamine and 2 mg Xylazine per ml; 0.1 ml per 20 grams body weight). One month after surgery each female received an implant (sc) of estradiol benzoate (EB) dissolved in sesame oil (50 ug in 0.025 ml) infused into a Silastic Implant (1.98mm id/3.17mm od), and sealed with Silastic adhesive. Two to five hours prior to the final 1-2 tests an injection (sc) of progesterone (P; 500ug) was given. After the completion of all behavioral tests EB implants were removed. One week later females were sacrificed and tissues prepared for immunocytochemical analysis.

Behavioral Testing: Pair Mating Tests

All tests were conducted in the dark, under red light illumination, between 1300-1700h. Each female (n=9+/+; n=8-/-) was tested (2-4 times) for sexual receptivity with a stud male. After EB-implantation each female received up to two tests. If the male ejaculated on the first test, the female was not retested. The two

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Received : 10/04/96

tests were conducted three days apart. All females were retested 4-7 days later. An injection of P was given 2-5 hours before testing. Under this regime of combined EB (given by Silastic implant) and P (given by injection) each female was tested 1-2 times. If a second test was required it was conducted 6 days after the first test, each test was preceded by a single injection of P.

Pair mating tests were conducted in clear plexiglass cages (18x38cm) placed on a mirror stand to allow ventral viewing and permit the observer to distinguish between mounts that did and did not include penile insertion into the vagina (intromissions). Stud males were placed into the test cage 2-3 hours prior to the introduction of the female. The tests lasted for 30 minutes, or until the female received an ejaculation. If the pair was engaged in mounting at the end of 30 minutes the test was continued until either the male ejaculated or the pair stopped interacting for 5 minutes. During the tests an observer (blind to the genotype of the female) recorded the latencies and numbers of each attempted mount, mounts with intromissions, and ejaculation. For each mount attempt, it was noted whether the female stood still. A lordosis quotient (LQ) was calculated by dividing the number of mounts during which the female stood still, in a lordosis posture, by the total mount attempts and multiplying by 100. A receptivity score for each female was also determined based on normal female behaviors such as squeaks, attempts to avoid mounts, rearing and kicking (8). For data analysis the tests which included ejaculations were used. If the female did not allow ejaculations on either test, under a given hormonal treatment, data for both trials were averaged.

Preference Tests

Two weeks after the final receptivity test female attractivity was assessed. The plexiglass test box was divided into three areas. The two areas on each end were of equal size (31.5x25.5cm), the middle "neutral" section was the smallest area (10.5x25.5cm). A tethered wild-type female was randomly assigned to one end of the test box. An ERKO was tethered on the other end. The stimulus OVX females (n=4 of each genotype) were treated with EB implants. Ten stud males were used as subjects. Each had prior experience in the testing box. Males were placed in the middle section at the start of the 10-minute trial. The males thus had free access to the entire test box while the stimulus females were confined to their separate ends. Placement of the tethered females was reversed for every other test. The number of visits to each side of the test box, and the total amount of time spent on each side was recorded.

Statistics

Data were analyzed with Student t-tests, unless they failed to meet the requirements for normality, in those cases Mann-Whitney U tests were employed. Fisher exact tests were used to compare proportional data.

Immunocytochemistry

Animals were deeply anesthetized with sodium pentobarbital (6 mg I.P) prior to perfusion through the aorta with heparinized saline, followed by modified Zamboni's fixative. Brains were removed and cryoprotected in 20% sucrose and then quickly frozen. Serial coronal sections (30 μm) were collected and the tissue was processed for immunocytochemistry using the monoclonal ER antibody H222 (generously provided by Abbott Labs). The procedures and controls for these immunocytochemical technique have been previously described (9). Tissues were incubated in H222 (1:1,000) for 48 hours at 4°C. After rinsing the tissues were incubated twice through biotinylated rabbit anti-rat IgG (1:500; Vector Labs), and Avidin Biotin Complex (1:1,000; Vector labs) to intensify the final reaction product. Immunoreactivity was visualized with nickel intensified DAB and 0.001% hydrogen peroxide.

Results

ER is Required for Behavioral Receptivity

Female ERKO mice were significantly less sexually receptive to males as compared with wild-type litter mates. None of the ERKO animals allowed males to mate to ejaculation. In contrast, 55% of the wild-type females received ejaculations during their tests after EB-priming alone, and 78% mated when EB was given with P ($p<0.02$). Under both hormone priming conditions, females lacking a functional estrogen receptor had significantly lower receptivity and lordosis quotient scores ($p<0.004$, Figure 1) as compared with wild-type females. Further, LQ and receptivity scores improved in wild-types females after P was given ($p<0.01$), but there was no response to P in the ERKO females ($p>1.0$).

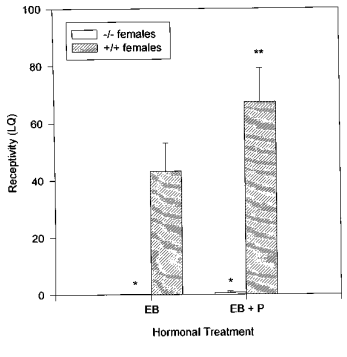

Fig 1: Receptivity as measured by LQ (mean ± SEM) in wild-type (+/+) and ERKO (-/-) mice. * Significantly different from +/+ receiving identical hormone treatment ($p<0.004$). ** Significantly different from +/+ treated with EB ($p<0.01$).

ERKO Mice are Attractive to Males

Female attractivity was not affected by genotype. During the pair mating tests, under both hormone treatments, stud males attempted to mount ERKOs as rapidly as they mounted wild-type females ($p>0.3$). The ERKO females received as many mount attempts as did wild-type animals ($p>0.7$). However, males tested with wild-type females were able to attain intromissions. Despite the fact that males persisted in mounting, the ERKO females did not stand during mounts, thus, males were unable to achieve penile insertion ($p<0.02$, Figure 2). In addition, in preference tests stud males spent an equivalent amount of time with OVX, EB treated, wild-type versus ER deficient females. Males also visited females of both genotype with equal frequencies ($p>0.1$).

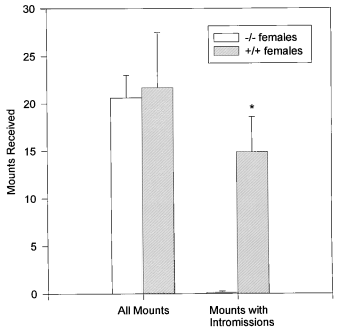

Fig 2: Attempted mounts and intromissions (means ± SEM). All OVX mice were treated with EB and P. * Significantly different ($p<0.003$) as compared with tests including ERKOs

ER immunoreactivity is reduced in ER knockouts

The immunocytochemistry revealed either a complete absence, or greatly reduced ER immunoreactivity (ER-ir) in the hypothalami of individual ERKO mice (Figure 3).

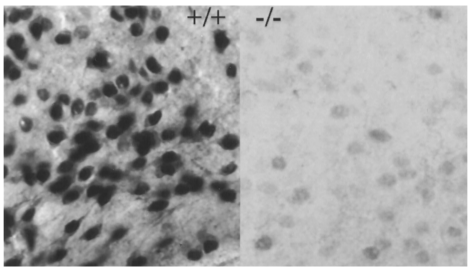

Fig 3: A high magnification photomicrograph of ER-ir in the lateral ventromedial hypothalamus of two OVX females. The section on the left is from a wild-type female, on the right is a section from an ERKO.

Discussion

Our results provide conclusive support for the hypothesis that ER is essential for the display of receptivity in female mice. These findings support and confirm data collected in a vast number of studies conducted in a variety of vertebrate species which show, albeit indirectly, that ER is required for normal female sexual behavior (1). Moreover, the present study offers several new and important insights. First, past studies rely on the use of pharmacological agents, brain surgeries and/or steroid hormone implants into brain. Many commonly used estrogen receptor blockers have estrogenic actions (10). In surgical experiments sham-operated control animals are typically compared with animals that receive lesions or implants. Yet, secondary effects of surgery on behaviors can never be completely eliminated. Thus, results of these types of studies need to be interpreted with caution. The use of the ERKO for this work obliviates these pharmacological and/or surgical confounds.

Recently, a second full-length estrogen receptor has been cloned and sequenced (5). This discovery leaves open the possibility that the ERβ, not the ER, regulates sexual receptivity. None of the previous data, collected with other experimental models, discriminates between these two ERs. The ERKO mice lack functional estrogen receptors, but, ERβ mRNA is present in ERKO tissues (Couse, Gustafsson and Korach, unpublished data). However, the ERβ, if it is present in ERKO brain, is not able to substitute for ER, which is essential for female receptivity.

It is possible that the faint, but detectable, levels of ER-ir in hypothalami of some ERKO females represents the smaller variant ER which is present in very low levels in ERKO uterine tissues (14). The antibody we employed for the ER-ir (H222) is a monoclonal made against the ligand-binding region of the ER. This antibody will recognize both the variant and wild-type forms of ER (14), and perhaps ERβ, conclusive results on ERβ reactivity with H222 have not been reported.

In the present study, wild-type mice displayed enhanced receptivity when they were treated with both EB and P. In contrast, there was no synergistic effect of EB and P in the ER knockouts. In several rodent species if estradiol is given alone after OVX, pharmacological doses must be used to elicit receptivity. Progesterone treatment alone has no effect, it is unable to induce receptivity (1,12). Many biochemical and histological studies have confirmed that the hypothalamic neurons that contain ER also contain progesterone receptor (PR) protein (12). In ERKOs treatment of uterine tissue with estradiol does not promote PR transcription (14). It is likely that the same is true in brain, thus ERKOs do not respond to

P injections because they lack neural PR. Like the ERKOs, PR knockout mice do not show receptive behavior (13). A very low dose of EB was used to treat the PR knockouts, one that did not facilitate receptivity in wild-type females. In our study, and in past work on other mouse strains, high doses of estradiol alone have been shown to restore receptivity (8). It would be useful to know if large doses of EB could promote some sexual receptivity in PR knockouts. This would give us additional insight into the role of ER in controlling receptivity, particularly through pathways that do not involve PR.

The fact that the ERKO mice were as attractive to males as the wild-type females is an important finding. First, it makes us confident that our assessments of receptivity are accurate. If the males were not attracted to the ERKO females we would not be able to evaluate female receptivity. The finding also supports the hypothesis that different aspects of female sexual behavior are regulated by different neural substrates (11). Additional studies are currently underway to assess aspects of proceptive behavior in the ERKOs.

Estrogen receptor knockout females have ovaries that contain immature and atretic follicles, immature reproductive tracts and elevated levels of estradiol in plasma (14). In addition, female ERKOs have elevated concentrations of androgens in plasma (15). Excess androgens have behavioral effects in both sexes, independent of ER. For these reasons, behavioral experiments conducted on neuroendocrine knockout mice should utilize gonadectomized animals given steroid hormone replacement. A final caveat to interpretation of behavioral data collected on ERKOs is that the mice are deprived of estrogen's actions on brain both developmentally and in adulthood. Thus, behavioral alterations detected in adults can be caused by the lack of both organizational effects during early development and/or activational effects of estrogens.

Acknowledgements

We thank Drs. S. Wersinger and X. Li for technical assistance, and Abbott Labs for the H222 antibody (H222) used in this work. This work was supported by NSF grant IBN 94-12605 and NIMH RSDA award K02 MH01349 (EFR).

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