

# A previously uncharacterized role for estrogen receptor $\beta$ : Defeminization of male brain and behavior

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Communicated by Elwood V. Jensen, University of Cincinnati College of Medicine, Cincinnati, OH, February 14, 2005 (received for review January 1, 2005)

Sex differences in brain and behavior are ubiquitous in sexually reproducing species. One cause of sexual dimorphisms is developmental differences in circulating concentrations of gonadal steroids. Neonatal testes produce androgens; thus, males are exposed to both testosterone and estradiol, whereas females are not exposed to high concentrations of either hormone until puberty. Classically, the development of neural sex differences is initiated by estradiol, which activates two processes in male neonates; masculinization, the development of male-type behaviors, and defeminization, the loss of the ability to display female-type behaviors. Here, we test the hypothesis that defeminization is regulated by estrogen receptor  $\beta$  (ER $\beta$ ). Adult male ER $\beta$  knockout and WT mice were gonadectomized, treated with female priming hormones, and tested for receptive behavior. Indicative of incomplete defeminization, male ER $\beta$  knockout mice showed significantly higher levels of female receptivity as compared with WT littermates. Testes-intact males did not differ in any aspects of their male sexual behavior, regardless of genotype. In olfactory preference tests, males of both genotypes showed equivalent preferences for female-soiled bedding. Based on these results, we hypothesize that ER $\beta$  is involved in defeminization of brain and behavior. This aspect of ER $\beta$  function may lead to developments in our understanding of neural-based sexually dimorphic human behaviors.

developmental neurobiology | neuroendocrinology | sexual differentiation

**M**ales undergo two processes during development that affect their adult behavior. Masculinization refers to the underlying neural circuitry and behavioral patterns that are exhibited to a greater degree by males than females. For example, males of many species display a set of sex specific courtship and copulatory behaviors. In addition, a separate process, defeminization, reduces the likelihood that males will display female-typical behaviors in adulthood, such as display of the receptive mating posture, lordosis. Many sexual dimorphisms in brain and behavior are caused by developmental sex differences in steroid hormones that act on nuclear receptors (1). Specifically, neonatal testes produce testosterone for a finite period beginning at the end of gestation until shortly after birth (2). Testosterone is aromatized neurally to estradiol (E2) and binds to two known estrogen receptors (ER $\alpha$  and ER $\beta$ ) (3). Depriving males of their testes, or steroids produced by the testes, during this developmental period results in demasculinization and feminization (4–6).

The mechanism by which estradiol affects both masculinization and defeminization is unknown. Here, we test the hypothesis that these processes are regulated by different ERs. We hypothesize that ER $\beta$  has a specialized function in the development of a sexually differentiated behavior and is essential for defeminization. This hypothesis is supported by the report of sex differences in ER $\beta$  in neonatal mice; during late gestation and the first 2 weeks after birth, males have significantly more ER $\beta$  mRNA

than females in the medial basal hypothalamus including the medial preoptic area (POA) (7).

To test our hypothesis, we used male ER $\beta$  knockout (ER $\beta$ KO) mice along with their WT littermates (8). Adult male and female ER $\beta$ KO mice can perform sex-typical sexual behaviors (9–11), and males are fertile (8). We predicted that if ER $\beta$  is exclusively involved in defeminization of the male brain, male ER $\beta$ KO mice would fail to undergo complete defeminization during development and, as a consequence, when tested in adulthood they would display more female-type receptivity than WT littermates. However, the lack of ER $\beta$  should have no impact on the masculinization process and, thus, male ER $\beta$ KO mice should display equivalent masculine sexual behavior as compared with WT males. We assessed a second sexually dimorphic social behavior, olfactory preferences. Male mice show a strong preference for female-soiled bedding over male-soiled bedding (12, 13). We hypothesized that WT and ER $\beta$ KO males would both preferentially investigate female-soiled bedding vs. male-soiled or clean bedding.

## Methods

**Animals.** The mice were generated by mating heterozygous carriers of the disrupted ER $\beta$  gene (8), and the offspring genotype (ER $\beta$ KO or WT) was determined by PCR amplification of tail DNA, as described in ref. 14. The individuals were of a mixed 129/SvJ and C57BL/6J background, backcrossed into the C57BL/6J strain for five generations (making them, on average, 97% similar to the inbred C57BL/6J). All males were weaned between 18 and 20 days of age and group-housed until they were between 50 and 60 days old. Males were individually housed either after castration (experiments 1 and 3) or beginning 3 days before testing (experiment 2) for the entire duration of testing, with food (Purina mouse chow no. 5001) and water available ad libitum. All individuals were kept on a 12-h light:dark cycle with lights off at 1200 hours.

**Experiment 1. Female sexual behavior.** Thirty-eight adult male mice (70–100 days of age) were used in this study, 16 WT and 22 ER $\beta$ KO. Mice were gonadectomized under general anesthesia (100 mg/kg ketamine and 10 mg/kg xylazine injected i.p.). Seven to 10 days after surgery, males were injected s.c. with estradiol benzoate (EB) (0.5  $\mu$ g dissolved in 0.05 ml of sesame oil). Two days later, progesterone (400  $\mu$ g in 0.03 ml of sesame oil) was administered s.c. 3–4 h before the onset of a sexual behavior test. Animals were tested seven times with 4–5 days between trials.

**Receptivity tests.** All sexual behavior tests were conducted starting 2 h after lights off, under red-light illumination. All testing was

Abbreviations: EB, estradiol benzoate; ER, estrogen receptor; KO, knockout; LQ, lordosis quotient; POA, preoptic area; PR, progesterin receptor.

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conducted in 18 × 38-cm Plexiglas test chambers that were placed on a mirror stand to allow for ventral viewing. Sexually experienced C57BL/6J and DBA/2J males were used as testing partners. Each stud male was habituated for 1 h in the test chamber before the introduction of the hormone-primed experimental male. All tests were terminated after the experimental males received 20 mounts from the stud male (defined as both forepaws on the hind region) or after 15 min, whichever occurred first. We defined lordosis by using the following stipulations: all four paws were grounded, the hind region was elevated off the floor of the test chamber, and the back was slightly arched. The lordosis quotient (LQ; lordosis number/number of mounts) was scored for each subject.

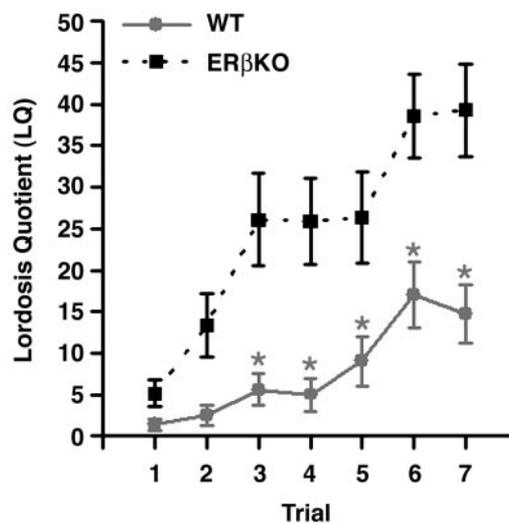
**Experiment 2. Male sexual behavior.** Twenty-five adult (60–120 days of age), gonad-intact males, 11 WT and 14 ERβKO, were used. Males received social exposure (15) for 3 consecutive days before sexual behavior testing commenced. Each male was then tested three times for male sexual behavior with an interval of 2–3 days between each consecutive test. However, once the male performed an ejaculation during a test, his testing sequence ended. One week after the final test, animals underwent olfactory preference testing (described below).

**Stimulus females.** Stimulus females (C57BL/6J) were ovariectomized after 40 days of age and implanted with a Silastic implant (1.96-mm i.d. × 3.18-mm o.d.) filled with EB (50 μg dissolved in 30 μl of sesame oil). Three to 5 h before the tests, the females were injected s.c. with progesterone (1 mg dissolved in 60 μl of sesame oil), and they were prescreened with a sexually experienced male to ensure receptivity.

**Male sexual behavior tests.** Sexual behavior tests were conducted in the same test boxes and at the same time of day as described in experiment 1. Each subject was habituated to the testing box for 30 min before a hormone-primed receptive female stimulus animal was introduced. Tests lasted for 30 min if no sexual behavior was displayed. If a mounting episode was displayed within the first 30 min, the test was extended for 60 additional min, or until the male performed an ejaculation, whichever occurred first. If a subject displayed an ejaculation, the test was terminated and the male was not tested again. The variables recorded included latencies to mount, thrust, intromit, and ejaculate and the number of mounting bouts and thrusts per mounting bout. Latency scores were cumulative over each test period. Frequency data were analyzed from the test during which the maximum amount of sexual behavior occurred.

**Experiment 3. Olfactory preference tests.** Males tested for sexual behaviors in experiment 2 were also tested for olfactory preferences. The detailed methods of our olfactory preference test are published in ref. 16. Tests were conducted between 3 and 8 h after room lights went out under red-light illumination. In brief, the experimental setup consisted of three small plastic containers filled with bedding located equidistant from each other inside the standard Plexiglas test box. For the habituation period, all containers contained clean bedding. Next, mice were removed from the test box, and three different containers containing different types of bedding (i.e., male-soiled, female-soiled, and clean) were placed in the box. Tests lasted for 10 min, and the time spent actively sniffing each container (the male's nose had to be in direct contact with the bedding) was recorded. The observer was blind to the content of each container.

**Stimulus bedding.** Twenty-four hours before bedding was collected, group-housed (four per cage) gonadectomized females that had s.c. EB-filled Silastic implants (1.96-mm i.d. × 3.18-mm o.d.; 50 μg of EB dissolved in 30 μl of sesame oil) were placed into a cage with clean bedding. The females received a progesterone injection 6 h before bedding collection. Soiled bedding from adult



**Fig. 1.** Mean (±SEM) LQ for castrated WT ( $n = 16$ ) and ERβKO ( $n = 22$ ) male mice. Males received s.c. injections of EB and progesterone 48 and 3 h, respectively, before each behavior test. Tests were conducted at 4- to 5-day intervals. \*, WT males displayed significantly lower levels of lordosis as compared with ERβKO males on each trial ( $P < 0.05$ ).

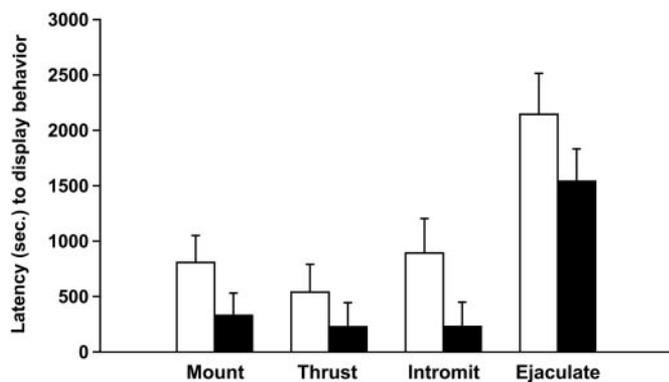
males (C57BL/6J) individually housed on clean bedding for 24 h was obtained from four cages.

**Data Analysis.** Female sex behavior data were analyzed by using a repeated-measures ANOVA with genotype and trial as the factors. Comparisons among trials and genotypes were analyzed by using Bonferroni multiple comparison tests, which correct for the number of comparisons made. In experiment 2, the frequencies of males of each genotype performing various behaviors were compared with Fisher exact tests. We analyzed male sexual behaviors, only including data from males that displayed the behaviors, with one-way ANOVA. A repeated-measures ANOVA with bedding as the repeated measure and genotype as the factor was used to analyze the data from the olfactory preference tests. Bonferroni multiple comparison tests were used for the post hoc analysis. In all cases, significance was reported at  $P < 0.05$  or less.

**Results**

**ERβKO Males Display Enhanced Female Sex Behavior.** Castrated ERβKO males treated with EB and progesterone displayed female-like receptivity, defined as holding the lordosis posture and allowing males to mount and thrust. A one-way repeated-measures ANOVA revealed effects of genotype [ $F_{(1,265)} = 18.37$ ;  $P < 0.001$ ] and trial [ $F_{(6,265)} = 13.14$ ;  $P < 0.01$ ]. The interaction between genotype and trial approached significance [ $F_{(6,265)} = 2.10$ ;  $P = 0.054$ ]. Overall, ERβKO males displayed significantly higher LQs as compared with WT littermates (Fig. 1), and as with female mice, the LQ scores of ERβKO males increased over trials; LQ on trials 6 and 7 were significantly greater than on trials 1–4 ( $P < 0.05$ ). Furthermore, post hoc analysis with Bonferroni multiple comparison tests revealed that ERβKO males exhibited a higher LQ compared with WT males on every trial ( $P < 0.05$ ) except for trials 1 and 2.

**Male Sexual Behavior Is Unaffected by Lack of Functional ERβ.** The majority of the males tested, 10 (of 14) ERβKO and 8 (of 11) WT mice exhibited sexual behavior in at least one test. These proportions were not significantly different ( $P = 0.94$ ). In total, eight ERβKO and five WT individuals achieved complete sequences of sexual behavior, including ejaculation, during the



**Fig. 2.** Mean ( $\pm$ SEM) latency to first mount, thrust, intromission, and ejaculation for testes-intact WT and ER $\beta$ KO mice in male sexual behavior tests. Latency to first mount was calculated from the onset of the testing period; all other latencies were calculated from the onset of the first mount displayed. No significant differences were found between WT and ER $\beta$ KO males. White histograms are data from WT, and black histograms are data from ER $\beta$ KO males.

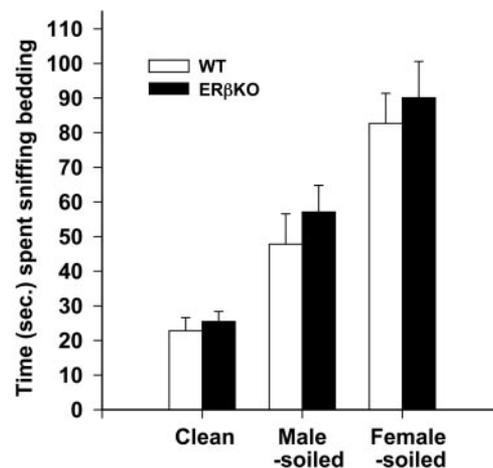
testing series. There were no differences between the genotypes in the latencies to mount [ $F_{(1,20)} = 2.29$ ], thrust [ $F_{(1,19)} = 0.88$ ], intromit [ $F_{(1,16)} = 3.02$ ], or ejaculate [ $F_{(1,11)} = 1.64$ ] (Fig. 2). The number of intromissions displayed before ejaculation, the latency between the first intromission and ejaculation, and the average number of thrusts with intromissions per mounting bout were not significantly different between the groups [ $F_{(1,11)} = 0.18$ , 0.71 and  $F_{(1,16)} = 0.46$ , respectively] (Table 1). When the total number of mounting episodes were normalized according to the amount of time that each subject had been tested, the differences remained nonsignificant [ $F_{(1,20)} = 1.80$ ] (Table 1).

#### Olfactory Preferences Are Equivalent Between WT and ER $\beta$ KO Males.

Olfactory preference tests conducted in sexually experienced, testes-intact mice revealed a significant effect of bedding type [ $F_{(2,69)} = 35.71$ ;  $P < 0.0001$ ] but not of genotype, and there was no significant interaction between the two factors. The post hoc analysis showed that all males preferred to spend significantly more time sniffing soiled bedding compared with the clean bedding used as a control, and both genotypes showed a significant preference for bedding previously soiled by estrous females over bedding soiled by males ( $P < 0.05$ ) (Fig. 3).

#### Discussion

Our major finding is that male mice lacking functional ER $\beta$  are incompletely defeminized. When treated with the appropriate hormonal priming, male ER $\beta$ KO mice display significantly more female-like sexual receptivity than WT littermates. Yet, lack of functional ER $\beta$  does not impair normal expression of adult masculine sexual behavior or olfactory preference in testes-intact males



**Fig. 3.** Mean ( $\pm$ SEM) time in seconds spent chemoinvestigating one of three bedding choices (clean, male-soiled, and female-soiled). Males were testes-intact and sexually experienced (10 WT and 13 ER $\beta$ KO). Although no genotype effect was found, males did spend significantly different amounts of time sniffing the three bedding type: the least time was spent investigating clean bedding, then male-soiled bedding, and, finally, the most time was spent sniffing female-soiled bedding ( $P < 0.05$ ).

(Table 2). When standard tests for male sexual behavior were performed, WT and ER $\beta$ KO males showed equivalent latencies to perform the various components of copulatory behavior, and the frequencies with which they displayed each behavior were also similar. Both WT and ER $\beta$ KO males have a distinct preference for female-soiled bedding over male-soiled or clean bedding. Olfactory preferences may be an assay of sexual interest and/or motivation (12), and, thus, our findings indicate that ER $\beta$ KO mice have normal interest in female olfactory cues. In summary, we find no evidence that masculinization is deficient in ER $\beta$ KO males; however, we propose that the defeminization process is incomplete in these animals.

The mechanisms by which ER $\beta$  affects defeminization are unknown. However, the idea that masculinization and defeminization may be uncoupled in males is not new (17). Castrated adult male ferrets can display female-like receptive behavior upon receipt of the appropriate priming hormones, but they also display typical male sexual behavior when tested with normal circulating levels of testosterone or with their testes intact (18, 19). Thus, male ferrets are masculinized but not defeminized, similar to the ER $\beta$ KO mouse. Lesions of the sexually dimorphic nucleus (SDN) in the male ferret POA/AH block female-like preferences for stud males but have little impact on male sexual behavior (20), suggesting that this region is involved in defeminization. This steroid-sensitive nucleus in the ferret is anatomically similar to the SDN of the POA found in rats and other mammals (20–22). However,

**Table 1. Values (mean  $\pm$  SEM) for variables recorded on the tests for masculine behavior**

Variable	WT	ER $\beta$ KO
Number of thrusts with intromissions per mounting episode	22.92 $\pm$ 8.25 (6)	29.76 $\pm$ 5.83 (12)
Total number of thrusts with intromissions preceding ejaculation	367.38 $\pm$ 59.05 (5)	326.80 $\pm$ 74.69 (8)
Total number of mounting episodes	29.92 $\pm$ 6.32 (9)	19.56 $\pm$ 7.60 (13)
Number of mounting episodes preceding ejaculation	28.50 $\pm$ 6.57 (5)	32.60 $\pm$ 8.31 (8)

The numbers of animals in each group is given in parentheses. No significant differences were registered between the groups.

**Table 2. Summary of behavioral differences between WT and ER $\beta$ KO male mice**

Behavior	WT	ER $\beta$ KO
Masculine sexual	+	+
Masculine olfactory	+	+
Feminine sexual	-	+

mice have fewer documented sexual dimorphisms in brain than rats, and C57BL/6J mice do not possess a SDN (23, 24). Yet, in both neonatal and adult mouse brains, sex differences in ER $\beta$  have been demonstrated (7, 25). Specifically, between embryonic day 17 and postnatal day 15, males had more ER $\beta$  mRNA than did females (7), and in adult mice, castrated C57BL/6J males have more ER $\beta$  immunoreactive cells throughout the medial POA than do adult ovariectomized females (25). We suspect that this sex difference in ER $\beta$  is related to defeminization of the male brain. The influence of ER $\beta$  on defeminization may involve the progesterin receptor (PR). In adult male C57BL/6J mice, but not female C57BL/6J mice, ER $\beta$  is involved in estradiol-regulation of PRs. Specifically, maximal PR induction in male mice requires at least one functional copy of ER $\beta$  (25). Because PR is also essential for the expression of female receptivity in rodents (26), we speculate that normal defeminization involves PR expression, which may be regulated in males by ER $\beta$ .

In adulthood, ER $\beta$ KO males and females can display normal sex-specific copulatory behaviors (9–11). In fact, ER $\beta$ KO females display more regular estrous cycles and enhanced receptivity as compared with WT littermates (9, 11, ¶). After equivalent treatment with estradiol, ER $\beta$ KO females tend to have more PR-immunoreactive neurons in the ventromedial nucleus of the hypothalamus than WT females (14). Thus, a lack of ER $\beta$  could enhance feminization in females. Female mice display varying degrees of feminine behavior as adults, and this may be attributed to differences in exposure to steroid hormones *in utero* (27). Depending on uterine position, some female embryos can be exposed to androgens from their male siblings. This testosterone may be aromatized to E2 in the brain and, via ER $\beta$ , may have a mild defeminizing effect in females. In males, sexual behavior in adult ER $\beta$ KO mice is equivalent to WT males, but transient developmental differences in both aggressive and sexual behaviors during puberty have been observed in male ER $\beta$ KO mice (10, 28). It is possible that these behavioral differences are based on the same neural circuitry that produces the propensity for male ER $\beta$ KO mice to display enhanced lordosis behavior.

Data from KO mice suggest that, in females, ER $\alpha$  is essential for normal sexual behavior and fertility (29). Females lacking ER $\alpha$  or both ER $\alpha$  and ER $\beta$  are infertile (30) and fail to display receptivity after hormone priming (9, 31, 32). Because the lack of functional ER $\alpha$  has such pronounced effects, ER $\alpha$ KO mice cannot be used to explore the role of ER $\beta$  in female reproduction. However, data from female rats support the possibility that ER $\beta$  may affect female reproduction, at least in some brain areas. Treatment of female rat pups for the first 12 days of life with estradiol, an ER $\alpha$ , or an ER $\beta$ -selective agonist decreases the number of neurons in the sexually dimorphic anteroventral periventricular region (AVPV) (33). In that area, all estrogen treatments reduced the number of neurons per unit area, thus making the region more male-like than female-like (i.e., defeminized). Rat brains display a sex difference in ER $\beta$  message in AVPV starting on postnatal day 7

and continuing into adulthood (34); females have more ER $\beta$ -mRNA-containing cells than do males, and this difference can be reversed by early treatment of females with estrogen or castration of male pups. Thus, the rat data suggest that ER $\beta$  may modulate cell numbers in the AVPV and, by inference, interfere with reproduction. Presently, we are treating neonatal mice with ER $\alpha$  and ER $\alpha$ -selective agonists and examining adult sexual behavior. Our preliminary data show a defeminizing effect of the ER $\beta$  agonist, but not an effect of the ER $\alpha$  agonist, on adult female sexual behavior (A.E.K. and E.F.R., unpublished data). Clearly, differences exist between mice and rats, and ER $\beta$  may not have precisely the same role in each species. However, the collective data from both species suggest that ER $\beta$  activation during development can influence defeminization in brain.

Another line of evidence that supports our hypothesis that ER $\beta$  is involved in defeminization comes from studies investigating the effects of early exposure to phytoestrogens on adult behavior. Phytoestrogens bind preferentially to ER $\beta$  (35) and generally have anti-estrogenic actions in female rats. Female rat pups injected with the phytoestrogen genistein daily for the first 5 days of life showed a reduction in lordosis behavior in adulthood (36). Female sexual behavior is suppressed in aromatase enzyme KO mice (37). However, this finding was only replicated when females were raised on a phytoestrogen-rich diet (38). Again, the mechanism may be that phytoestrogens activate ER $\beta$  and, thus, defeminize receptive behavior.

The complementary process to defeminization is masculinization. Based on data presented here and on unpublished data in which female ER $\beta$ KO mice treated with T displayed normal levels of mounting and thrusting behavior when tested with receptive females (A.E.K. and E.F.R., unpublished data), we suggest that ER $\beta$  is not required for masculinization. It is tempting to speculate that ER $\alpha$  instead is responsible for masculinization. Mice lacking functional ER $\alpha$  show a number of behavioral impairments including the failure of both males and females to exhibit masculine sexual behavior when they are tested with the appropriate hormones in adulthood (15, 39, 40). Male ER $\alpha$ KO mice show low levels of masculine sexual behavior (40, 41) and have no preferences for awake females versus males, anesthetized females versus males, or female versus male-soiled bedding (42). Although these data are consistent with the hypothesis that masculinization requires ER $\alpha$ , it is not yet possible to determine whether the actions of ER $\alpha$  are essential during development and/or in adulthood. The test of this hypothesis awaits development of mouse models with temporal control of ER $\alpha$  expression.

In summary, we hypothesize that ER $\beta$  plays an essential role in sexual differentiation of brain and behavior. Whether defeminization also requires ER $\alpha$  is not clear, but interactions between the two receptors within several brain nuclei, including those involved in sexual behavior, have been well documented (14, 43, 44). Moreover, the pattern of response to estradiol in the ventromedial nucleus of the hypothalamus and POA of male ER $\beta$ KO mice is feminized for both ER $\alpha$  and PR regulation (14), and it is likely that both of these receptors are involved in expression of receptivity. Past work suggesting neural (14, 45, 46) and behavioral (10, 47, 48) deficiencies in ER $\beta$ KO mice need to be evaluated within this hypothesis in mind. This evaluation could lead to new applications of estrogen-based treatments for sexually dimorphic neurological diseases.

We thank Aileen Wills and Savera Shetty for expert technical assistance and Alexander Kauffman for constructive comments on an earlier draft of this paper. This work was supported by National Institutes of Health Grants R01 MH57759 and K02 MH01349 (to E.F.R.). A.E.K. was supported by Individual National Research Service Award Predoctoral Fellowship F31MH70092.

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