

# Estrogen Response Element-Independent Estrogen Receptor (ER)- $\alpha$ Signaling Does Not Rescue Sexual Behavior but Restores Normal Testosterone Secretion in Male ER $\alpha$ Knockout Mice

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Estrogen receptor (ER)- $\alpha$  mediates estradiol ( $E_2$ ) actions in the male gonads and brain and is critical for normal male reproductive function. In the classical pathway, ER $\alpha$  binds to estrogen response elements (EREs) to regulate gene transcription. ER $\alpha$  can also regulate gene transcription independently of EREs via protein-protein interactions with transcription factors and additionally signal via rapid, nongenomic pathways originating at the cell membrane. This study assessed the degree to which ERE-independent ER $\alpha$  signaling can rescue the disrupted masculine sexual behaviors and elevated serum testosterone (T) levels that have been shown to result from ER $\alpha$  gene deletion. We utilized male ER $\alpha$  null mice that possess a ER knock-in mutation (E207A/G208A;

AA), in which the mutant ER $\alpha$  is incapable of binding to DNA and can signal only through ERE-independent pathways (ER $\alpha^{-/AA}$  mice). We found that sexual behavior, including mounting, is virtually absent in ER $\alpha^{-/-}$  and ER $\alpha^{-/AA}$  males, suggesting that ERE-independent signaling is insufficient to maintain any degree of normal sexual behavior in the absence of ERE binding. By contrast, ERE-independent signaling in the ER $\alpha^{-/AA}$  mouse is sufficient to restore serum T levels to values observed in wild-type males. These data indicate that binding of ERs to EREs mediates most if not all of  $E_2$ 's effects on male sexual behavior, whereas ERE-independent ER $\alpha$  signaling may mediate  $E_2$ 's inhibitory effects on T production. (*Endocrinology* 148: 5288–5294, 2007)

MALE FERTILITY IS dependent on the sex steroid hormones testosterone and 17 $\beta$ -estradiol. Testicular testosterone (T) secreted during pre- and perinatal periods is necessary to masculinize the external genitalia and brain. Although there is evidence for the requirement of the androgen receptor (AR) in the development and function of the male reproductive tract and masculine behaviors (1), T's effects are exerted largely through its conversion to estradiol ( $E_2$ ) by aromatase and subsequent signaling through the estrogen receptor (ER) (2, 3). The importance of  $E_2$  action in male fertility is demonstrated by descriptions of testicular dysfunction and behavioral deficits in ER $\alpha$  knockout (ER $\alpha$ KO) mice. Although prenatal development of the reproductive tract does not depend on ER, ER $\alpha$ KO males are infertile due to atrophy of the testes and seminiferous tubules, tubule dysmorphogenesis, reduced sperm counts, and impaired copulation and other sexually motivated behaviors (4–7). ER $\alpha$ KO males also display elevated serum T levels, reflecting the absence of ER-mediated enhancement of ste-

roidogenesis in the neonatal period (8). In contrast to ER $\alpha$ , deletion of ER $\beta$  does not impair testicular function, spermatogenesis, or normal masculine sexual behavior in adult mice (9–12); however, ER $\beta$  may influence the timing of puberty (11) and appears to play a role in behavioral defeminization (12, 13). This would suggest a greater requirement for ER $\alpha$  in the development of normal male fertility; however, it remains a possibility that ER $\alpha$  and ER $\beta$  regulate reproductive function together because ER $\alpha$  and ER $\beta$  often form heterodimers and interactions between them have been documented in several tissues (14–19).

In the classical pathway of estrogen action,  $E_2$  binds to the ligand binding domain of ER, inducing conformational changes that allow the receptor to interact with coactivator or corepressor molecules. The ligand-receptor complex ultimately binds as a dimer to estrogen response elements (EREs) in the promoter region of target genes to either activate or repress gene expression (20–23). Whereas  $E_2$  acts predominantly via this pathway, other mechanisms of  $E_2$  action have also been described, such as rapid, nongenomic effects through a membrane-associated ER (24–28). Emerging evidence supports the existence of another pathway in which ER can regulate genes that lack an ERE via protein-protein interaction with other transcription factors, such as c-Fos/c-Jun B [activator protein 1 (AP-1)], specificity protein-1, and nuclear factor- $\kappa$ B (29–38). Whether  $E_2$  regulates

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Abbreviations: AA, ER $\alpha$  mutation E207A/G208A; AR, androgen receptor; CV, coefficient of variance;  $E_2$ , estradiol; ER, estrogen receptor; ERE, estrogen response element; ER $\alpha$ KO, ER $\alpha$  knockout; T, testosterone.

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sexual behavior or the neuroendocrine system via ERE-independent pathways such as these remains to be determined.

The generation of ER $\alpha^{-/AA}$  mutant mice by Jakacka *et al.* (31) provided a unique opportunity to distinguish between ERE-dependent and ERE-independent mechanisms of E<sub>2</sub> action *in vivo*. These mice have a mutation (E207A/G208A; AA) in the DNA recognition sequence of ER $\alpha$ , which selectively eliminates ER $\alpha$  signaling through ERE binding and activation of ERE-containing reporter genes. This mutant receptor can signal normally through protein-protein interactions, as demonstrated by active ER regulation of reporter genes containing AP-1 response elements and ER interaction with Jun *in vitro* (31, 39). Although heterozygote ER $\alpha^{+/AA}$  males are fertile, heterozygote females display ovarian, uterine, and mammary gland defects and are consequently infertile (31).

The goal of the present study was to examine the relative role of ERE-dependent and ERE-independent ER $\alpha$  actions in masculine sexual behavior and the hypothalamic-pituitary-gonadal axis. We used complete ER $\alpha$  null (ER $\alpha^{-/-}$ ) animals and compound heterozygotes (ER $\alpha^{-/AA}$ ), which lack ERE-dependent ER $\alpha$  signaling on both alleles, and thus, E<sub>2</sub> action can occur only through ERE-independent mechanisms. Previous studies demonstrated that the mutant ER $\alpha$  allele in ER $\alpha^{-/AA}$  mice can at least partially rescue some of the phenotypes that result from ER $\alpha$  deletion, including elevated trabecular bone mineral density (40), loss of negative feedback on gonadotropin secretion in the female (41), and tubular dysmorphogenesis and spermatogenesis in the testis (our unpublished observations). In the present studies, we assessed the degree to which the knock-in of this mutated allele, and thus the introduction of ERE-independent ER $\alpha$  signaling, could similarly rescue the impaired masculine sexual behavior and abnormal serum hormone levels observed in ER $\alpha^{-/-}$  males.

## Materials and Methods

### Animals

All animal procedures were conducted in accordance with protocols approved by Northwestern University's Animal Care and Use Committee. The ER $\alpha$  null (ER $\alpha^{-/-}$ ) and ER $\alpha^{+/AA}$  mutant mice were generated as previously described (31, 39, 42). Breeders were backcrossed for eight to 13 generations onto the C57BL/6 line. Compound heterozygotes (ER $\alpha^{-/AA}$ ) were generated by mating heterozygote ER $\alpha^{+/AA}$  males with heterozygote ER $\alpha$  null females (ER $\alpha^{+/-}$ ). ER $\alpha^{-/-}$  mice were generated by mating ER $\alpha^{+/-}$  males and ER $\alpha^{+/-}$  females. All mice were genotyped at weaning. DNA was isolated by digestion of tail tissue and amplified in two separate PCRs to determine the presence or absence of the wild-type ER $\alpha$  and the presence or absence of the knock-in mutation.

Adult male mice were individually caged at weaning and housed under a reversed 12-h light, 12-h dark cycle (lights off at 1000 h) with food and water available *ad libitum*. All males remained individually caged throughout the extent of the study and were not tested for behavior until they reached sexual maturity.

Stimulus female mice were group caged, ovariectomized under isoflurane anesthesia, and given sc injections of estradiol benzoate and progesterone to ensure maximum sexual receptivity. Estradiol benzoate (10  $\mu$ g) was injected 48 and 24 h before testing; progesterone (500  $\mu$ g) was injected 3–5 h before testing.

### Sexual behavior testing

In a protocol modified from Ogawa *et al.* (43), intact male mice were tested twice for masculine sexual behavior (naïve and experienced), a minimum of 3 d apart. All tests were conducted under dim red light

illumination during the dark phase of the light cycle, beginning 2 h after lights off. Stimulus females were first screened with nonexperimental, stud males before being placed in the experimental male's home cage for 30 min. Mounts and intromissions were scored according to the descriptions of McGill and colleagues (44, 45). Repeated, rapid, and shallow pelvic thrusting motions were scored as mounts, whereas deeper thrusts that occurred with a slower rate of pelvic thrusting than mounts were scored as intromissions. An observer blind to genotype recorded the following measures: number of mounts, number of intromissions, mount latency, intromission latency, and ejaculation latency. After the completion of the two 30-min tests, some animals ( $n = 12$ ) were tested in three additional sessions to determine whether repeated sexual experience had an effect on behavior.

### Hormone measurements

Animals were deeply anesthetized with ketamine and xylazine ip, and blood was withdrawn via cardiac puncture at 1500 h. Blood was centrifuged and serum stored frozen at  $-20^{\circ}\text{C}$  until RIA. Serum from each animal was assayed for LH, T, and FSH. Serum LH levels were determined using RP-3 standard and S-11 antibody, generously provided by the National Institute of Diabetes and Digestive and Kidney Diseases; the sensitivity and intraassay and interassay coefficients of variance (CVs) were 0.01 ng/tube, 4.87, and 8.20%, respectively. Serum FSH levels were determined using RP-2 standard and S-11 antibody, also from National Institute of Diabetes and Digestive and Kidney Diseases; the sensitivity and intraassay and interassay CVs were 0.05 ng/tube, 19, and 14.6%, respectively. Serum T levels were measured using a RIA kit from MP Biomedicals (Orangeburg, NY); the sensitivity and intraassay and interassay CVs were 0.02 ng/ml, 8.28, and 13.9%, respectively.

### Statistics

The proportion of animals in each experimental group that exhibited mounts, intromissions, and ejaculation was analyzed using  $\chi^2$  tests. Mount and intromission frequencies for each 30-min test (naïve and experienced) and serum hormone data were initially analyzed with a Bartlett's test of equal variances. If variances differed significantly, the Kruskal-Wallis test and Dunn's multiple comparisons *post hoc* test were used. If the variances did not differ significantly, a one-way ANOVA and Newman-Keuls *post hoc* test were used. The behavioral frequency data represent all test subjects whether or not they engaged in copulation (*e.g.* a male that did not mount was assigned a score of zero for mount frequency). Behavioral latencies, on the other hand, were calculated only for those males that displayed the behavior. Consequently, the sample sizes for ER $\alpha^{-/-}$  and ER $\alpha^{-/AA}$  males were too small to perform statistical analyses on behavioral latencies.

## Results

### Masculine sexual behavior

The proportion of animals exhibiting masculine sexual behavior was significantly different between genotypes in both tests (Fig. 1). Almost all wild-type (ER $\alpha^{+/+}$ ) males mounted in both the first (94%) and second (100%) tests, most intromitted (test 1, 71%; test 2, 82%), and some ejaculated (test 1, 6%; test 2, 18%). In contrast, only one of 14 ER $\alpha^{-/-}$  males (7%) displayed any sexual behavior; this male mounted three times over the course of two tests and achieved a single intromission. Similarly, only three of 17 ER $\alpha^{-/AA}$  males mounted (test 1, 18%; test 2, 12%); two of these males intromitted (test 1, 12%; test 2, 6%) and none ejaculated.

In both behavioral tests, mount frequency was significantly lower in ER $\alpha^{-/-}$  and ER $\alpha^{-/AA}$  males, compared with ER $\alpha^{+/+}$  male counterparts ( $P < 0.001$ ), as was intromission frequency ( $P < 0.01$ , Fig. 2). Wild-type males demonstrated almost twice as many mounts in the second behavioral test as in the first test, whereas ER $\alpha^{-/-}$  and ER $\alpha^{-/AA}$  males did

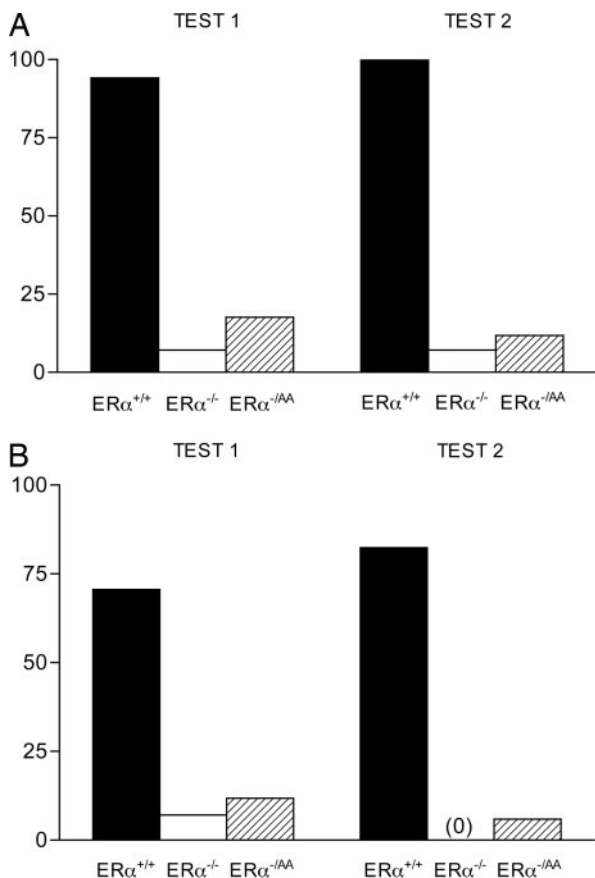


FIG. 1. Proportion of male mice displaying mounts and intromissions. Wild-type (ER $\alpha^{+/+}$ ), ER $\alpha$ KO (ER $\alpha^{-/-}$ ), and heterozygote mutant ER $\alpha^{-/\Delta\Delta}$  male mice were tested in two 30-min tests with a sexually receptive stimulus female. Males were sexually naïve in the first test and sexually experienced in the second test. In both tests significantly fewer ER $\alpha^{-/-}$  and ER $\alpha^{-/\Delta\Delta}$  males mounted (A) or intromitted (B) than expected ( $P < 0.001$ ,  $\chi^2$  analysis).

not appear to be affected by sexual experience. To determine whether this was due to a delay in their response to experience, mice were tested in three additional sessions (data not shown). ER $\alpha^{+/+}$  males continued to display robust sexual behavior, whereas ER $\alpha^{-/-}$  and ER $\alpha^{-/\Delta\Delta}$  males failed to show any improvements with additional experience. In fact, both mounting and intromissive behaviors were almost completely absent in these males, even in the fifth test.

Although statistical analyses on behavioral latencies could not be performed due to the small sample sizes, the few ER $\alpha^{-/-}$  and ER $\alpha^{-/\Delta\Delta}$  males that did display mounts appear to do so with longer latencies relative to wild types in both the first test (ER $\alpha^{+/+}$ , 12.60  $\pm$  1.31 min,  $n = 16$ ; ER $\alpha^{-/-}$ , 21.82 min,  $n = 1$ ; ER $\alpha^{-/\Delta\Delta}$ , 17.55  $\pm$  3.29 min,  $n = 3$ ) and second test (ER $\alpha^{+/+}$ , 9.17  $\pm$  1.52 min,  $n = 17$ ; ER $\alpha^{-/-}$ , 20.95 min,  $n = 1$ ; ER $\alpha^{-/\Delta\Delta}$ , 27.06  $\pm$  0.44 min,  $n = 2$ ).

#### Serum hormone levels

Basal T levels were significantly elevated in ER $\alpha^{-/-}$  males (2.70  $\pm$  0.77 ng/ml;  $n = 13$ ; Fig. 3A), compared with ER $\alpha^{+/+}$  (0.91  $\pm$  0.36 ng/ml;  $n = 13$ ;  $P < 0.05$ ) and ER $\alpha^{-/\Delta\Delta}$  animals (0.72  $\pm$  0.52 ng/ml;  $n = 9$ ;  $P < 0.01$ ). Plasma T levels of ER $\alpha^{-/\Delta\Delta}$  males were similar to those of wild-type males.

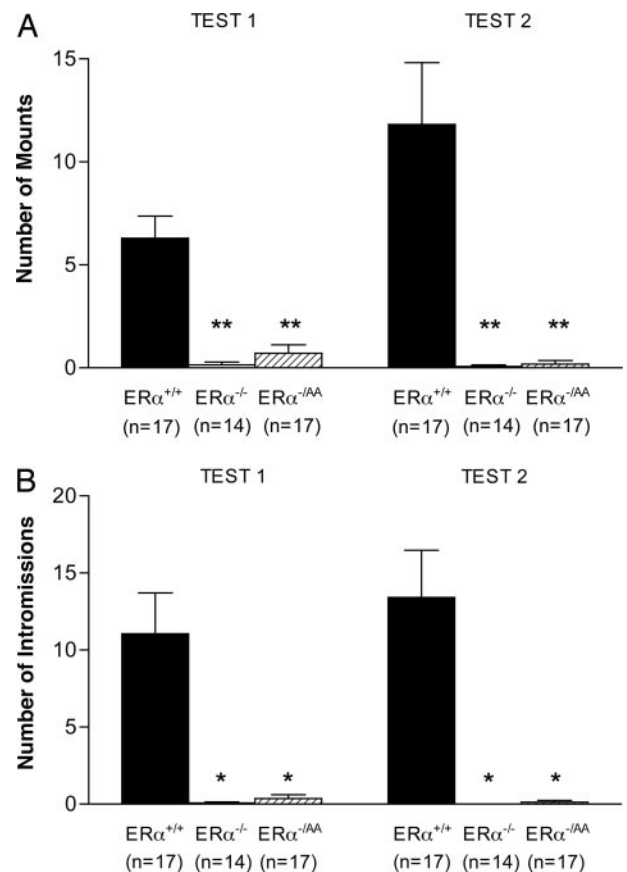


FIG. 2. Mount and intromission frequency. Male mice were tested twice with a sexually receptive female; mice were sexually naïve in the first test and sexually experienced in the second test. In both tests ER $\alpha^{-/-}$  and ER $\alpha^{-/\Delta\Delta}$  males display significantly fewer mounts (A) and intromissions (B) than ER $\alpha^{+/+}$  males (\*,  $P < 0.01$ , \*\*;  $P < 0.001$ , compared with ER $\alpha^{+/+}$ , Kruskal-Wallis with Dunn's multiple comparisons test). Values shown are mean  $\pm$  SEM.

This approximately 3-fold increase in T levels in ER $\alpha^{-/-}$  males is consistent with previous reports (4, 7). There was no effect of genotype on LH levels ( $P > 0.05$ ; ER $\alpha^{+/+}$ , 0.94  $\pm$  0.54 ng/ml,  $n = 13$ ; ER $\alpha^{-/-}$ , 0.46  $\pm$  0.12 ng/ml,  $n = 13$ ; ER $\alpha^{-/\Delta\Delta}$ , 0.95  $\pm$  0.66 ng/ml,  $n = 9$ ; Fig. 3B). Basal FSH levels were significantly lower in ER $\alpha^{-/\Delta\Delta}$  males (16.77  $\pm$  1.99 ng/ml;  $n = 9$ ), compared with ER $\alpha^{+/+}$  (22.20  $\pm$  0.95 ng/ml,  $n = 13$ ) and ER $\alpha^{-/-}$  males (24.02  $\pm$  1.19 ng/ml,  $n = 13$ ; Fig. 3C).

## Discussion

These studies assessed the physiological and behavioral roles played by ERE-independent ER $\alpha$  signaling in the male reproductive axis. Our findings reveal that ERE-independent ER $\alpha$  signaling is sufficient to establish a normal level of T secretion in male mice, but it does not by itself maintain any aspect of masculine sexual behavior. Whereas the majority of wild-type male mice mounted and intromitted in our tests, sexual behavior was almost completely absent in ER $\alpha^{-/-}$  and ER $\alpha^{-/\Delta\Delta}$  male mice. The few ER $\alpha^{-/-}$  and ER $\alpha^{-/\Delta\Delta}$  males that did display some sexual behavior demonstrated significantly lower behavioral frequencies than wild-type males. Because there were no significant differences in behavior between ER $\alpha^{-/\Delta\Delta}$  males and ER $\alpha^{-/-}$  males, we con-

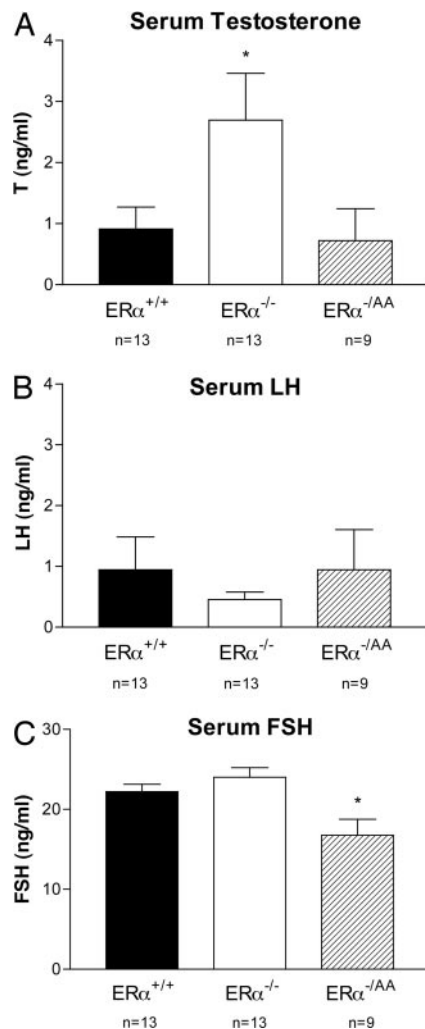


FIG. 3. Serum hormone levels. A, Serum T levels are significantly elevated in ER $\alpha$ <sup>-/-</sup> males, compared with ER $\alpha$ <sup>+/+</sup> ( $P < 0.05$ ) and ER $\alpha$ <sup>-/AA</sup> males (\*,  $P < 0.01$ ). B, There is no effect of genotype on serum LH levels. C, Serum FSH levels are significantly lower in ER $\alpha$ <sup>-/AA</sup> males, compared with ER $\alpha$ <sup>+/+</sup> and ER $\alpha$ <sup>-/-</sup> males (\*,  $P < 0.01$ ). Values shown are mean  $\pm$  SEM.

cluded that one ERE-independent ER $\alpha$  mutant allele is not sufficient, and ERE-dependent ER $\alpha$  signaling is essential, to maintain normal male behavior.

Our results suggest that sexual behavior depends on ER $\alpha$ -DNA binding and are consistent with previous *in vivo* studies that demonstrate the importance of genomic actions of E<sub>2</sub> on masculine sexual behavior. Reinstating copulation after castration with steroid hormones usually takes days to weeks, which suggests that longer-term genomic effects are necessary. This is supported by findings that the protein synthesis inhibitor anisomycin blocks the effects of T (and E<sub>2</sub>) on copulatory behavior when implanted into the male rat preoptic area (46). The mutant ER $\alpha$  in our ER $\alpha$ <sup>-/AA</sup> model can alter gene transcription at non-ERE sites by tethering to other transcription factors such as Jun (39). The lack of mounts and intromissions in these animals, however, suggests that transcription through non-ERE sites is not sufficient to maintain sexual behavior. Thus, gene targets of E<sub>2</sub> action that contribute to the expression of behavior likely contain EREs.

There is a variety of non-ERE-mediated mechanisms that have been proposed for E<sub>2</sub> action. As just one example, E<sub>2</sub> can signal via rapid, nongenomic actions originating at the cell surface. It is not entirely clear whether a membrane-associated ER $\alpha$  plays a role in the central control of sexual behavior, although E<sub>2</sub> can have rapid actions on neuronal firing in male preoptic area slices (47). Presumably, the mutant ER $\alpha$  in our ER $\alpha$ <sup>-/AA</sup> model is capable of translocating to the plasma membrane and mediating nongenomic effects of E<sub>2</sub>, as the knock-in mutation is specific to the DNA-binding domain and leaves the membrane-localization domain intact (39). Whereas further analyses are needed to obtain direct evidence that this is possible, the fact that ER $\alpha$ <sup>-/AA</sup> males display severely impaired behavior compared with ER $\alpha$ <sup>+/+</sup> counterparts would suggest that if there are any nongenomic actions of E<sub>2</sub> occurring through a membrane-associated ER $\alpha$ , they are not sufficient to maintain normal masculine sexual behavior in the absence of ERE-dependent pathways.

It is possible that the absence of sexual behavior in ER $\alpha$ <sup>-/AA</sup> males is due to the fact that only one allele of the mutant ER $\alpha$  is present and is therefore insufficient to elicit significant effects. Other studies of ER $\alpha$ <sup>-/AA</sup> mice support the idea that one mutant allele is enough to at least partially rescue the phenotypes generated by the deletion of the wild-type ER $\alpha$ . For example, ER $\alpha$ <sup>-/-</sup> animals display elevated trabecular bone mineral density, whereas ER $\alpha$ <sup>-/AA</sup> animals have levels similar to those of wild-types (40). In the female, the knock-in mutation restores negative feedback on gonadotropin secretion, which is absent in ER $\alpha$ <sup>-/-</sup> females (41). Finally, testicular degeneration, epididymal dysfunction, and increased T secretion are all observed in ER $\alpha$ <sup>-/-</sup> males but not young ER $\alpha$ <sup>-/AA</sup> males (our unpublished observations; the present study). However, different physiological processes can have different gene expression requirements, and two copies of the mutant ER $\alpha$  may be necessary for the restoration of sexual behavior. Accordingly, a role for ERE-independent ER $\alpha$  signaling in male sexual behavior cannot be ruled out completely. It would therefore be especially interesting to study the AA mutation in the homozygous state to determine whether gene dosage does in fact have an effect on sexual behavior. Unfortunately, the apparent infertility of heterozygous females (ER $\alpha$ <sup>+ /AA</sup>) makes generating these animals difficult (31).

It is important to note that some studies suggest that E<sub>2</sub> and ER signaling may not be essential for the expression of sexual behavior in adulthood. Despite evidence that neonatal E<sub>2</sub> may play a role in masculinization of sexual behavior, male aromatase knockouts, which cannot convert T to E<sub>2</sub>, can sire litters when they are young adults but demonstrate reduced fertility with age (48–50). ER $\alpha$ KO males display normal sexual behavior when given the dopamine agonist apomorphine, suggesting that the brain is sufficiently organized and that ER $\alpha$  is not necessary during development or adulthood for the expression of male sexual behavior. Furthermore, other studies support AR-dependent masculinization. For example, gonadally intact AR knockout males exhibit no male sexual behavior. Although E<sub>2</sub> treatment resulted in recovery of mounts and intromissions in AR knockout males, ejaculation was not restored and any E<sub>2</sub>-induced recovery was only about 50% of that observed in wild-type mice (1).

The ability of dihydrotestosterone treatment to restore mounts and intromissive behaviors in castrated ER $\alpha^{-/-}$  males also suggests the importance of androgen signaling (1). However, our study suggests that AR is not sufficient to maintain normal behavior in intact animals because T conversion to dihydrotestosterone and AR stimulation can occur in our ER $\alpha^{-/-}$  and ER $\alpha^{-/AA}$  males, yet sexual behavior was virtually absent. Similarly, the present study further demonstrates that ER $\beta$  is not sufficient for the expression of copulation in our ER $\alpha^{-/-}$  males under these conditions. This is in contrast to the findings of Ogawa *et al.* (51), who proposed that, because ER $\alpha$ βKO males do not display behavior, either one of the ERs is sufficient for the expression of simple mounting in male mice, indicating a redundancy of function.

Although ERE-independent mechanisms alone do not appear to play a role in sexual behavior, our results show that they may mediate E<sub>2</sub> action on serum T levels. It is well documented that prenatal and early postnatal E<sub>2</sub> treatment permanently disrupts morphogenesis and function of the male reproductive tract (52) and that E<sub>2</sub> treatment in adulthood can reduce serum T levels (53). Consistent with previous reports (4, 7), we observed that ER $\alpha$  deletion results in elevated T levels. We did not observe an elevation in T in ER $\alpha^{-/AA}$  males and serum levels were not significantly different from those of ER $\alpha^{+/+}$  counterparts. We conclude from these data that ERE-independent signaling introduced by the knock-in mutation was sufficient to restore serum hormone T levels in the adult.

In general, elevations in serum T levels may be attributed to increased stimulation by LH or by increased steroidogenesis. It does not appear that deletion of ER $\alpha$  disrupts negative feedback, causing a consequent rise in LH because the present study and others fail to show elevated LH in ER $\alpha^{-/-}$  males (4, 54). In contrast, others have reported approximately 2-fold greater LH levels in intact ER $\alpha^{-/-}$  males; however, it is important to note that those levels are not as high as those in castrated wild-type males. Moreover, castration of ER $\alpha^{-/-}$  males resulted in a significant rise in LH, suggesting that negative feedback on LH by T may be mediated at least in part by mechanisms independent of ER $\alpha$  (55). Clearly ER $\alpha$  is critical for negative feedback in the female, as demonstrated by extremely high LH levels in ER $\alpha^{-/-}$  female mice (56); however, the AR may play the predominant physiological role in the male (54). Our results would suggest that AR, or perhaps ER $\beta$ , is sufficient to mediate negative feedback on LH in the absence of ER $\alpha$ . Furthermore, the apparent rescue of serum T by the AA mutation is independent of changes in LH because serum levels were not affected by genotype.

ER $\alpha$  is present in the fetal Leydig cells of rodents in which it regulates steroidogenesis (57). Delbes *et al.* (58) demonstrated that *ex vivo* T production is elevated in fetal ER $\alpha^{-/-}$  mouse testis, independent of LH stimulation, whereas other reports suggest that the increased T levels observed in adult ER $\alpha^{-/-}$  mice can be attributed to both a direct effect of E<sub>2</sub> on the testis and stimulation by high levels of LH (59). In ER $\alpha$ -deficient fetal and adult testes, increased androgen biosynthesis is due in part to Leydig cell hypertrophy, increased steroidogenic enzyme gene expression (*e.g.* steroidogenic acute regulatory protein, cytochrome P450 17 $\alpha$ -hydroxy-

lase/17–20 lyase (P40<sub>17 $\alpha$</sub> ), 17 $\beta$ -hydroxysteroid dehydrogenase type III), and increased steroidogenic enzyme activity (*e.g.* P450<sub>17 $\alpha$</sub> , 17 $\beta$ -hydroxysteroid dehydrogenase) (58, 59). Whether ERE-independent signaling through ER $\alpha$  has a direct role in Leydig cell steroidogenic function remains to be determined. As exposure to environmental compounds with estrogenic activity has become a major concern in recent years, uncovering the mechanisms of E<sub>2</sub>'s actions will be useful for generating new strategies for treating testicular development disorders and adult male infertility.

ERE-independent ER $\alpha$  signaling also appears to play a role in the regulation of serum FSH levels, as demonstrated by significantly lower levels in intact ER $\alpha^{-/AA}$  males, compared with both ER $\alpha^{+/+}$  and ER $\alpha^{-/-}$  males. This is particularly interesting because both copies of ER $\alpha$  are capable of signaling via non-ERE mechanisms in wild-type animals, yet the AA mutation has a significant suppressive effect in the absence of ERE-dependent pathways. This suggests that perhaps ERE-dependent mechanisms antagonize the ERE-independent mechanisms that modulate E<sub>2</sub> effects on FSH. In support of our findings, transcriptional repression of the ovine FSH $\beta$  gene by E<sub>2</sub> appears to be mediated via receptor-protein interactions with basal transcription factors, independent of direct DNA binding by ER (60). Specifically, ovine FSH $\beta$  transcription can be regulated by c-Jun and c-Fos proteins via two AP-1-like sites in the ovine FSH $\beta$  proximal promoter, which appear to be important for the regulation of FSH production *in vivo* (61). It remains a possibility that ERE-independent ER $\alpha$  signaling does not act on FSH synthesis directly but rather through another mechanism, such as by increasing testicular inhibin or reducing activin. Further studies will be required to determine the mechanisms involved in the suppression of serum FSH in ER $\alpha^{-/AA}$  males.

In summary, we have demonstrated that ERE-independent signaling via ER $\alpha$  in ER $\alpha^{-/AA}$  mice is not sufficient to recover masculine sexual behavior in the absence of ERE-dependent mechanisms, indicating that signaling through EREs mediates most if not all of E<sub>2</sub>'s effects on male sexual behavior. In contrast, ERE-independent mechanisms are sufficient to restore serum T levels, suggesting that EREs are not necessary to mediate E<sub>2</sub>'s inhibitory actions on steroidogenesis. Understanding the molecular mechanisms by which ER $\alpha$  mediates its effects in specific physiological systems will ultimately be helpful in the development of pharmacological therapies that differentially modulate ERE-dependent and -independent processes.

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## References

- Sato T, Matsumoto T, Kawano H, Watanabe T, Uematsu Y, Sekine K, Fukuda T, Aihara K, Krust A, Yamada T, Nakamichi Y, Yamamoto Y, Nakamura T, Yoshimura K, Yoshizawa T, Metzger D, Chambon P, Kato S 2004 Brain masculinization requires androgen receptor function. *Proc Natl Acad Sci USA* 101:1673–1678
- Meisel RL, Sachs BD 1994 The physiology of male sexual behavior. In: Knobil E, Neill J, eds. *The physiology of reproduction*. 2nd ed. New York: Raven; 3–105
- Davidson JM 1969 Effects of estrogen on the sexual behavior of male rats. *Endocrinology* 84:1365–1372
- Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB, Korach KS 1996 Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology* 137:4796–4805
- Wersinger SR, Sannen K, Villalba C, Lubahn DB, Rissman EF, De Vries GJ 1997 Masculine sexual behavior is disrupted in male and female mice lacking a functional estrogen receptor  $\alpha$  gene. *Horm Behav* 32:176–183
- Wersinger SR, Rissman EF 2000 Dopamine activates masculine sexual behavior independent of the estrogen receptor  $\alpha$ . *J Neurosci* 20:4248–4254
- Rissman EF, Wersinger SR, Taylor JA, Lubahn DB 1997 Estrogen receptor function as revealed by knockout studies: neuroendocrine and behavioral aspects. *Horm Behav* 31:232–243
- Delbes G, Levacher C, Habert R 2006 Estrogen effects on fetal and neonatal testicular development. *Reproduction* 132:527–538
- Krege JH, Hodgins JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor  $\beta$ . *Proc Natl Acad Sci USA* 95:15677–15682
- Ogawa S, Chan J, Chester AE, Gustafsson JA, Korach KS, Pfaff DW 1999 Survival of reproductive behaviors in estrogen receptor  $\beta$  gene-deficient ( $\beta$ ERKO) male and female mice. *Proc Natl Acad Sci USA* 96:12887–12892
- Temple JL, Scordalakes EM, Bodo C, Gustafsson JA, Rissman EF 2003 Lack of functional estrogen receptor  $\beta$  gene disrupts pubertal male sexual behavior. *Horm Behav* 44:427–434
- Kudwa AE, Bodo C, Gustafsson JA, Rissman EF 2005 A previously uncharacterized role for estrogen receptor  $\beta$ : defeminization of male brain and behavior. *Proc Natl Acad Sci USA* 102:4608–4612
- Kudwa AE, Michopoulos V, Gatewood JD, Rissman EF 2006 Roles of estrogen receptors  $\alpha$  and  $\beta$  in differentiation of mouse sexual behavior. *Neuroscience* 138:921–928
- Greco B, Allegretto EA, Tetel MJ, Blaustein JD 2001 Coexpression of ER $\beta$  with ER $\alpha$  and progesterin receptor proteins in the female rat forebrain: effects of estradiol treatment. *Endocrinology* 142:5172–5181
- Temple JL, Fugger HN, Li X, Shetty SJ, Gustafsson J, Rissman EF 2001 Estrogen receptor  $\beta$  regulates sexually dimorphic neural responses to estradiol. *Endocrinology* 142:510–513
- Rissman EF, Heck AL, Leonard JE, Shupnik MA, Gustafsson JA 2002 Disruption of estrogen receptor  $\beta$  gene impairs spatial learning in female mice. *Proc Natl Acad Sci USA* 99:3996–4001
- Lindberg MK, Moverare S, Skrtic S, Gao H, Dahlman-Wright K, Gustafsson JA, Ohlsson C 2003 Estrogen receptor (ER)- $\beta$  reduces ER $\alpha$ -regulated gene transcription, supporting a “ying yang” relationship between ER $\alpha$  and ER $\beta$  in mice. *Mol Endocrinol* 17:203–208
- Nomura M, Korach KS, Pfaff DW, Ogawa S 2003 Estrogen receptor  $\beta$  (ER $\beta$ ) protein levels in neurons depend on estrogen receptor  $\alpha$  (ER $\alpha$ ) gene expression and on its ligand in a brain region-specific manner. *Brain Res Mol Brain Res* 110:7–14
- Kudwa AE, Gustafsson JA, Rissman EF 2004 Estrogen receptor  $\beta$  modulates estradiol induction of progesterin receptor immunoreactivity in male, but not in female, mouse medial preoptic area. *Endocrinology* 145:4500–4506
- Glass CK 1994 Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. *Endocr Rev* 15:391–407
- McKenna NJ, Lanz RB, O'Malley BW 1999 Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev* 20:321–344
- Smith CL, O'Malley BW 2004 Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocr Rev* 25:45–71
- Tsai MJ, O'Malley BW 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* 63:451–486
- Ronnekleiv OK, Kelly MJ 2005 Diversity of ovarian steroid signaling in the hypothalamus. *Front Neuroendocrinol* 26:65–84
- Wade CB, Robinson S, Shapiro RA, Dorsa DM 2001 Estrogen receptor (ER) $\alpha$  and ER $\beta$  exhibit unique pharmacologic properties when coupled to activation of the mitogen-activated protein kinase pathway. *Endocrinology* 142:2336–2342
- Navarro CE, Abdul Saeed S, Murdock C, Martinez-Fuentes AJ, Arora KK, Krsmanovic LZ, Catt KJ 2003 Regulation of cyclic adenosine 3',5'-monophosphate signaling and pulsatile neurosecretion by Gi-coupled plasma membrane estrogen receptors in immortalized gonadotropin-releasing hormone neurons. [republished in *Mol Endocrinol* 2003;17:1792–1804]. *Mol Endocrinol* 17:1792–1804
- Lu Q, Pallas DC, Surks HK, Baur WE, Mendelsohn ME, Karas RH 2004 Striatin assembles a membrane signaling complex necessary for rapid, nongenomic activation of endothelial NO synthase by estrogen receptor  $\alpha$ . *Proc Natl Acad Sci USA* 101:17126–17131
- Pedram A, Razandi M, Levin ER 2006 Nature of functional estrogen receptors at the plasma membrane. *Mol Endocrinol* 20:1996–2009
- Coleman KM, Smith CL 2001 Intracellular signaling pathways: nongenomic actions of estrogens and ligand-independent activation of estrogen receptors. *Front Biosci* 6:D1379–D1391
- Cato AC, Nestl A, Mink S 2002 Rapid actions of steroid receptors in cellular signaling pathways. *Sci STKE* RE9
- Jakacka M, Ito M, Martinson F, Ishikawa T, Lee EJ, Jameson JL 2002 An estrogen receptor (ER) $\alpha$  deoxyribonucleic acid-binding domain knock-in mutation provides evidence for nonclassical ER pathway signaling *in vivo*. *Mol Endocrinol* 16:2188–2201
- Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, Webb P 2000 Estrogen receptor pathways to AP-1. *J Steroid Biochem Mol Biol* 74:311–317
- Safe S 2001 Transcriptional activation of genes by 17  $\beta$ -estradiol through estrogen receptor-Sp1 interactions. *Vitam Horm* 62:231–252
- Ray A, Prefontaine KE, Ray P 1994 Down-modulation of interleukin-6 gene expression by 17 $\beta$ -estradiol in the absence of high affinity DNA binding by the estrogen receptor. *J Biol Chem* 269:12940–12946
- Aronica SM, Kraus WL, Katzenellenbogen BS 1994 Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. *Proc Natl Acad Sci USA* 91:8517–8521
- Webb P, Lopez GN, Uht RM, Kushner PJ 1995 Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. *Mol Endocrinol* 9:443–456
- Castoria G, Barone MV, Di Domenico M, Bilancio A, Ametrano D, Migliaccio A, Auricchio F 1999 Non-transcriptional action of oestradiol and progesterin triggers DNA synthesis. *EMBO J* 18:2500–2510
- Gaub MP, Ballard M, Scheuer I, Chambon P, Sassone-Corsi P 1990 Activation of the ovalbumin gene by the estrogen receptor involves the fos-jun complex. *Cell* 63:1267–1276
- Jakacka M, Ito M, Weiss J, Chien PY, Gehm BD, Jameson JL 2001 Estrogen receptor binding to DNA is not required for its activity through the nonclassical AP1 pathway. *J Biol Chem* 276:13615–13621
- Syed FA, Modder UI, Fraser DG, Spelsberg TC, Rosen CJ, Krust A, Chambon P, Jameson JL, Khosla S 2005 Skeletal effects of estrogen are mediated by opposing actions of classical and nonclassical estrogen receptor pathways. *J Bone Miner Res* 20:1992–2001
- Glidewell-Kenny C, Hurley LA, Pfaff L, Weiss J, Levine JE, Jameson JL 2007 Nonclassical estrogen receptor  $\alpha$  signaling mediates negative feedback in the female mouse reproductive axis. *Proc Natl Acad Sci USA* 104:8173–8177
- Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M 2000 Effect of single and compound knockouts of estrogen receptors  $\alpha$  (ER $\alpha$ ) and  $\beta$  (ER $\beta$ ) on mouse reproductive phenotypes. *Development* 127:4277–4291
- Ogawa S, Lubahn DB, Korach KS, Pfaff DW 1997 Behavioral effects of estrogen receptor gene disruption in male mice. *Proc Natl Acad Sci USA* 94:1476–1481
- McGill TE 1962 Sexual behavior in three inbred strains of mice. *Behaviour* 19:341–350
- McGill TE, Blight WC 1963 Effects of genotype on the recovery of sex drive in the male mouse. *J Comp Physiol Psychol* 56:887–888
- McGinnis MY, Kahn DF 1997 Inhibition of male sexual behavior by intracranial implants of the protein synthesis inhibitor anisomycin into the medial preoptic area of the rat. *Horm Behav* 31:15–23
- Silva NL, Boulant JA 1986 Effects of testosterone, estradiol, and temperature on neurons in preoptic tissue slices. *Am J Physiol* 250:R625–R632
- Honda S, Harada N, Ito S, Takagi Y, Maeda S 1998 Disruption of sexual behavior in male aromatase-deficient mice lacking exons 1 and 2 of the cyp19 gene. *Biochem Biophys Res Commun* 252:445–449
- Fisher CR, Graves KH, Parlow AF, Simpson ER 1998 Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. *Proc Natl Acad Sci USA* 95:6965–6970
- Robertson KM, Simpson ER, Lacham-Kaplan O, Jones ME 2001 Characterization of the fertility of male aromatase knockout mice. *J Androl* 22:825–830
- Ogawa S, Chester AE, Hewitt SC, Walker VR, Gustafsson JA, Smithies O, Korach KS, Pfaff DW 2000 Abolition of male sexual behaviors in mice lacking estrogen receptors  $\alpha$  and  $\beta$  ( $\alpha\beta$ ERKO). *Proc Natl Acad Sci USA* 97:14737–14741
- Akingbemi BT 2005 Estrogen regulation of testicular function. *Reprod Biol Endocrinol* 3:51
- Robaire B, Ewing LL, Irby DC, Desjardins C 1979 Interactions of testosterone and estradiol-17 $\beta$  on the reproductive tract of the male rat. *Biol Reprod* 21:455–463
- Wersinger SR, Haisenleder DJ, Lubahn DB, Rissman EF 1999 Steroid feedback on gonadotropin release and pituitary gonadotropin subunit mRNA in mice lacking a functional estrogen receptor  $\alpha$ . *Endocrine* 11:137–143
- Lindzey J, Wetsel WC, Couse JF, Stoker T, Cooper R, Korach KS 1998 Effects of castration and chronic steroid treatments on hypothalamic gonadotropin-

- releasing hormone content and pituitary gonadotropins in male wild-type and estrogen receptor- $\alpha$  knockout mice. *Endocrinology* 139:4092–4101
56. **Couse JF, Yates MM, Walker VR, Korach KS** 2003 Characterization of the hypothalamic-pituitary-gonadal axis in estrogen receptor (ER) null mice reveals hypergonadism and endocrine sex reversal in females lacking ER $\alpha$  but not ER $\beta$ . *Mol Endocrinol* 17:1039–1053
57. **O'Donnell L, Robertson KM, Jones ME, Simpson ER** 2001 Estrogen and spermatogenesis. *Endocr Rev* 22:289–318
58. **Delbes G, Levacher C, Duquenne C, Racine C, Pakarinen P, Habert R** 2005 Endogenous estrogens inhibit mouse fetal Leydig cell development via estrogen receptor  $\alpha$ . *Endocrinology* 146:2454–2461
59. **Akingbemi BT, Ge R, Rosenfeld CS, Newton LG, Hardy DO, Catterall JF, Lubahn DB, Korach KS, Hardy MP** 2003 Estrogen receptor- $\alpha$  gene deficiency enhances androgen biosynthesis in the mouse Leydig cell. *Endocrinology* 144:84–93
60. **Miller CD, Miller WL** 1996 Transcriptional repression of the ovine follicle-stimulating hormone- $\beta$  gene by 17 $\beta$ -estradiol. *Endocrinology* 137:3437–3446
61. **Strahl BD, Huang HJ, Sebastian J, Ghosh BR, Miller WL** 1998 Transcriptional activation of the ovine follicle-stimulating hormone  $\beta$ -subunit gene by gonadotropin-releasing hormone: involvement of two activating protein-1-binding sites and protein kinase C. *Endocrinology* 139:4455–4465

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