

Interactive report

Sex with knockout models: behavioral studies of estrogen receptor α ¹

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Abstract

Estrogens are an important class of steroid hormones, having multiple targets, in the body and brain, and exerting ubiquitous effects on behavior. At present, two estrogen receptors (ER α and β) have been cloned and sequenced in mammals. In the brain these receptors are regionally specific, but both have widespread distributions, which are largely non-overlapping. Given the newly emerging complexities of estrogen's mechanisms of action it is important to distinguish which pathways are involved in modifying which behaviors. We use a knockout mouse, lacking functional copies of the estrogen receptor α (ER α) gene, to study the mechanisms by which estrogens mediate behaviors. There are pronounced ramifications of ER α gene disruption on behavior. First, female ER α knockout (ER α KO) mice do not display normal feminine sexual behavior. Second, treatment of adult mice with androgens promotes masculine sexual behavior in both sexes. However, male-typical sexual behavior is severely compromised in male and female ER α KOs. Third, male ER α KOs do not exhibit the same social preferences for female mice as do wildtype (WT) littermates. Thus, the ER α is essential for normal expression of sexual behaviors. In addition, gonadectomized ER α KO and WT mice rapidly learn to escape from the Morris water maze. Exogenous estrogen treatment prevents WT females from learning this task, yet, has no effect in ER α KO mice, suggesting that estrogens effects on learning in adult females involves the ER α . Based on these data we hypothesize that ER α mediates many of the effects of estrogen on sexual behavior, learning, and memory. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The steroid hormone, estrogen, is produced primarily by the ovaries in females, and the testes in males. The final step in estrogen (E) biosynthesis is the aromatization of androgens into E. This process occurs in the gonads, adrenal glands, adipose tissue, and in the brain. Because testosterone (T) can act either at the androgen receptor (AR; directly or after conversion to dihydrotestosterone), or the estrogen receptor (ER) behavioral neuroendocrinologists have asked whether it is the activation of the AR, ER, or both that ultimately stimulates behavior. It is now clear that E regulates many social and reproductive behaviors [36–39,58]. Even masculine sexual behavior requires estrogen [39]. However, it is unknown whether E activates behavior via the ER α , ER β , or another pathway. The

ER α KO mouse offers a unique opportunity to differentiate the role of ER α from these other ERs in behavior.

In the past ten years the study of estrogen's influence on behavior has expanded as it has been demonstrated that non-reproductive behaviors can also be modified by this hormone (see *Hormones and Behavior* vol. 34, Dec., 1998). As human populations age and women experience natural estrogen decline during menopause, the effects of this hormone, or its absence, on cognitive function have been noted [38,63]. Estrogen replacement therapy is commonly prescribed to ameliorate the effects of estrogen withdrawal on behavior, bone integrity, and tissue elasticity. However, there are risks associated with long-term administration of estrogen, particularly cancer cell growth. Thus, over the past few years the study of behavioral action of estrogens has been transformed from a specialty issue to one of general concern for public health. The discovery of multiple estrogen receptors has opened the door for the formulation and development of specific agonists and antagonists, which could act on brain versus bone versus breast and other reproductive tissues. However, before these drugs

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can be developed, basic research needs to delineate which estrogen receptors mediate which of estrogen's many effects.

In our laboratory we study neuroendocrine regulation of behavior. We are using the ER α KO mouse [34] to assess which of the behavioral effects of estrogen are mediated by the ER α . In this review we describe the work we have conducted with this knockout (KO) model and speculate about the specific role of the ER α in specific behaviors.

2. Estrogen receptors and the ER α KO mouse

The ER is a member of the steroid receptor superfamily, and until recently, was thought to be essential for embryonic survival, in part because no natural mutations (in any vertebrate species) of the gene had been found. The discovery five years ago of an adult man with estrogen resistance caused by a disruptive ER gene mutation [74] indicated that ER mutations were not necessarily lethal. In addition, at that same time a transgenic mouse model [34]

was developed to study ER function. In this mouse exon 2 of the ER gene was disrupted by the insertion of a neomycin-resistance gene. The resulting 'ER α -knockout' or 'ER α KO' mouse lacked functional copies of the only ER gene isolated at that time (now designated the ER α). In contrast to expectations, the mice are born live and survive to adulthood with normal gross external and internal phenotypes [9,12,15,16,34]. However, both male and female homozygotes are infertile and there are subtle differences between KO and WT littermates [10,15,16,62].

Until a few years ago, it was believed that estrogens acted through a single nuclear receptor, and all ER function was attributed to this single receptor (see Fig. 1). The classically accepted mechanism of ER function was one of ligand-activated transcription of specific target genes [2]. The notion of a single ER was challenged when alternately spliced forms of ER α , either having sequence changes upstream of the exon 4/5 boundary [20,69], or lacking exon 4 were discovered [27,73]. In 1996 a second full-length estrogen receptor, ER β , with a high binding affinity for 17 β -estradiol, was cloned and sequenced from rat

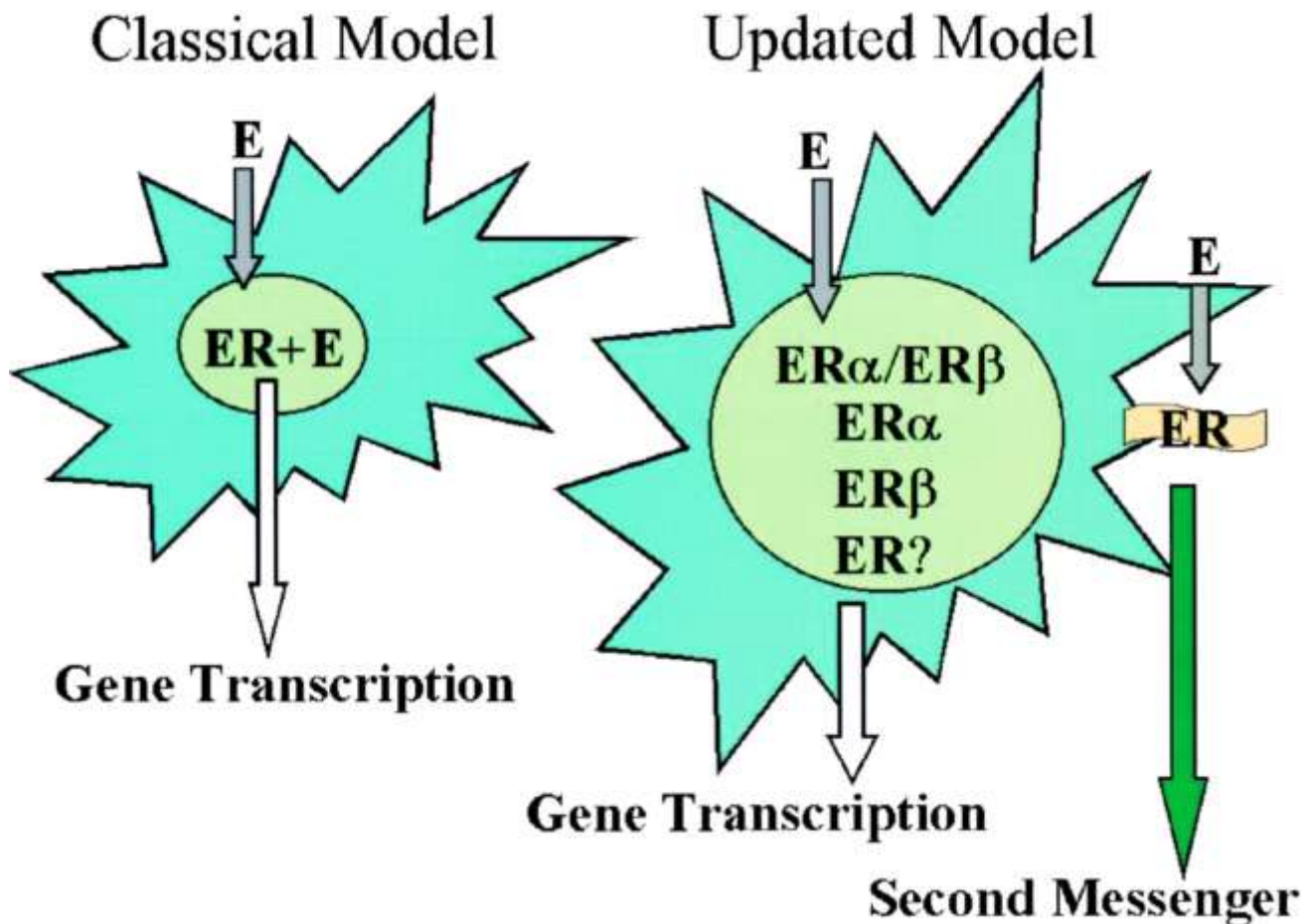


Fig. 1. A representation of the classical model of estrogenic action (left) and an updated model (right). The classical model includes a single nuclear receptor which, when bound to ligand, activates gene transcription. The updated model includes the ER α and ER β receptors, the possibility that these receptors for heterodimers, and the potential for as yet unidentified nuclear ER receptors. All of these would act on gene transcription. In addition, a membrane bound ER would act on a second messenger to alter cellular processes.

prostate and ovary [31]. Estrogen receptor β has since been found in other tissues, including brain, and in other species, including humans [6,30,32,43,64,77].

The roles of and relationship between the two estrogen receptors has yet to be determined. Estrogen receptor α and β mRNA are expressed in nearly equal amounts in rat brain, although proportions of each receptor type differ in specific regions [30,65]. In the rat brain, the majority of ER α mRNA and protein is present in limbic nuclei, the preoptic area (POA), the hypothalamus and midbrain nuclei that have reciprocal connections with the hypothalamus (HT [65]). Estrogen receptor β mRNA and protein are found in many of the same areas, and are present in the olfactory bulb, hippocampus, cortex, and cerebellum [32,65]. Recently a subpopulation of cells in the POA, bed nucleus of the stria terminalis (BNST), and the medial amygdala have been shown to express ER α protein and ER β mRNA [68]. Finally, *in vitro*, ER α and β can form both homodimers and heterodimers (Fig. 1; [55,57]). This opens yet another genomic pathway by which estrogen can affect gene transcription. Thus, ER α (and ER β) KO mouse models play an increasingly important role in distinguishing the effects of the various ERs on estrogen-mediated behaviors.

Recently it has become clear that the classical genomic mode of E action does not fully explain E's action *in vivo*. Several reports describe responses to estrogens that are difficult to explain with a genomic mechanism [19,25,42,60,75]. These studies suggested that an estrogen responsive protein could be present in the membrane, coupled to a second messenger signaling mechanism (see Fig. 1). In addition, the newly described ara70 protein, which allows estrogen to activate the AR provides yet another mechanism by which E can act on transcription [88]. The physiological significance of all of these different E binding proteins has not yet been determined, but characterizations of the independent and dependent actions of different ER proteins are beginning [8,12,40,77]. Finally, it is also possible that estrogen is metabolized into other neuroactive forms that act via a mechanism which is independent of E binding proteins and therefore is not blocked by antiestrogen treatment [12]. Thus, the number of mechanisms by which E can exert biological effects is ever expanding. Given the dearth of specific agonists and antagonists for these various pathways, the ER α KO model is one of the best ways to specifically test the role of the ER α in a given process.

3. Caveats specific to the ER α KO mouse

The ER α KO mouse presents a clean ablation of an important steroid receptor system. Use of these mice avoids complications introduced by neural lesions, variable spread of drugs or hormones at the site of release, or antagonists, which may have estrogenic effects under some conditions

(e.g. tamoxifen). With the ER α KO mouse there is permanent suppression of gene activity, unlike the short-term effects obtained with anti-sense oligonucleotide treatment. There are no drug side-effects or concerns about compounds that do or do not cross the blood–brain barrier (for example the anti-estrogen ICI 182,780). At present there are no estrogen agonists or antagonists that act selectively on a specific ER protein, thus, the ER α KO mouse is the optimal model to distinguish the actions of estrogens on multiple receptors. Further, the recently engineered ER β KO mice [29], when studied alongside the ER α KO, and the inevitable production of double mutants will provide an increasingly accurate understanding of the complex roles of these two receptors.

However, as with any model system there are important caveats, which have to be considered when experiments are planned and data are interpreted. First of all, as is true for any gene KO model, important pleiotropic effects of the disrupted gene can be discovered. Estrogen, as we have stated, has multiple targets and functions. One feature of this steroid is that it is involved in maintenance of the brain–pituitary–gonad feedback axis. Thus, ER α KO mice differ from WT littermates in levels of plasma concentrations of endogenous sex steroid, and pituitary hormones, neural expression of steroid receptors, and quantity of neurotransmitters; particularly those involved in the steroidal regulation of gonadotropin releasing hormone [9,33,62,82,83]. The second caveat is that steroid hormones are involved in brain development [76]. The ER α KO mice lack functional ER α at all times, and from all tissues which makes it difficult to distinguish if a behavior is disrupted because of a developmental failure in estrogen-regulated neuronal formation, or a failure in adult estrogen-activated circuitry. Finally, in the ER α KO mouse there is transcription within the neomycin-disrupted region of the ER α gene and translation of a truncated protein [10]. This protein appears to bind estrogen. Although in theory this mutant ER should lack DNA binding function, this has not yet been demonstrated *in vivo*.

The ER α is an integral part of the hypothalamic–pituitary–gonad feedback axis. This was demonstrated early on simply by assessment of plasma levels of estradiol (E₂) and luteinizing hormone (LH) in ovary-intact ER α KO females [9]. Female ER α KO mice have elevated levels of both E₂ and LH as compared with controls. In addition, testosterone (T) levels are elevated in plasma of both sexes of ER α KO mice [62]. More recently work in both male and female ER α KO mice demonstrates that steroid negative feedback on LH is mediated by ER α in females and that the ability of the androgen receptor (AR) to mediate feedback in males is disrupted by the ER α mutation [33,83]. Because levels of circulating steroid hormones are significantly affected by this gene disruption it is important that behavioral studies are conducted in animals with equivalent levels of hormones. Decades of research have demonstrated that there are dose-dependent effects of sex

steroids on sexual, aggressive, maternal, and a host of other behaviors. Unfortunately, simply giving equal doses of a hormone to WT and ER α KO littermates does not guarantee that the brain is exposed to the same concentrations of the steroid. For example, the ER α disruption may influence the half-life of the steroid, production of steroid-binding proteins, and/or the activity levels of enzymes (such as 5 α -reductase and aromatase), which catalyze the conversion of steroids in the biosynthetic pathway. An additional manner in which the same dose of a steroid may have different effects in the brains of ER α KO and WT mice is via effects on steroid receptors. For example, we have shown that after equivalent treatment with testosterone WT mice have more androgen receptor (AR) immunoreactivity in the bed nucleus of the stria terminalis (BNST) as compared with ER α KO mice [85]. Finally, steroid hormones regulate expression and production of many neurotransmitters [13,71]. For example, there is a sexual dimorphism in the rodent vasopressin (AVP) system [13]. In mice, males have many-fold higher densities of AVP immunoreactivity, and concentrations of AVP protein, in limbic regions as compared with females. In ER α KO mice this sex difference is just barely detectable since both males and females have very low levels of immunoreactive AVP [14]. We have speculated that this reduction in AVP may be responsible, in part, for diminished sexual behavior in ER α KOs [85].

As mentioned, the current ER α KO mouse cannot be used to differentiate developmental versus adult (also called activational) effects of ER α on behavior. As noted in other mammals male mice experience a surge in T late in gestation [44,78]. Castration on the day of birth impairs expression of male mouse aggressive behavior in adulthood [43]. Likewise, injections of T to newborn female pups facilitate adult mounting behavior and aggression [17,18]. Many of these T-activated effects are likely caused by E, after it is aromatized from T [39,79]. Thus, an ER is activated during development, however, it is not clear if it is the same ER or another ER that is activated in adulthood to facilitate the expression of behaviors. In the near future, inducible expression of ER α will allow us to determine which ERs are essential during which critical periods.

A final characteristic of this particular KO is that truncated, mutant ER protein, is produced from the disrupted ER α gene [10]. Two mutant proteins (ERKO-E1 and ERKO-E2) have been identified in ER α KO uterus [10], along with ER β [30]. Several lines of evidence suggest that the disrupted ER α gene may also be transcribed and translated in brain. When we examined ER-immunoreactivity (ER-ir) in the brains of ER α KO females using two ER antibodies which were raised against peptide from the ligand binding region of the ER α , immunoreactivity was noted in the same neural regions that contain ER α in WT mice [41,61]. The presence of a truncated receptor does not mean it is functional, but this is an important consideration, particularly since estradiol in-

duces progesterone receptor (PR) immunoreactivity and mRNA in brain [41,66]. Induction of PR-ir (40% of that noted in WT females) was observed in the ventral medial hypothalamus (VMH), a region that is nearly devoid of ER β mRNA in ER α KO brain [67]. Reverse transcriptase-PCR revealed the 3' end, but no 5' end of the ER α gene in the VMH, along with ER β mRNA [41].

4. General methods

Our original breeding pairs of mice which were heterozygotic for the ER α gene disruption were generously provided to us by Dr. Dennis Lubahn (University of Missouri). The ER α KO mice were generated from a mixed 129/J and C57BL/6J background and our colony is in the 7th generation of crosses into the C57BL/6J genetic background. Genetic background is an important consideration since mouse strains differ both in respect to expression of behaviors and also in steroid regulation of behavior [70]. For example, the first generation of ER α KO mice were produced with ES cells (129/J) and C57BL/6J founders, these offspring had a 50:50 representation of genes from each parental strain. Subsequent generations have been backcrossed into the C57BL background. By the 10th generation their genome is 99% identical to that of the C57BL parental strain. Thus, it is possible that if the parental strains display differences in their behavioral endocrinology some of the data collected from the F₂ versus the F₁₀ KO mice could be significantly different based on the difference in genetic representation of the 129 versus C57BL genomes.

We generate mice for our studies by pairing heterozygotic animals and genotyping their offspring by PCR analysis of tail DNA [34]. Homozygotic mice, either wild-type (WT, +/+), or knockout mice which possess two copies of the disrupted ER α gene (ER α KO, -/-) are used for our experiments. We use heterozygotes (with one normal and one disrupted copy of the ER α gene) as stimulus animals. At weaning (day 20), and throughout the studies described here, each mouse was housed individually. The mice were supplied with lab mouse chow and water ad lib., and maintained on a 12:12 light:dark cycle with lights off at 1300 (EDT). In all of our studies subjects are at least 50 days of age (adults) at the time of gonadectomy and hormone replacement.

5. Female sexual behaviors

Decades of study has shown that estrogen is required for female sexual behavior; ovariectomy (OVX) eliminates this behavior, estrogen replacement restores it, estrogen receptor antagonists, or treatment with ER-antisense oligonucleotides counteract the effects of the steroid [58]. However, given the newly discovered ER β , other ER isoforms,

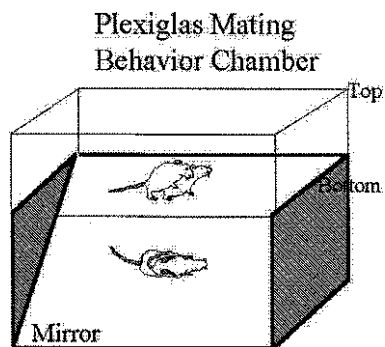


Fig. 2. A drawing of our sex behavior testing box. This Plexiglas rectangular box is placed on a mirror stand that allows ventral viewing.

and the potential for a membrane ER, it is not clear which responsive element(s) mediates estrogens' effects on female sex behavior. Our studies show that one aspect of female sexuality is mediated by ER α ; receptivity. However ER α does not mediate a second aspect of sexual behavior, the female's attractivity.

Ovariectomized WT and ER α KO littermates were treated with 17 β -estradiol benzoate (EB) in Silastic implants [61]. All females were tested for sexual behavior twice after EB treatment alone, and two more times, after treatment with EB supplemented with progesterone (P). Tests were conducted under red light illumination during the dark phase of the light cycle. Stud males were habitu-

ated to an empty, clear Plexiglas testing box prior to the introduction of the female. Test boxes were placed on a mirror stand to allow ventral viewing (Fig. 2). Under these condition observers, blind to the genotypes of the mice, tested females for 30 min, or until they received an ejaculation (whichever occurred first).

Female ER α KOs failed to show receptive postures in response to mounting attempts from studs, whereas 80% of the WT littermates treated with the same hormones were receptive. Males mounted females of both genotypes an equal number of times but only attained penile intromissions and ejaculations with WT females. These results suggest that one component of female sexual behavior, receptivity, was diminished by ER α gene disruption. However, another aspect of the females' sexuality, her attractivity to a male, was unaffected by disruption of the ER α . This conclusion is based on the stud males' behavior during the mating tests. When tested with females that received either EB only, or EB and P, stud males attempted to mount ER α KOs as rapidly as they mounted WT females. The ER α KO females received as many mount attempts as did WT animals. Despite the fact that males persisted in mounting, the ER α KO females did not stand still during mounts and the males were unable to achieve penile intromissions. Our findings on sexual behavior in ER α KO females have been replicated by Ogawa et al. [50].

Social Preference Chamber

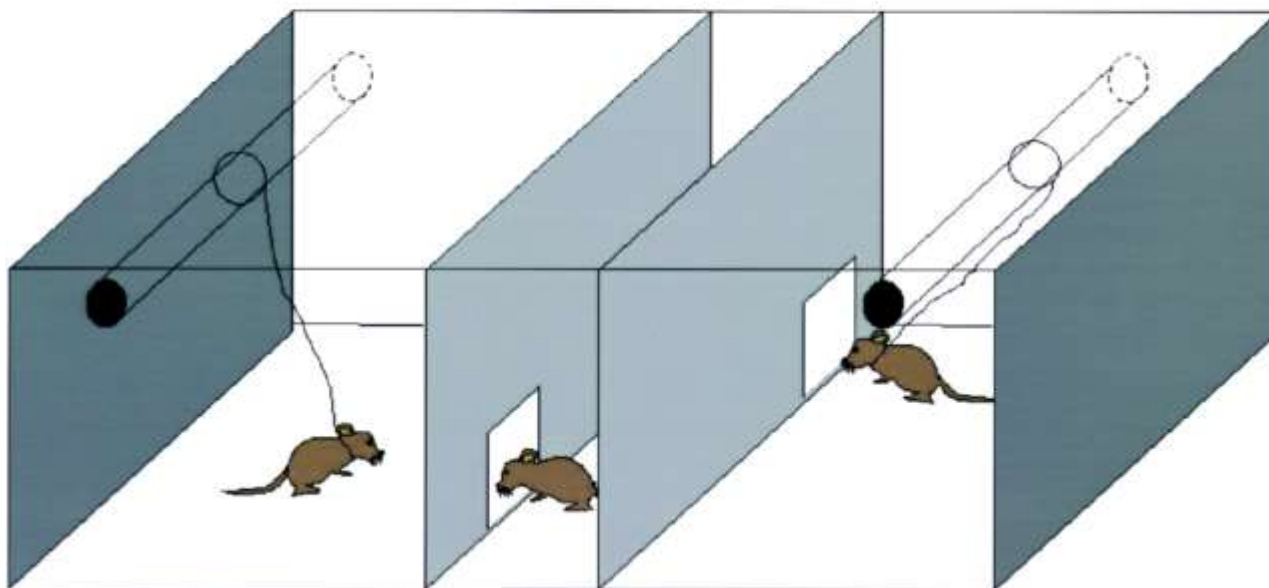


Fig. 3. A drawing of our social preference testing box. This three chamber box has a middle neutral area where the subject can escape from either stimulus animal. The two stimulus animals are tethered in the two end sections. Stimulus mice can move freely within their compartment but cannot move into other sections of the box.

Attractivity was also unaffected by the ER α gene disruption when we tested males' social preferences. To assess whether stud males were equally attracted to WT versus ER α KO females, females (ER α KO versus WT) were tethered in opposite ends of a test box [61]. The box was divided into three sections; the areas on each end were of equal size (31.5 \times 25.5 cm) and were separated by a smaller (10.5 \times 25.5 cm) middle 'neutral' section. The females were leashed with lightweight nylon-line attached to a collar that was securely fastened around their neck. The other end of the line was secured loosely to a bar across the top of the testing chamber. Thus, the tethered females could move about their compartment but could not exit (Fig. 3). Stud males spent an equal amount of time during a 10-min test with OVX, EB-treated WT and ER α KO females. These data indicate that females of both genotypes were equally attractive to males. The fact that the ER α KO mice were as attractive to males as their WT littermates is important, since it allows us to be confident that our assessments of receptivity are accurate. If the males were not attracted to the ER α KO females we would not be able to accurately evaluate female receptivity. The finding also supports the hypothesis that different aspects of female sexual behavior are regulated by different neural substrates [58].

6. Masculine sexual behaviors

Castration and hormone replacement studies show that adult male sexual behavior is dependent on testicular hormones, although the behavior can take many weeks to attenuate after castration [39,81]. Male mice display a rich assortment of courtship behaviors including; attraction to female chemosignals, vocalizations to females and their odors, and territorial scent marking. The steroids required for these behaviors have been examined, as has the hormonal basis of copulation. In mice, E, T, or other aromatizable androgens can reinstate male copulation after castration [35,81], however, strain differences in behavior are notorious. As mentioned above, the ER α KO mice were generated from a mixed 129/J and C57BL/6J background. Previous research on C57BL/6J \times AKR/6 hybrids demonstrates that male courtship behaviors are reduced by castration and reinstated by T and E, but typically not by DHT treatment [3,28,46,47]. Intracranial implants of T in the medial preoptic (mPOA), anterior hypothalamus (AH) or the ventromedial hypothalamus (VMH), but not in the septum partially reinstate urine marking to pre-castration rates [48]. Testosterone implants in the mPOA, but not in any other brain region restore ultrasonic calling. Interestingly, E₂ implanted in the septum or the mPOA increases frequencies of both urine marking and ultrasonic calls. In a few strains (other than C57BL/6J) the AR, not an ER, appears to regulate masculine behavior

[35,49]. A spontaneous point mutation in AR renders this receptor non-functional [7] in so called Tfm/Y (now called the AR^{tfm}) mice. These genetic males have a female phenotype and fail to display masculine sexual behavior when gonad-intact. After castration, and subsequent treatment with high doses of E₂, with or without DHT, about 25% of these mice will mate to ejaculation [54]. The data indicate that male mouse courtship and copulation are likely to be regulated by ER, however, the form of ER that is operational is not yet known. Masculine copulatory behavior is also expressed by female mice after they are ovariectomized and given T replacement [17]. Little is known about which T metabolites and steroid receptors regulate masculine sexual behavior in females.

We have shown that the ER α plays a critical role in expression of masculine sexual behavior ([62,85], Rissman, unpublished data). In the majority of our studies we use castrated males. Each male received an equivalent dose of T in the form of a Silastic capsule implanted on the day of castration. We have tested both sexually experienced and naive males. Tests were typically given over a 3–4 week interval, once every 3–5 days. Depending on the study tests lasted for between 30 min and 4 h each time. The stimulus females (heterozygotes from the ER α KO colony) were OVX and implanted with EB. Three to five hours before testing each female was injected with P (500 μ g) and screened for receptivity with a stud male. Behavior tests were conducted in the same Plexiglas boxes described earlier for tests of female sexual behavior (see Fig. 2).

Under the testing conditions we employed, between 80 and 100% of WT males mate to ejaculation. By contrast, we have never seen an ER α KO male (either gonad-intact or gonadectomized and treated with T) mate to ejaculation ([62,85], Rissman, unpublished data). All WT males that ejaculate mount and intromit with females prior to the ejaculation. By contrast, between 0 and 40% of ER α KO males mounted females and 0–12.5% intromitted ([62,85], Rissman, unpublished data). Recently Ogawa et al. [52] have published data very similar to our own. In their study gonadectomized WT and ER α KO males received daily injections of testosterone propionate (TP).

In addition, we have examined masculine sexual behavior in female mice and have shown that similar to the studies in males, mounting and thrusting behavior is virtually absent in ER α KO females. WT and ER α KO mice were OVX and given T replacement. We tested females under very similar conditions to those described for males. WT females treated with T mount and thrust in tests with receptive females. ER α KO females showed significantly less masculine behavior than WT littermates [85]. This finding emphasizes that display of masculine sexual behavior, regardless of the genetic sex of the subject, relies on the ER α . By contrast, one report [51] on aggressive behavior in the female ER α KO states that aggression 'masculinized', yet clearly the ER α KOs are deficient in masculine sexual behavior.

There are several mechanisms that may explain the reduction in sexual behavior observed in ER α KO mice. One that we are presently examining is that ER α KO and WT males differ in their responsiveness to androgens. We have recently conducted an extensive dose–response study in castrated males treated with one of several replacement doses of T. We found that seminal vesicles in WT males are more sensitive to identical T doses than in ER α KO males (Rissman, unpublished data). This might be the case in brain as well. Our finding that AR immunoreactivity is reduced in the ER α KO BNST could explain the decrease in androgen sensitivity in the ER α KO animals. In a study in which one very high dose of T given to castrated mice, LH was suppressed to a greater extent in WT than in ER α KOs [83].

Alternatively, the ER α gene disruption could affect the peripheral nervous system, and /or the motoneurons in the spinal cord that innervate penile musculature. Although no data have been published on the musculature of the penis in the ER α KO males, the gubernaculum and cremaster muscles are deficient in both ER α KO and heterozygotic males [15]. This may partially explain the low sperm counts and infertility in the ER α KO mice, although heterozygotes are as fertile as WT males [16]. However, several lines of evidence fail to support this hypothesis. First, this explanation cannot be used to explain lack of masculine sexual behavior in female ER α KOs. Since the penis and its musculature are not developed in either WT or ER α KO females (Rissman, unpublished data), there should not be any sensory feedback in females of either genotype. Second the behavioral data in both sexes [52,85] do not support this hypothesis. In rats, penile anesthesia has no effect on the latency with which males attempt to mount females. Further the number of times males, without sensation in the penis, mount increases relative to controls. Thus, lack of peripheral feedback enhances some copulatory behaviors [39]. In contrast the ER α KO males take a longer time than WT males to mount and intromit, and the frequency of these behaviors per test is reduced [52,85], suggesting reduced sexual motivation.

7. Male sexual preferences

Sexual behavior is complex and can be subdivided into different aspects. Although most studies examine performance of sexual behavior in mice, another important feature is sexual motivation. Our data suggest that sexual performance is reduced in male ER α KO in large part due to a motivational deficit. Sexual motivation has been studied in male rats and is androgen dependent [39]. In addition the neurotransmitter, dopamine is involved with both copulatory performance and motivation [5,26]. Some portions of the dopamine system are sexually dimorphic and this dimorphism is reversed in ER α KO mice [72].

Male rodents that are gonad-intact or have T replacement preferentially interact with females versus males. When given a choice between a hormone deplete (OVX) or a hormone-primed female the female with replacement hormones is typically preferred. We tested male preferences in the three-chambered test boxes described above (Fig. 3). In our initial experiment [62] we tested sexually naive ER α KO and WT males after castration and T hormone implant administration. The stimulus animals (all heterozygotes) were stud males, and OVX females treated with an EB implant. These animals were tethered on opposite ends of the test box for 10 min testing bouts. Stimulus animals, and their positions in the test box, were switched after every 4–5 trials. WT males spent significantly more time with female stimulus animals and less time in the neutral section of the box, ER α KOs exhibited no social preferences.

In a similar study WT and ER α KO males were castrated and given T-implants (Rissman, unpublished data). First they were given a choice of associating with another male, or an OVX female treated with an E-implant as in Experiment 1. Again, WT males spent significantly more time with either a female, or a male, as compared to time spent alone, but, there was no difference in the amount of time or numbers of visits to a male versus a female. As we reported earlier, ER α KO males, however, did not display any significant preferences in terms of time spent in each chamber of the arena. Next, males were given a choice of associating with an OVX, or an OVX hormone-primed (EB + P) female. WT and ER α KO males both displayed a significant difference in the amount of time spent with either stimulus animal versus alone. Wildtype males spent the most time with receptive females, then with OVX females and the least amount of time alone. The ER α KO males spent more time with receptive females than alone, but they spent equivalent amounts of time with OVX and hormone primed females. In addition, WT males spent significantly more time with receptive females and less time alone as compared with ER α KO males.

In a third study we used a variation on this testing design and gonad-intact WT and ER α KO males [84]. This time we used anesthetized, untethered, stimulus animals in the three-chambered box to control for differences in the interactions that WT and ER α KO have with the stimulus animals, in particular with hormone-primed females. Our logic was that if ER α KO males were attracted to a hormone-primed female, but aspects of their interactions with them were aversive, the male may not display the association preference. In the first three trials, males were given a choice between a male and an OVX + E female. In three additional trials an OVX female replaced the stimulus male. WT and ER α KO males spent significantly more time in a chamber with a social partner than in the neutral compartment. In addition, all males spent a similar amount of time in chambers containing the female versus the male. Likewise, when given a choice between an OVX and an

OVX + E female, males of both genotypes spent significantly more time in chambers containing a social partner than alone. The aspect of ER α KO versus WT male behavior that did differ was the amount of time they spent sniffing the bodies of the anesthetized stimulus animals. WT males spent significantly more time engaged in chemo-investigatory behavior directed at the female as compared with the male. Likewise, WTs also spent significantly more time sniffing the OVX + E than the OVX female. By contrast, ER α KO males spent on average less than 10 s (out of a 10 min test) displaying chemo-investigatory behavior toward any of the stimulus animals and did not prefer to investigate one stimulus animal more than the other [84].

These findings lead us to examine the olfactory abilities of ER α KO as compared with WT males. We used behavioral and neuroendocrine methods and concluded that both main and accessory olfactory bulb function in ER α KO males is normal [84]. Thus, the chemo-investigatory deficits in the ER α KO males are not caused by a perceptual impairment.

8. Learning in the Morris water maze

It is clear that estrogens can both improve and impair memory. The specific outcome of treatment depends on the task employed and the treatment regimen. In women, a rapid rise in estrogen levels during the menstrual cycle is associated with spatial memory deficits [63]. Similarly, in rodents, an acute rise in E₂ is associated with impaired learning on the Morris water task, a task that requires proper hippocampal-dependent memory function [21, 23,80], however see [4,56]. The impairment in hippocampal-dependent memory may relate to the elevated level of E₂ [24], to rapid fluctuations in other associated hormones (e.g. progesterone) that act to modify hippocampal neural activity [42], or to hormone related rewiring of hippocampal circuits [38,87].

The ER α KO mouse provides a tool to help elucidate the mechanism by which estrogen acts to influence spatial learning. We employed the Morris water maze to test the hypothesis that high levels of E₂ suppress learning through an ER α related mechanism [21]. Adult male and female, WT and ER α KO, mice were gonadectomized and half of the mice in each of the four groups were treated with estradiol benzoate (EB), while the other half received vehicle injections. Injections were administered 28 h and 4 h prior to the first training trial of the cue or spatial discrimination version of the water maze task. Subsequent injections were given 4 h before the first trial of each test day. For the spatial version, mice were trained and tested in a black circular pool (120 cm in diameter), located in a well-lighted room containing an assortment of 2- and 3-dimensional cues. Pool water was maintained at a temperature of 23 \pm 2°C and a black escape platform (10.5 cm

in diameter) was located approximately 1.5 cm beneath the water level. The escape platform was located in the center of one of four quadrants of the pool and remained in the same location relative to distal cues in the room throughout testing. However, for different squads of mice tested, the platform was located in different quadrants to control for possible location biases. A series of 12 trials with rest periods between each was conducted for four consecutive days. We recorded latency to find the hidden platform for each trial. The cue version protocol was identical to that of the spatial version except that the black platform was made visible by attaching a white flag to the top and affixing a white band around the rim which extended 1.5 cm above the water level. Black curtains were hung around the pool to limit distal cues and no intentional cues other than the platform were available to the subject during this phase of training. In addition to randomizing the start locations, the quadrant, which contained the escape platform, was randomized across trials.

A decrease in escape latency over the course of training is routinely used as an indication that animals have acquired a successful strategy for escaping from the pool. In our study mice in 7 of the 8 treatment groups exhibited a decrease in escape latency over training trials and thus, learned to navigate and escape from the water maze. The only exception was seen in the WT females treated with EB, these animals did not show a decrease in escape latency over the course of training. However, when tested in the cued version of the maze WT females treated with EB rapidly learned to find the platform, in fact their behavior was no different from that of WT females treated with oil. This result excludes the notion that motivational or sensory-motor impairments were responsible for the lack of acquisition for WT females treated with EB on the spatial version of this task.

The fact that ER α KO mice can acquire the spatial discrimination task suggests that ER α is not required for learning on this task. Furthermore, since all mice were gonadectomized, the results indicate that changes in behavior were specific to E. There was a non-significant tendency for better performance by oil-treated WT as compared to ER α KO females. Importantly, a genotype difference was not observed for male mice suggesting that the absence or presence of a functional ER α gene is not a marker for background genetics, and as such, behavioral differences are not due to testing two different strains of mice [11]. Our data show that a functional ER α gene is required for E₂ to exert effects on learning in females. In addition, learning differences and the ability of E₂ to modify learning may be due in part to the lack of functional ER α gene during development. For example, E₂ treatments to newborn female rats, result in enhanced learning on spatial tasks during adulthood [53,86]. The effects are likely due to the classic organizational effects of sex steroids on brain structure and function during development.

9. Conclusions and future directions

Rapid advances are being made in our understanding of the mechanisms of steroid actions. Ligand dependent and independent effects of steroid receptors are under study, as is the role of coactivators, and the presence of membrane associated receptors [1,37,59,60]. Much of this work has been conducted in vitro and unfortunately studies of behavior cannot be done in this manner. Thus a gap is present between theoretical mechanisms of action and in vivo validation that these possible pathways act on behavior. The development of genetically engineered mouse models is essential for testing new hypothetical mechanisms. The ER α KO mouse is the first member of what will be a cast of new players to use to study behavioral neuroendocrinology. We are using these animals to assess the role of ER α in motivational and performance aspects of sexual behavior. We are also interested in other behaviors that are regulated by estrogens, in particular learning and memory.

Our work on females shows that sexual behavior requires ER α . However, 40% of the normal complement of progesterone receptors (PR) induced by E are present in the ER α KO VMN [41]. Detailed analysis of this subpopulation will help us to determine if these PR containing cells are completely without function in the sexual behavior circuit or if other E-induced factors are also absent in the ER α KO VMN and these are required along with PR. We have noted new interactions between the ER α and AR which may influence the organism's sensitivity to androgens given in adulthood. We have also shown that ER α gene disruption impairs sexual motivation in males. These findings will help us to distinguish neural pathways involved in copulatory motivation versus performance.

To continue our studies of learning we are employing additional learning tasks that do and do not use spatial memory. We are also asking if the effects of E on learning depend on E-dose. We have found that medium and low doses of estrogen can facilitate some learning in females while high levels of estrogen are inhibitory. ER α KO females appear to learn the relatively complex Morris water maze [21], yet they are not as good as WT when it comes to simple inhibitory avoidance (IA). We have found that low doses of estrogen can facilitate IA learning in ER α KO females [22]. This may be the first indication that the ER β , which is expressed in high levels in the hippocampus, may play a role in learning and memory.

We look forward to the next generation of ER α KO mice, which will be made with Cre-Lox regulation of the ER α . This will allow us to determine when the ER α gene has to be expressed to either organize and/or activate behaviors. The ER β KO mouse and ER α KO \times ER β KO double mutants will be used to examine overlap and independence of function between these two ER proteins. These mice will also assist in the discovery of other ERs. Given that estrogen plays a role in the ontogeny and

development of many diseases (cancer, osteoporosis, heart disease, Alzheimer's, to name a few) there will not be a shortage of interesting mouse models. The challenge for behavioral neuroendocrinologists will be to recognize the virtues, and limitations, of these knockouts and transgenics and gain insight and appreciation into the opportunities these models offer.

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