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Effect of ER-β gene disruption on estrogenic regulation of anxiety in female mice

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ABSTRACT

It has been shown that long-term estrogen treatment in gonadectomized female mice increases anxiety levels. On the other hand, a recent study has reported that estrogen may down-regulate the levels of anxiety by acting through estrogen receptor (ER) β. In the present study, we investigated the role of ER-β in the regulation of anxiety levels in female mice after long-term estrogen treatment. Gonadectomized ER-β knockout (βERKO) female mice and their wild type (βWT) littermates were implanted several different doses (experiment 1: 2.0 μg/day, experiment 2: 1.0, 0.4, 0.2 or 0.1 μg/day) of an estradiol benzoate (EB) or placebo pellet. Ten days after pellet implant, behavioral tests commenced to measure the anxiety levels (experiment 1: light–dark transition test (LDT), experiment 2: LDT, elevated plus maze test (EPM) and social investigation test (SIT)). We found that, at higher-doses, long-term treatment of EB had anxiogenic effects in both βWT and βERKO mice as indicated by a decrease of the time spent in the light side and the number of transitions between two sides during LDT. In contrast, several behavioral measurements indicated that the lower-doses treatment of EB might reduce the anxiety levels possibly through ER-β. Particularly, the anxiolytic effects of EB in the SIT were more pronounced in βWT mice than βERKO mice. Together, the findings in the present study suggest that estrogen may have both anxiolytic and anxiogenic effects in female mice, and that ER-β gene disruption did not affect anxiogenic regulation by estrogen in female mice, but partially affected anxiolytic regulation.

Estrogens are known to affect anxiety or fear-related behavior. Symptoms of depression, anxiety and cognitive dysfunction in postmenopausal women have been associated with reduced levels of estradiol; estrogen replacement therapy improved the mood disturbance [1]. In rodents, lower circulating levels of estradiol were often associated with an increase in anxiety-related behaviors. Female rats [2,3] and mice [4] in the diestrus stage during their estrous cycle show shorter time spent in open arms of elevated plus maze (EPM) than females in the proestrus or estrus stage. Acute estrogen treatment of female rats in the diestrus stage, which mimics the hormonal level that occurs during the proestrus, reduced anxiety, as indicated by a larger percentage of the spent time in open arms of EPM [2]. These results strongly suggest that an increase in estradiol level in estrus females may be responsible for the reduction of their anxiety.

On the other hand, the effects of estrogen replacement in ovariectomized mice and rats on anxiety-related behaviors are still controversial. An acute single injection of estrogen to ovariectomized rats 3 h before behavioral tests had no effect on the number of entries to open arms nor the percentage of time spent in the open arms during EPM tests [3,5]. Three consecutive daily injections of estradiol benzoate (EB) to ovariectomized rats, however, enhanced the time spent in the open arms of EPM [5]. This discrepancy of the effects of estrogens may not be simply due to the difference in the number of injections (single vs three daily injections). Rather, it may be due to the interval between estrogens injection and behavioral tests. A single EB injection could increase open-field activity [6] and time spent in open arms during EPM test [7] in ovariectomized rats when they were tested 48 h after the injection.

In contrast to these anxiolytic results of bolus treatment, chronic estrogen treatment in ovariectomized female mice is reported to increase anxiety levels. Female mice implanted with a capsule containing EB for 7 days prior to behavioral tests showed higher levels of fear responses in a range of fear and anxiety-provoking situations than the animals treated with a vehicle-containing capsule [8,9]. The anxiogenic effects of chronic estrogen treatment were also founded in humans. Postmenopausal women chronically treated with 17β-estradiol on 3 month exhibited a significant increase in negative mood and anxiety [10], and 10 years treatment with estradiol implants in healthy postmenopausal women impaired their cognitive

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function [11]. These results suggest that the effects of chronic estrogen treatments on mental situation differ from that of acute one. Additionally, chronic enhancements of estrogen level may also relate to mood disorders in pregnant and postpartum women [12].

Since the difference between the bolus injections and the chronic treatment was not only a treatment period but also the amount volume of estrogens during the period, these results suggest that estrogen may have different effects on anxiety-related behavior depending on the dose treated to the animals. Indeed, for several memory tasks, bipolar effects of estrogen were reported. High-dose estradiol injection impaired, whereas low-dose estradiol injection facilitated, the performances of ovariectomized female rats on spatial memory tasks [13,14] and non-spatial working memory task [15]. Additionally, many endocrine factors have unusual dose–response relationship, including U-shaped and inverted U-shaped curve, by interacting with the other endocrine mechanism. It may be possible that the dose effects of estrogens on anxiety-related behavior show nonlinear form.

Recent studies using genetically modified mice reported that estrogen receptor (ER) β might play an important role in the regulation of anxiety levels. Krezel et al. [16] reported that gonadally-intact female mice with disrupted ER-β gene spent shorter time in the open arms of the EPM. Ovariectomized female ER-β knockout (βERKO) mice also showed shorter spent time in the outer half of the open arms than their wild type, regardless of presence or absence of estrogen treatment [17]. Additionally, injection of diarylpropionitrile (DPN), which is a selective ER-β agonist, reduced anxiety-related behavior in ovariectomized female rats [18,19]. These results suggested that ER-β is involved in anxiolytic effects of estrogen.

In contrast, it is not known whether ER-β may also mediate anxiogenic effects of estrogen reported in chronically treated animals. In the present study, we investigated the role of ER-β in the regulation of anxiety levels in female mice after chronic EB treatment using female βERKO mice (experiment 1). Since it is still possible that differences in the behavioral effects of estrogen in animals treated with bolus injections and those treated chronically may be due to differences in the amount of estrogen administered, we tested the mice with several different doses of EB (experiment 2). In addition, we measured anxiety-related behaviors in several different behavioral paradigms in experiment 2, because the effects of estrogen on anxiety level may depend on the experimental context, such as testing paradigms. Measurements in behavioral tests commonly used to measure anxiety levels, such as open-field and EPM tests, are often confounded with general activity levels of animals and are affected by testing conditions, such as brightness of illumination [20]. In contrast, animals' behaviors in light–dark transition tests (LDT) are known to be relatively independent of activity levels and differences in testing conditions. Therefore, in the present study we tested the same animals both in EPM and LDT to compare the behavioral effects of various doses of estrogen and ER-β gene disruption in two types of anxiogenic situations. We also performed social investigation tests (SIT) to assess social anxiety levels, which were measured as responses to an intruder mouse presented in experimental animals' home cage.

1. Methods

1.1. Mice

βERKO female mice and their wild type (βWT) littermates were used. They were obtained from the βERKO breeding colonies maintained at the Rockefeller University by mating heterozygous male and female mice. Original breeding pairs (in C57BL/6J background) were obtained from the National Institute of Environmental Health Sciences [21]. From weaning to 10 days before the first behavioral tests, subjects were housed with same-sex littermates in plastic cages (30×20×12 cm). At the age of 9–17 weeks, mice were ovariectomized and received subcutaneous implants with either a β-estradiol 3-benzoate (EB) pellet or a placebo pellet (Innovative Research of America, Toledo, OH) under anesthesia with isoflurane inhalation. All pellets were on a 21-day release schedule and average amount released per day was calculated based on the total amount of EB in the pellets. In experiment 1, mice in each genotype were divided into two groups, (1) placebo (βWT: $n=11$, βERKO: $n=12$) and (2) EB 2.0 μg/day pellet (βWT: $n=12$, βERKO: $n=13$). In experiment 2, mice were treated with five different doses of EB, (1) EB 0 μg/day (placebo, βWT: $n=10$, βERKO: $n=11$), (2) EB 0.1 μg/day (βWT: $n=10$, βERKO: $n=9$), (3) EB 0.2 μg/day (βWT: $n=7$, βERKO: $n=7$), (4) EB 0.4 μg/day (βWT: $n=10$, βERKO: $n=9$), or (5) EB 1.0 μg/day (βWT: $n=9$, βERKO: $n=11$). After ovariectomy and pellet implants, all mice were individually housed in plastic cages (30×20×12 cm), except during the period of social investigation test described below. They were kept on a 12/12 h light/dark cycle (light off at noon) at constant temperature (22 °C) throughout the extent of the studies. Food and water were available *ad libitum*.

1.2. Behavioral tests

Starting 10 days after pellet implantation, all mice were tested for anxiety-related behaviors. In experiment 1, mice were tested once for light–dark transition on the 10th day. In experiment 2, mice were tested in three different behavioral paradigms, in the order of LDT (Day 10), EPM (Day 13) and SIT (Day 16). All behavioral tests were performed starting 2 h after lights off.

1.2.1. Light–dark transition (LDT) test

The test apparatus consisted of a clear plastic box (50×50×35 cm) with a black (light opaque) covered-plastic box (50×25×25 cm) in one side (the dark side). The black box had an open doorway (2×5 cm) that led to the light side of the apparatus, which was illuminated by a 40-W white bulb (about 420 lx on the floor). Mice were moved from the living room to the testing room at least 1 h before the test. At the beginning of the tests, mice were removed from their cages, gently placed at the doorway, and released into the dark side of the apparatus. A Digiscan analyzer and software (Digiscan Model RXYZCM, Accuscan Instruments, Columbus, OH) were used to collect and store horizontal-activity data, which was monitored by infra-red beams. For each mouse, the following measurements were recorded for 10 min: total moving time and total activity (total number of beam breaks) in each compartment, total time spent in the dark compartment, total time in the light compartment, number of transitions between the dark and light compartments, and the latency to the first emergence from the dark to the light compartment. After the test, the amount of defecation was counted. Between the tests, the apparatus was thoroughly wiped clean.

1.2.2. Elevated plus maze (EPM) test

Mice were tested in a maze consisting of a 5×5 cm center platform from which extended four acrylic arms in a cross formation, with two opposing arms enclosed by side walls and two left open (Rockefeller University Instrument Shop, New York, NY). Arms were 30×5 cm, with the enclosed arms having 15-cm-high opaque acrylic walls. The entire maze was elevated 40 cm above the floor and illuminated by a 50-W red light bulb suspended 100 cm above the maze. At the beginning of the test, mice were placed in the center of the maze, facing to one of the open arms. Number of entries into and time spent in open arms and closed arms were recorded manually during 5 min tests.

1.2.3. Social investigation test (SIT)

One day before the test, mice were transferred to larger housing cages (45×23×15.5 cm). They were tested against gonadally intact male mice of C57BL/6J strain for 10 min. Male stimulus mice were

placed in a clear perforated (28 small holes with a 5 mm diameter on the bottom 3 cm) plexiglass cylinder (7 cm diameter × 12 cm height) at least 10 min before the testing. They were then introduced into the center of females' housing cages. Total moving distance of female mice in the housing cage, and total number and cumulative duration of females' sniffing toward perforated parts of the cylinder were analyzed by a Macintosh computer using a video tracking software, ImageSI (tube) version 1.13 (O'Hara & Co., Ltd., Tokyo, Japan) developed based on the NIH Image. All tests were performed in a testing room illuminated with a red ceiling light.

1.3. Measurement of serum estradiol levels

On the day after the SIT test (Day 17) of experiment 2, animals were sacrificed and their blood samples were collected. Serum estradiol levels were determined using Coat-A-Count Total Estradiol Kit (DPC, Los Angeles, USA) according to the manufacturer's instruction. All samples were run in a single assay. The cumulative intra-assay CV was 11.3%. Detection limit was 10 pg/ml. We provisionally substituted zero for the values under the limit.

1.4. Statistics

All data are presented as a mean+SEM, except estradiol levels which are indicated median+quartile deviation. All behavioral data were analyzed by two-way ANOVAs, for the main effects of genotype and EB doses, and their interaction. Significant main effects and interactions were tested with Bonferroni post hoc comparisons in order to identify significant variations among the variables. In SIT, we excluded three mice (one of each from the groups of βERKO-placebo, βWT-placebo and βWT-0.1 μg/day) from the analysis due to an equipment failure.

2. Results

2.1. Experiment 1

Overall, there were no main effects of genotype in any measurements during the LDT tests; the time spent in the light compartment (Fig. 1a), the number of transitions between two compartments (Fig. 1b), and the latency to the first emerge to the light compartment (Fig. 1c). EB treatment at the dose of 2.0 μg/day similarly affected these behavioral measurements in βWT and βERKO mice. Both the time spent in the light compartment [$F(1, 44)=8.75$, $P<0.01$] and the number of transitions [$F(1, 44)=16.53$, $P<0.001$] were significantly reduced by EB treatment compared to the placebo control groups regardless of genotype. EB treatment also tended to increase the latency to emerge to the light compartment in both genotypes, but did not reach a statistically significant level [$F(1, 44)=2.27$, $P=0.14$].

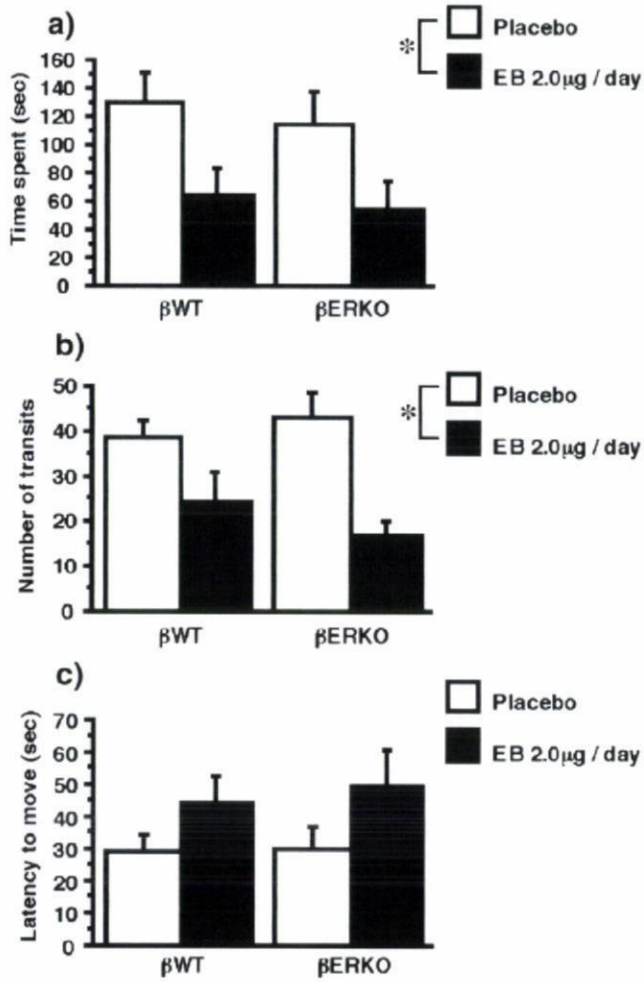

Fig. 1. The results (mean+SEM) of the light/dark transition (LDT) test in experiment 1. Regardless of genotype, mice treated with EB at the dose of 2.0 μg/day (βWT: $n=12$, βERKO: $n=13$) showed significantly higher levels of anxiety compared to the placebo treated mice (βWT: $n=11$, βERKO: $n=12$), as indicated by a large reduction of the total time spent in the light side of LDT apparatus (a) and the number of transitions between the dark and light sides (b). The latency to the first emergence from the dark to the light compartment also tended to increase in the EB treated groups. (*$P<0.05$).

2.2. Experiment 2

2.2.1. LDT

In contrast to anxiogenic effects of EB at a higher dose (2.0 μg/day) in experiment 1, EB treatment at lower-doses reduced the levels of anxiety. Overall, the mice implanted with a 0.2 μg/day EB pellet spent longer time in the light compartment than those implanted with a placebo pellet (Fig. 2a; main effect of EB treatment [$F(4, 83)=2.72$, $P<0.05$]). Though these behavioral effects of EB were more pronounced in βWT than βERKO mice, there was no main effect of genotype [$F(1, 83)=0.57$, $P=0.45$] and interaction between genotype and EB treatment [$F(4, 83)=1.24$, $P=0.30$]. Lower doses of EB treatment also increased the number of transitions between two compartments. The mice treated with an EB pellet at the doses of 0.2 μg/day showed a significantly higher number of transitions than those treated with a placebo pellet (Fig. 2b; main effect of EB treatment [$F(4, 83)=2.65$, $P<0.05$]). Again, there was no apparent genotype difference in terms of these anxiolytic effects of EB on this measurement. In contrast to these two measurements, the main effect of EB dose was not statistically significant in the latency to the first exit to the light compartment (Fig. 2c). Instead, there was a significant overall genotype difference: βERKO mice showed a significantly longer latency than βWT mice (Fig. 2c; main effect of genotype [$F(1, 83)=4.62$, $P<0.05$]. The genotype differences were more apparent in the mice treated with a placebo, 0.1, or 0.2 μg/day EB pellet compared to those in the other treatment groups, although the interaction between genotype and EB treatment was not statistically significant [$F(4, 83)=0.90$, $P=0.47$].

2.2.2. EPM

Most of the mice hardly enter open arms as indicated in the time spent in the open arms, and the main effects of genotype, EB treatment, and the interactions between them were not statistically significant in any behavioral measures (data not shown).

2.2.3. SIT

In this test, genotype specific dose-dependent effects of EB treatment were more clearly seen in all three measurements. Particularly,

a statistically significant interaction between genotype and EB treatment [$F(4, 80) = 3.06$, $P < 0.05$] was found in the moving distance (Fig. 3a). Post hoc analysis revealed that the moving distance was highest in βWT mice treated with a 0.2 μg/day EB pellet [$P < 0.05$ vs placebo group] and lowest in those treated with a 1.0 μg/day EB pellet [$P < 0.05$ vs placebo or 0.2 μg/day EB pellet groups]. In βERKO mice, on the other hand, all five groups of mice showed similar levels of moving distance. Inverted U-shape effects of EB treatment were also seen in two other measurements, the number (Fig. 3b) and the total duration (Fig. 3c) of sniffing bouts, in βWT mice but not in βERKO mice, although the interaction between genotype and EB treatment did not reach a statistically significant level.

2.2.4. Plasma levels of estradiol

The plasma levels of estradiol of the animals treated with 1.0 μg/day EB pellet were 76 (+99) pg/ml and 114 (+124) pg/ml in βERKO and βWT mice, respectively. Those of the animals treated with 0.4 μg/day EB were 31 (+24) pg/ml (βERKO) and 11 (+35) pg/ml (βWT). In the mice treated with lower doses of estrogen (0.2 and 0.1 μg/day EB), the plasma concentrations were not at a detectable level. There were no genotype differences at any dose.

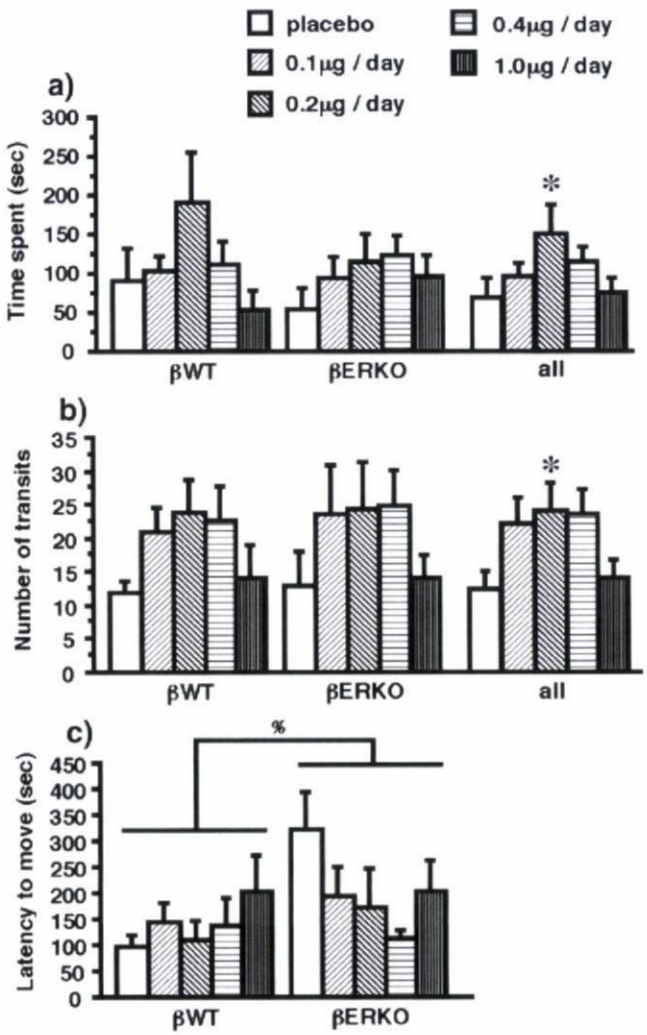

Fig. 2. Total time spent in the light side of the LDT apparatus (a), the number of transitions between the dark and light compartments (b) and the latency of the first move from the dark to the light compartment (c) in experiment 2 (*$P < 0.05$, compared to the placebo group). In the latency to move from the dark to the light compartment, main effect of genotype was also significant (%$P < 0.05$). All: each treatment group was collapsed across genotype. Placebo (βWT: $n = 10$, βERKO: $n = 11$), EB 0.1 μg/day (βWT: $n = 10$, βERKO: $n = 9$), EB 0.2 μg/day (βWT: $n = 7$, βERKO: $n = 7$), EB 0.4 μg/day (βWT: $n = 10$, βERKO: $n = 9$), and EB 1.0 μg/day (βWT: $n = 9$, βERKO: $n = 11$).

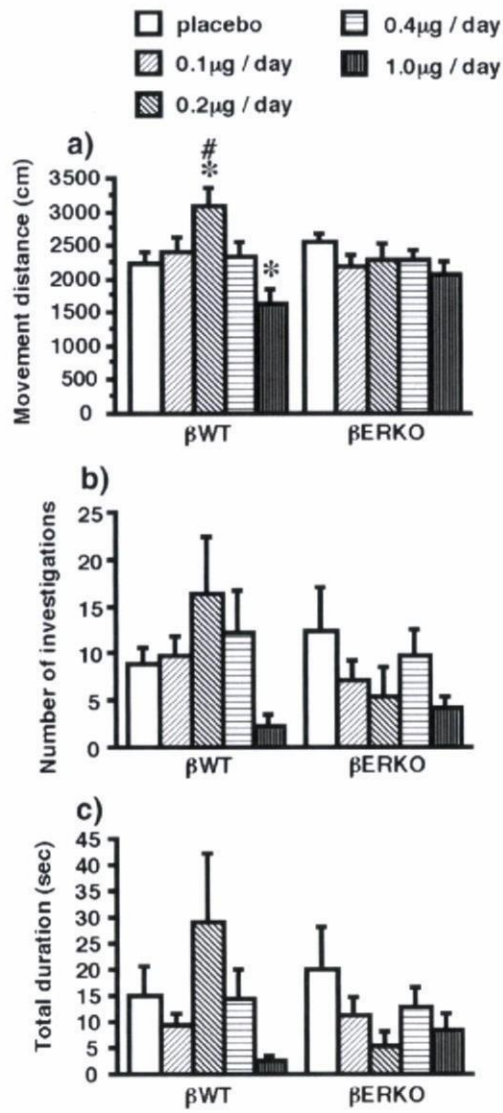

Fig. 3. The total moving distance of the subjects in the apparatus (a), total number (b) and duration (c) of investigation of stimulus mice during the SIT (*$P < 0.05$, compared to placebo group of the same genotype; #$P < 0.05$, compared to the 1.0 μg/day group of the same genotype). Placebo (βWT: $n = 9$, βERKO: $n = 10$), EB 0.1 μg/day (βWT: $n = 9$, βERKO: $n = 9$), EB 0.2 μg/day (βWT: $n = 7$, βERKO: $n = 7$), EB 0.4 μg/day (βWT: $n = 10$, βERKO: $n = 9$), and EB 1.0 μg/day (βWT: $n = 9$, βERKO: $n = 11$).

3. Discussion

In the present study, we examined the effects of estrogen on the levels of anxiety in both non-social (LDT and EPM) and social (SIT) situations. In experiment 1, we tested animals in the LDT apparatus and found that EB treatment at the dose of 2.0 μg/day decreased the time spent in the light side and the number of transitions between the dark and light sides. These results indicate that long-term EB treatment at a relatively high-dose, among the treatment dose used in this study, increases the anxiety level of the female mice. Especially, it is worthy to note that the EB treatment affected the time spent in the light side. That is considered a reliable index of the animal's anxiety level, because that does not directly depend on the animal's activity level. Actually, the total moving distance in the dark side, that is considered a direct index of the animal's activity level, did not show

a statistical difference between the EB-treated and placebo groups in experiment 1 (data not shown). Although we did not conduct EPM nor SIT in experiment 1, the results in the LDT tests strongly led us to hypothesize that estrogen might have anxiogenic effects, rather than anxiolytic effects, at higher-doses. Additionally, our findings of anxiogenic EB effects are consistent with those of previous studies reporting an increase of fear responses in female mice implanted with a silastic capsule containing EB (25–75 μg/capsule) 7 days before behavioral tests [8,9]. Since both βWT and βERKO mice responded similarly to EB treatment in experiment 1 (Fig. 1), ER-β may not be responsible for the behavioral effects of estrogen at this dose. On the other hand, ER-α may possibly play a crucial role for the anxiogenic regulation of estrogen, because βERKO mice have almost the same distribution of ER-α as βWT [22].

In contrast to the results in experiment 1, the mice implanted with a 0.2 μg/day EB pellet spent a longer time in the light compartment and showed a higher number of transitions between two compartments during the LDT tests than those implanted with a placebo pellet. Because these behavioral measures were commonly used for index of animal's anxiety level as described above, the enhancement in the mice implanted with a 0.2 μg/day EB pellet suggests that the chronic treatment of relatively lower-doses EB decreased the anxiety level of the mice. Similar inverted U-shape dose–response curve of estrogen effects were also shown in the SIT. For instance, the moving distance was highest in animals treated with a 0.2 μg/day EB pellet compared to those in four other treatment groups. Moving distance is directly affected by an activity level of animal. However, under ambiguous danger stimuli, or anxiogenic stimuli, depressing moving activity generally seems to be adaptive for the animal. While, less anxious animals may move more freely even when an anxiogenic stimulus such as a gonadally intact strange male mouse was introduced into their home cage. Actually, it was reported that the general activity level measured by running wheel in the OVX female mice treated with EB was enhanced compared to the animal treated with a vehicle oil, but the moving distance of the animals in the center area of open-field, that meant anxious situation, was decreased [9]. These results suggest that the moving distance in an anxious situation does not reflect general activity level of animals. Therefore, we hypothesize that moving distance in SIT also reflects animals' anxiety levels in social situations. It should be noted that the inverted U-shape dose–response curve with anxiolytic effects at lower-doses was only seen in βWT, but not βERKO, mice in this index. These results collectively suggest that ER-β activation may be more crucially involved in the regulation of animal behavior in social situations. While, the amount of sniffing to an intruder male mouse, more direct measurements of responsiveness to anxiogenic social stimulus, in the βWT mice treated with the lower-doses EB were not statistically different from the other groups of the genotype. The amount of sniffing may reflect the mechanism of a social attraction, or motivation of social investigation. Therefore, the anxiolytic effects of EB treatment on the moving distance on SIT in the present study cannot be explained directly from the mechanism of social attraction, or motivation.

Overall, the results in the present study suggest that the regulatory effects of chronic estrogen treatment on anxiety-related behaviors were dose-dependent; estrogen is anxiogenic at higher doses, whereas it has anxiolytic effects at lower doses. Only in the SIT of experiment 2, we found the effects of gene disruption of ER-β on anxiolytic action of estrogen, as the βWT mice treated with a 0.2 μg/day EB pellet showed increased moving distance, but not βERKO mice. Therefore, we can conclude that the disruption of ER-β, at least partially, affected anxiolytic actions of estrogen, but not anxiogenic actions. Recent studies have provided significant evidence to indicate that ER-β plays an important role in decreasing anxiety levels. βERKO mice showed higher anxiety-related behavior than βWT in EPM and open-field [16,17], and the injection of a selective ER-β agonist reduced anxiety-related behavior in ovariectomized female rats in several behavioral tests including EPM and open-field [18,19]. In the present study, there was a significant overall genotype difference between βERKO and βWT mice in the emergence latency to the light side of LDT, and the result also suggested overall higher anxiety of βERKO than βWT mice. Therefore, ER-β is possibly related to regulation of initial anxiety level of animal.

It is assumed that a number of mechanisms regulated by estrogen in an ER-β dependent manner may be responsible for behavioral effects of ER-β gene disruption found in the present study. It has been shown that oxytocin mRNA levels in the hypothalamic paraventricular nucleus are up-regulated by estrogen via ER-β [23]. Oxytocin is known to have anxiolytic effects since intracerebroventricular injections of oxytocin reduced anxiety levels [24–26] and disruption of oxytocin gene enhanced anxiety-related behaviors [27]. In addition, it is reported that the anxiolytic effects of oxytocin are enhanced by estrogen treatment [24,25]. Therefore, it is possible that ER-β activation suppresses the anxiety-related behaviors in female mice by regulating hypothalamic oxytocin levels. Moreover, it may be worth pointing out that in the present study, the clearest relationship between ER-β deletion and a lack of anxiolytic effects of estrogen treatment was shown in the SIT, which measured the levels of social anxiety. In the previous studies, we also found that social recognition was disrupted in both ER-β knockout mice and oxytocin knockout mice [28,29].

The brain serotonergic system is also implicated in the regulation of anxiety-related behaviors [30]. There is some evidence suggesting that estrogen regulates the mesencephalic ascending serotonergic pathway via ER-β. In mice, more than 90% of ER-β immunopositive cells in the dorsal raphe nuclei (DRN) co-express tryptophan hydroxylase (TPH), the rate-limiting enzyme of serotonin synthesis, whereas only 20% of ER-α positive cells express TPH [22]. Estrogenic regulation of serotonin contents in the DRN is greatly affected by ER-β gene disruption [17]. Therefore, it is possible that ER-β activation may modulate anxiety-related behavior by regulating the dorsal raphe-serotonergic pathway in female mice. In the DRN, ER-β, but not ER-α, plays a crucial role in the estrogenic induction of progestin receptors in TPH-positive cells [31]. Therefore, it is possible that ER-β dependent anxiolytic effects of estrogen may be further accentuated by progesterone administration.

It is also possible that ER dependent estrogenic regulation of neuronal activity in the medial amygdala may be responsible for the behavioral effects found in the present study. It has been reported that systemic EB treatment increased Fos immunoreactivity in the medial amygdala [32]. Furthermore, intra-amygdala EB administration decreased anxiety and fear related behaviors in ovariectomized rats [7]. In this brain region, both ER-α and ER-β are localized [33,34] and may possibly be involved in the estrogenic regulation of anxiety-related behaviors. While, a recent study [35] showed that hippocampus played an important role for anti-anxiety and anti-depressive effects of ER-β, but not ER-α, by administering the ER-α or ER-β selective agonists to the hippocampus, directly. These several mechanisms that were described above may act in compensate, rather than exclusive, manner for regulating the anxiety-related behavior.

One of the most important and remaining problems in the present study is the actual circulating level of estradiol responsible for the anxiolytic or anxiogenic effects. At the dose we found the most prominent anxiolytic effects, i.e., 0.2 μg/day, plasma levels of estradiol were not at a detectable level in the present study. Estradiol levels in mice treated with 0.4 μg/day of EB pellet were 31 (+24) pg/ml for βERKO and 11 (+35) pg/ml for βWT and similar to those found in estrus stage of naturally cycling mice (17 (+4) pg/ml) in our preliminary study using the same assay protocol. During pregnancy, females are exposed to higher level of estrogen for longer period, and the anxiety level in pregnant female was also reduced [36]. It was reported the serum level of estradiol in the pregnant female rodents

was approximately 30 pg/ml [37,38]. The estradiol level of pregnant female is lower than that of mice treated with 1.0 μg/day of EB pellet in our study, rather similar to that of mice treated with 0.4 μg/day of EB pellet. On the other hand, those in the mice treated with 1.0 μg/day EB were much higher than physiological levels, i.e., 76 (+99) pg/ml and 114 (+124) pg/ml in βERKO and βWT mice, respectively. Although we did not measure the plasma levels of estradiol in the mice which were treated with 2.0 μg/day EB pellet and showed high anxiety levels (experiment 1), the findings in the present study suggest that circulating levels of estradiol at the levels similar to or lower than those during estrus and/or pregnant female may have anxiolytic behavioral effects while super-physiological levels of estrogen may induce anxiogenic effects.

Another problem of this study is inconsistency of the results among the behavioral tests. In experiment 2, the effects of ER-β gene deletion on anxiety-related behavior showed in the SIT, but were not clear in LDT. The inconsistencies of anxiety-related behavior depend on the test paradigms also showed in many other studies [39,40]. These results may involve that anxiety and emotionality have multidimensional components and situation-dependant aspects. Especially, since ER-β is considered to play an important role for regulating social recognition mechanism [28,29], it is possible that such mechanisms underlie the background of the anxiolytic effects of EB treatment in social situation.

The differences of basal level of behavior between experiments were also a technical, but critical, problem. The animals treated with a placebo pellet in experiment 2 showed higher anxiety level compared to those of experiment 1, as indicated in shorter time spent in the light side, lower number of transitions, and longer latency to the first emerge in the LDT test. However, we do not think that the difference of basal level of the behavior is critical for our conclusion, since the anxiogenic effects of higher-dose EB treatment were also stable in experiment 2, as indicated that the animals treated with 1.0 μg/day EB pellet showed the highest anxiety level among the treatment groups in several behavioral measures.

In conclusion, our results suggest that estrogen may have both anxiolytic and anxiogenic effects in female mice and ER-β may be involved primarily in anxiolytic, but not anxiogenic, action of estrogen. We do not exclude the possibility, however, that ER-α may also be involved in the anxiolytic effects of estrogen. Indeed, in the LDT of experiment 2, the βERKO mice treated with a lower-dose of estrogen pellet also showed reduced levels of anxiety-related behaviors compared to those treated with placebo, as well as βWT. The exact role of ER-α in the regulation of anxiety levels needs to be determined in further studies.

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Behavioral/Systems/Cognitive

Estrogen Receptors in the Medial Amygdala Inhibit the Expression of Male Prosocial Behavior

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Studies using estrogen receptor α (ERα) knock-out mice indicate that ERα masculinizes male behavior. Recent studies of ERα and male prosocial behavior have shown an inverse relationship between ERα expression in regions of the brain that regulate social behavior, including the medial amygdala (MeA), and the expression of male prosocial behavior. These studies have lead to the hypothesis that low levels of ERα are necessary to "permit" the expression of high levels of male prosocial behavior. To test this, viral vectors were used to enhance ERα in male prairie voles (*Microtus ochrogaster*), which display high levels of prosocial behavior and low levels of MeA ERα. Adult male prairie voles were transfected with ERα in the MeA (MeA-ERα) or the caudate–putamen (ERα control) or luciferase (MeA-site-specific control), and 3 weeks later tested for spontaneous alloparental behavior and partner preference. Enhancing ERα in the MeA altered/reduced male prosocial behavior. Only one-third of MeA-ERα males, compared with all control males, were alloparental. MeA-ERα males also displayed a significant preference for a novel female. This is a critical finding because the manipulations of neuropeptides, oxytocin and vasopressin, can inhibit the formation of a partner preference, but do not lead to the formation of a preference for a novel female. The results support the hypothesis that low levels of ERα are necessary for high levels of male prosocial behavior, and provide the first direct evidence that site-specific ERα expression plays a critical role in the expression of male prosocial behavior.

Key words: estrogen receptor α; *Microtus ochrogaster*; transfection; viral vector; aggression; amygdala

Introduction

Estrogen plays a critical role in the expression of male social behavior. Studies using estrogen receptor (ER) knock-out mice indicate that ERα and ERβ regulate different aspects of male behavior. ERβ is involved in defeminization (Kudwa et al., 2005), anxiety, and cognition (Krezel et al., 2001), whereas ERα masculinizes behavior (Ogawa et al., 1998; Wersinger et al., 1997). Although some males display high levels of prosocial behavior and positive affiliative behavior, masculine behavior is typically associated with low levels of prosocial behavior and high levels of aggression. The expression of high levels of prosocial behavior requires a reduction of "typical" masculine behavior. It has been hypothesized that decreasing ERα within the social neural circuit is "necessary" for the expression of high levels of prosocial behavior (Cushing et al., 2004; Cushing and Wynne-Edwards, 2006).

Comparative studies support this hypothesis. Illinois prairie voles (*Microtus ochrogaster*) are highly social, forming pair bonds and providing biparental care. However, Kansas males are significantly less social (Cushing and Kramer, 2005a) and express significantly higher levels of ERα in the medial amygdala (MeA) and bed nucleus of the stria terminals (BST) than Illinois males (Cushing et al., 2004). Socially monogamous pine voles (*M. pinetorum*) express lower levels of ERα than the polygynous meadow (*M. pennsylvanicus*) and montane voles (*M. montanus*) (Cushing and Wynne-Edwards, 2006). Finally, male ERα in species of two dwarf hamsters (*Phodopus* sp.) that differ in social behavior mirrored ERα expression within prairie vole populations (Cushing and Wynne-Edwards, 2006).

Changes in ERα expression are correlated with prosocial behavior. In male prairie voles, neonatal castration eliminated alloparental behavior (Lonstein et al., 2002) and the ability of neuropeptides to stimulate pair bond formation (Cushing et al., 2003), whereas significantly increasing ERα in specific brain regions, including the MeA (Cushing and Kramer, 2005b). Long-day male Siberian hamsters (*Phodopus sungorus*) are nonaggressive, although aggression increases under short days, which is associated with increased ERα (Kramer et al., 2008). Finally, in mice male aggression has been associated with individual variation in ERα (Trainor et al., 2006). These findings are significant, but correlative. Therefore our goal was to increase ERα in the MeA of male prairie voles and test the prediction that this would disrupt prosocial behavior.

This study focused on the MeA. MeA ERα is inversely correlated with male prosocial behavior (Hnatczuk et al., 1994; Cush-

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ing et al., 2004; Cushing and Wynne-Edwards, 2006). Conspecific odors activate the vomeronasal organ, triggering the MeA and BST, leading to stimulation of the rest of the social neural circuit (Newman, 1999). Additionally, the MeA innervates the "reward" system via the ventral pallidum, and the reward system is critical for long-term social bond formation (Young et al., 2005). Finally, the MeA regulates social recognition/memory (Ferguson et al., 2001), which is a necessary for long-term social bond formation. We are not suggesting that the MeA is the primarily responsible for regulating specific social behaviors, but that it is critical for the initiation of social responses and therefore changes in MeA can and will impact many aspects of the social response.

Materials and Methods

Animal husbandry

Animals used in this study were laboratory-reared prairie voles that originated from wild stock trapped near Urbana, IL. Animals were housed under a 14 h/10 h light/dark cycle and provided high fiber rabbit chow and water *ad libitum*. Animals were housed in accordance with the United States Department of Agriculture and National Institutes of Health guidelines and all procedures were approved by the University of Illinois at Chicago Animal Care and Use Committee before undertaking any experimental manipulation.

Viral vector transfection

To achieve site-specific overexpression of ERα in the MeA, adult males (60–70 d of age) were stereotactically injected, bilaterally, with adenoassociated viral (AAV) vectors encoding human ERα. The site of the MeA was determined to be −1.25 mm anteroposterior, ±1.65 mm mediolateral, and −5.8 mm dorsal from bregma. Two controls were established. Site-specific controls were generated by transfecting the MeA of males with a similar vector encoding firefly luciferase cDNA, and ERα controls were generated by transfecting the caudate–putamen with ERα. Caudate neurons do not express ERα. The AAV vectors used in this study have been described previously in detail (Musatov et al., 2006). Briefly, the vectors express short hairpin RNA containing ERα (GGCATGGAGCATCTCTACA) or luciferase (CCGCTGGAGAGCAACTGCAT) target sequences under the control of the human H1 promoter. In addition, both vectors were designed to express enhanced green fluorescent protein (GFP) as a reporter to visualize transfection neurons and placed under a control of a hybrid cytomegalovirus/chicken-β-actin promoter to ensure stable long-term expression. Vector stocks were generated using a helper-free AAV-2 plasmid transfection system, purified by heparin affinity chromatography and dialyzed against PBS as described previously (Clark et al., 1999). AAV genomic titers were determined by quantitative PCR and adjusted to 109 particles per microliter. Three weeks after transfection males began behavioral testing. After completion of behavioral testing, males were killed to analyze the efficiency of gene transfer, and behavioral data were only analyzed in males in which transfection was verified.

Verification of transfection. After completion of the social preference test, brains from experimental animals were fixed using transcardial perfusion, sectioned at 30 μm on a freezing sliding microtome, and then the free-floating sections were stained for ERα using standard AB immunocytochemistry (for complete details, see Cushing et al., 2004). The human-specific primary antibody RM9101-s (Neomarkers; 1:1000 dilution) was used to label transfected ERα and then visualized using DAB. RM9101-s does not label prairie vole ERα. Therefore, all ERα observed with this antibody were the product of transfection. Successful transfection was determined qualitatively by visually examining ERα expression using a Nikon E-800 microscope. It should be noted that there were no animals that display only a few transfected ERα-immunoreactive cells, and transfected males either displayed no or a significant amount of transfected ERα. Figure 1*a* shows a typical level of transfected ERα expression, which is compared with nontransfected ERα expression seen in Figure 1*b*. Only animals that showed at least unilateral ERα expression resulting from transfection in the MeA, were used in subsequent analysis of behavior data. Luciferase expression was determined by examining tissue for GFP expression (see Fig. 1*c*).

Behavior

Alloparental test. Males participated in a standard alloparental test (Cushing and Kramer, 2005a; Trainor et al., 2006; Young et al., 2005). Males were placed in an alloparental test arena consisting of two cages (12 × 18 × 28 cm) connected by a plastic tunnel. Males were allowed 30 min to adapt to the arena before a 1- to 3-d-old pup was placed in one of the two cages. The behavior of the male was videotaped and analyzed for 10 min after entrance into the pup's cage, or for 30 min if the male failed to enter the pup cage. Males that spent a minimum of 3 min in contact with the pup, licking and grooming, or huddling with the pup were classified as alloparental. Because all control males were alloparental, for the purpose of analysis they were grouped.

Social preference. Five days after the alloparental test, treated males were tested for social preference. This was done using the standardized vole partner preference test (Liu et al., 2001; Cushing et al., 2003). Treated males were cohabited with an unrelated sexually naive adult female for 1 h. This female was classified as the familiar "partner." Female prairie voles do not undergo spontaneous estrus and require prolonged exposure to a male to become sexually receptive. Therefore, mating is not a factor in this test. Immediately after cohabitation, test animals participated in a social preference test. The social preference arena consisted of three polycarbonate cages (12 × 18 × 28 cm) in a modified Y shape. The two cages housing stimulus animals were in parallel and a third cage (neutral) was attached separately to each stimulus cage. The familiar female was gently tethered in one of the stimulus cages while an age- and size-matched sexually naive female (novel/"stranger") that was unrelated to both the familiar female and the subject was tethered in the other stimulus cage. Then, the experimental male was placed in the neutral chamber and allowed to move about freely for 3 h. The test was recorded using a time-lapse video (12:1 ratio) and then scored by an experimentally blind scorer. The data scored and analyzed include frequency of entrance, total time spent in the chambers, and time spent in physical contact with partner and stranger. It should be noted that without hormonal manipulation, such as central administration of arginine vasopressin, 1 h of cohabitation does not lead to the formation of preference for the familiar stimulus animal (DeVries et al., 1996; Cho et al., 1999); therefore, control males were predicted to spend equal amounts of time in the cages of and in physical contact with both females.

Statistical analysis. Based on the a priori assumption that the two control groups would not differ, preplanned comparisons were made between the two controls. If and only if there was no difference in any measure, they were grouped into a single control group for the purpose of analysis, which was the case in this study. Alloparental data represents count data with two possible outcomes; therefore the data were analyzed using 2 × 2 Fisher's exact probability. For the social preference test, an ANOVA was used to analyze between treatment effects, whereas a paired *t* test was used for within-treatment analysis.

Results

As indicated by immunoreactivity of transfected ERα 12 (eight bilateral and four unilateral) of the 20 males in which AAV-ERα was injected into the MeA were successfully transfected (Fig. 1*a*). There were a total of 19 control males (9 MeA luciferase and 10 caudate ERα). There was a significant treatment effect of increased ERα in the MeA on both the expression of spontaneous alloparental behavior and social preference. Enhancing ERα in the MeA inhibited alloparental behavior with only 4 of 12 (33%) MeA-ERα males displaying alloparental behavior, compared with 19 of 19 (100%) of the control males (Fisher's exact $p < 0.0001$) (Fig. 2). Nonalloparental behavior displayed by MeA-ERα males included attacking the pup ($n = 5$), attempting to mount and mate with the pup ($n = 2$), or ignoring the pup ($n = 1$). As predicted, control males did not display a preference for either the familiar or novel female. In contrast, MeA-ERα males

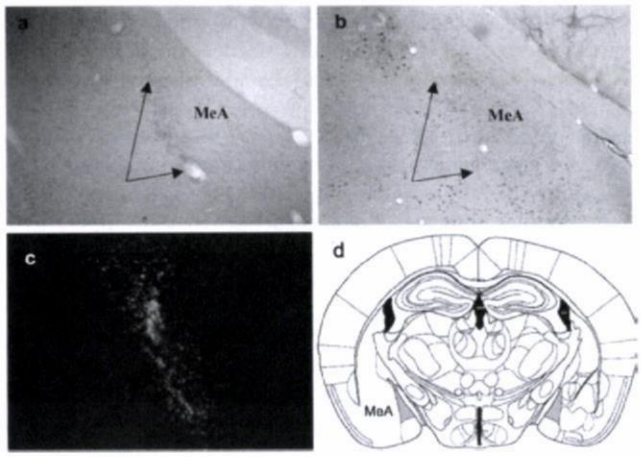

Figure 1. ***a***, Photomicrograph of AAV-mediated ERα overexpression. ***b***, Typical low levels of ERα immunoreactivity in the medial amygdala of male prairie voles. ***c***, GFP expression. ***d***, Graphic representation of the site of infusion (gray shaded area) (adapted from Paxinos and Franklin, 2001). Endogenous vole ERα (***a***) is labeled using the primary polyclonal antibody c1355 (Millipore; 1:10,000 dilution) and developed with nickel-DAB (purple staining) (Cushing et al., 2004), whereas transfected human ERα is labeled using the primary antibody RM9101-s (1:1000 dilution) and visualized using DAB (brown).

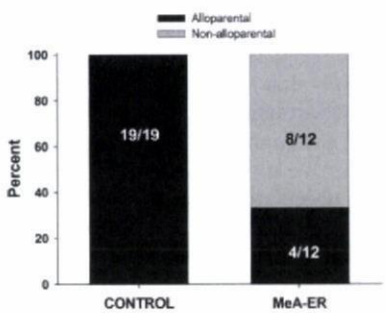

Figure 2. Results of treatment on the expression of spontaneous alloparental behavior. Enhancing EPα in the MeA (MeA-ERα) resulted in a significant decrease in alloparental behavior compared with control males. $p < 0.001$.

spent significantly more time in a novel female's cage compared with control males (ANOVA, $p < 0.05$) (Fig. 3). MeA-ERα males formed a "stranger" preference spending significantly more time in the cage of a novel female (pair *t* test, $p < 0.05$) and in physical contact with a novel female than with a familiar female (paired *t* test, $p < 0.05$) (Fig. 3).

Discussion

The results from this study indicate the importance of ERα in regulating male prosocial behavior providing direct evidence that ERα can inhibit the expression of male prosocial behavior. Furthermore, the results support the hypothesis that a reduction in ERα is necessary for the expression of high levels of male prosocial behavior. Increasing ERα in the MeA of the adult male prairie vole reduced or inhibited the expression of alloparental behavior and the initial formation of social preferences.

Unlike most male rodents, which are infanticidal or ignore pups, even inexperienced male prairie voles display high levels of spontaneous alloparental behavior, including licking, grooming, huddling, and retrieving unrelated pups. In the majority of males, enhancing ERα in the MeA produced a more "typical" male response that of pup-directed aggression, although in two males it produced a novel response: pup-directed sexual activity. The expression of spontaneous alloparental behavior is very difficult to disrupt in male prairie voles (Cushing and Kramer, 2005a), and although it has been suggested that alloparental behavior is controlled by neuropeptides, inhibition of endogenous neuropeptides does not disrupt the alloparental behavior in adult males. However, neonatal castration does disrupt male alloparental behavior (Lonstein et al., 2002) as well as significantly increasing ERα in the MeA (Cushing and Kramer, 2005b). These findings suggest that steroids play a major role in facilitating and perhaps organizing the male's response to pups. The fact that increasing ERα in adults and removal of testes from neonates both inhibit the expression of alloparental behavior suggests that a low threshold level of steroid is necessary for the expression of alloparental behavior, but that higher levels masculinize behavior.

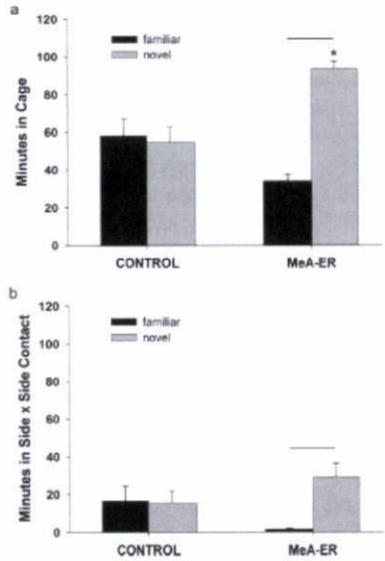

Figure 3. ***a***, Mean time (±SE) that males spent in the cage of the familiar or novel female by treatment. ***b***, Mean time (±SE) in side-by-side contact with the familiar and novel female by treatment. MeA-ERα males spent significantly less time in the familiar female cage and significantly more time in the novel female cage than control males ($p \leq 0.05$). Within-treatment analysis revealed that MeA-ERα males displayed a preference for the novel female spending significantly more time in the novel female cage than the familiar female cage, and more time in side-by-side contact with the novel female. *$p < 0.05$, significant between treatment difference; line above error bars shows significant difference within treatment, $p < 0.05$. Error bars indicate SEM.

Enhancing ERα in the MeA clearly altered alloparental behavior. However, there was variability in the response. Although the sample size was insufficient to determine the reason for the variability, it is possible that differential enhancement of ERα could explain part of the variation. Variation could have been related to unilateral versus bilateral enhancement of ERα. Although it has been hypothesized that the right and left amygdala may differentially regulate behavior (Cooke et al., 2007), empirical studies that have compared unilateral versus bilateral effects in the amygdala suggest that there is no difference as unilateral and bilateral lesions produce the same effect on behavior [MeA, Sheehan et al. (2001); basolateral, Floresco and Ghods-Sharifi (2007)]. Additionally, the degree of enhancement could have affected the response. It is possible that animals with more ERα responded differently than those with less. Finally, it could be a combination of the degree of enhancement and unilateral versus bilateral ERα. Future studies will be designed to determine whether the right and left side of the amygdala play different roles in regulating social behavior.

Although male prairie voles form long-term pair bonds, this process requires either extended cohabitation or central administration of the neuropeptide vasopressin (Insel and Young, 2001). Control males displayed the predicted response, spending an equal amount of time with the familiar and the novel female, whereas MeA-ERα males formed a preference for a novel female, spending significantly more time in contact with the novel female. This result is similar to observations of male mice in that after spending time with a nonreceptive female, males prefer a novel female when given a choice (Moy et al., 2004). The preference for a novel female is particularly relevant when considered in light of the numerous studies of social preference in prairie voles and the function of neuropeptides. In has been argued that neuropeptides are primarily responsible for the formation of pair bonds (Keverne and Curley, 2004; Young and Wang, 2004; Nair and Young, 2006). Vasopressin and oxytocin antagonists inhibited the formation of a partner preference or inhibited social contact, but did not result in the formation of a preference for a novel female (Cho et al., 1999). These results suggest that ERα expression significantly alters the normal steps involved in the formation of social preference. Interestingly, the only other study in which male prairie voles formed a preference for a novel female also involved steroidal manipulation through adrenalectomies followed by treatment with corticosterone (DeVries et al., 1996).

Although several mechanisms have been proposed to regulate the formation of pair bonds, the neuropeptide arginine vasopressin has been proposed to be the primary mechanism involved in regulating pair bond formation and parental behavior in male prairie voles (Young and Wang, 2004). Although we are not suggesting that the current results negate the role of vasopressin, they clearly demonstrate that there are critical aspects of social bonding that have not previously been investigated and that ERα plays a critical role. The results from this study bring into question the assumption that the pattern of the vasopressin V1a receptor (V1aR) is sufficient to explain the formation of pair bonds. Neonatal castration is one of the few reported methods to disrupt alloparental behavior in adult male prairie voles (Lonstein et al., 2002). Adult males that were castrated neonatally display a female pattern of ERα and females display low levels of alloparental behavior (Cushing and Kramer, 2005b). Neonatal castration also inhibited the subsequent ability of centrally administered vasopressin to stimulate partner preferences in adult males, but did not alter the expression of V1aR (Cushing et al., 2003), indicating that the lack of response was not caused by changes in the ability to respond to vasopressin. Chimeric male mice that express prairie vole V1aR display a prairie vole-like pattern of V1aR and an increase in general social affiliation (Young et al., 1999), but do not develop a partner/social preference. One possibility is that social preferences were absent in these transgenic mice because ERα expression was unchanged from the normal pattern, which includes high levels in the MeA. Prairie voles display variation in the length of a microsatellite in the V1aR promoter and length is positively correlated with the expression of prosocial behavior; additionally, this element in the V1aR of the polygynous montane and meadow voles is substantially shorter (Hammock and Young, 2004; Hammock et al., 2005). However, the assertion that the length of this microsatellite is the key element in prosocial behavior has recently been challenged with the finding that many other species in the genus *Microtus* that do not express high levels of social behavior also display significant variation in V1aR microsatellite length (Fink et al., 2006). This finding lead to the suggestion that other factors/genes must also be involved in the ultimate formation of long-term pair bonds. Although montane and meadow voles express the same V1aR microsatellite and the same pattern of V1aR in the brain, meadow voles display prosocial behavior in response to centrally administered vasopressin, whereas montane males do not (Young et al., 1999). They do, however, differ in ERα expression with meadow vole male expressing an intermediate pattern between montane and prairie (Cushing and Wynne-Edwards, 2006). We suggest that the current findings indicate that a reduction in ERα may also be necessary.

The formation of long-term social bonds is a complex process that has been proposed to involve at least two neural circuits, the social behavior neural circuit and the reward system (Young et al., 2005). It has been proposed that within these circuits the ventral pallidum (Lim et al., 2001) and lateral septum (Liu et al., 2001) play an essential role in pair bond formation in males, and the bed nucleus of the stria terminalis in male parental care (De Vries and Villalba, 1997). Here, we show that altering receptor patterns of a nucleus upstream of these regions is sufficient to alter the behavioral response to social stimuli. This does not negate the critical role of the lateral septum or reward system in the expression of social behavior, but clearly demonstrates that changes in the initial response to social stimuli can prevent potentiation of these areas. This also presents the possibility that variable responses can be produced from the same system by altering ERα expression, seasonally or otherwise, to change how the neural circuit regulates social behavior. The findings in this study do not imply that the MeA directly regulates pup-directed aggression or the formation of pair bonds, but that changing the receptor pattern can change the response of the whole circuit and dramatically alter the ultimate response. This conclusion is emphasized by the observation that MeA-ERα males mounted and thrust against the pup. Of the thousands of alloparental tests that have been run with prairie voles, this has never been reported, and supports the concept that altering receptor expression can result in misdirected behavior.

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Estrogen receptors α and β mediate different aspects of the facilitatory effects of female cues on male risk taking

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Summary
Male risk taking and decision making are affected by sex-related cues, with men making poorer and riskier decisions in the presence of females and, or their cues. In non-human species, female cues can also increase male risk taking, reducing their responses to predator threat. As estrogen receptors α and β (ERα and ERβ) are involved in the mediation of social and sexual responses, we investigated their roles in determining the effects of female-associated cues on male risk taking. We examined the effects of brief pre-exposure to the odors of either a novel or familiar estrous female on the avoidance of, and aversive responses to, predator threat (cat odor) in ERα and ERβ wild type (αERWT, βERWT) and gene-deleted (knockout, αERKO, βERKO) male mice. Exposure of αERWT and βERWT males to the odors of a novel, but not a familiar, estrous female mouse resulted in enhanced risk taking with the males displaying reduced avoidance of, and analgesic responses to, cat odor. In contrast, αERKO male mice failed to show any changes in risk taking, while βERKO males, although displaying greater risk taking, did not distinguish between novel and familiar females, displaying similarly reduced avoidance responses to cat odor after exposure to either a novel or familiar female odor. These findings indicate that the gene for ERα is associated with the sexual mechanisms (response to estrous female) and the

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genes for ERβ and ERα with the social (recognition of novel female) mechanisms underlying the effects of female cues on male risk taking.

1. Introduction

Sex-related cues have a significant impact on male behavior. Men are reported to make "poorer" and riskier decisions when female-related cues are present (Roney et al., 2003, 2007; Wilson and Daly, 2004; Ariely and Lowenstein, 2006; Van den Bergh and Dewitte, 2006). In non-humans, predation threat provides an ethologically relevant risk for examining male decision taking (Lima and Dill, 1990; Kavaliers and Choleris, 2001). For example, the presence of a female leads to a greater risk taking and boldness by male guppies towards a predator (Godin and Dugatkin, 1996).

Animals generally respond to the threat of predation risk with a number of defensive behaviors including either immobilization or fleeing, an increase in corticosterone levels, as well as a decrease in nociceptive sensitivity and the induction of analgesia (Blanchard et al., 1990, 1998; Kavaliers and Colwell, 1991; Kavaliers and Choleris, 2001). In rodents, where chemical signals play a key role in social behavior and communication (e.g. Hurst and Benyon, 2004; Hurst et al., 2001; Beynon and Hurst, 2003), male mice that are exposed to female odor show reduced fear responses and greater risk taking. Brief exposure to the urinary odors of a novel, though not a familiar, estrous female, enhances the risk taking and boldness displayed by male mice reducing their avoidance of predator odor as well as the predator-induced rises in corticosterone and analgesic responses (Kavaliers et al., 2001).

This risk-enhancing phenomenon is, thus, composed of two aspects. One is a sexual component involving a response to a sexually receptive female and her cues, while the other consists of a social response involving the distinction between a novel and familiar individual. Only a novel estrous female will induce this enhanced risk taking by a male. These sexual and social components allow males to selectively respond to sexually receptive and potentially accessible novel females, thereby, potentially increasing their reproductive fitness.

The neurobiological mechanisms that underlie social and sexual behaviors and responses (e.g. Choleris et al., 2003, 2004; Insel and Fernald, 2004; Keverne and Curley, 2004) as well as those that affect risk taking and boldness (e.g. Montague and Berns, 2002; Trepel et al., 2005; Ditto et al., 2006) are coming under increased scrutiny. There is mounting evidence that estrogens and estrogen receptors (ERs) have an important role in determining various aspects of social and sexual behavior in males as well as in females. Mice in which the genes encoding either estrogen receptor α (ERα) or estrogen receptor β (ERβ) had been disrupted (ER-knockout mice (ERKO, αERKO, βERKO)) were impaired in their olfactory-mediated social recognition (Imwalle et al., 2002; Choleris et al., 2003, 2006; Kavaliers et al., 2004). In addition, ERα has been associated with the mediation of male aggression and sexual behavior. ERα wild-type (αERWT) male mice displayed normal sexual behavior and mating with estrous females while αERKO males failed to do so. αERKO males were, however, reported to show normal responses to, and interests in, the odors of estrous females (Ogawa et al., 1998, 2000, 2002; see, however, Rissman et al., 1999; Wersinger and Rissman, 2000). In contrast to ERα, the lack of a functional ERβ, while affecting social recognition (Choleris et al., 2003), did not impair normal expression of adult sexual behavior or preferences for females by male mice. Both the wild-type (βERWT) and βERKO male mice expressed an interest in, and responses to, female olfactory cues and exhibited normal sexual behavior, with the βERKO mice also displaying enhanced inter-male aggression (Ogawa et al., 1999, 2000; Nomura et al., 2002). Thus, ERα seems to be involved in the mediation of both social responses and sexual behaviors, while ERβ seems to be involved with social responses but not sexual behaviors. These findings raise the possibility that the genes for ERα and ERβ may also be differentially involved in mediating the sexual and social components of the impact of female cues on male risk-related behaviors. Here we examined the effects of brief exposure to the odors of either a novel or a familiar female on the subsequent avoidance and aversive responses of αERWT, βERWT, αERKO and βERKO male mice to predator odor.

2. Methods

2.1. Animals

Gonadally intact male αERKO and βERKO mice and their wild-type (WT) littermates (αERWT and βERWT); 25–30 g; 7–12 months of age) were used. They were obtained from the breeding colony maintained at The Rockefeller University (New York, NY, USA) by mating heterozygous male and female mice. The genotype of each mouse was determined by PCR amplification of tail DNA. Both colonies of mice were developed in a mixed 129/SvJ and C57BL/6J background and back-crossed into C57BL/6J. The original breeding pairs were obtained from the National Institute of Environmental Health Sciences (Lubhan et al., 1993; Krege et al., 1998). Female Swiss Webster mice ((Charles River NY) 20–30 g, 2–3 months of age) were housed in a separate room. Prior to testing, all of the mice were individually housed for 2–3 weeks in Plexiglas cages under a 12 h:12 h light–dark cycle (lights off at 10:00 h) at 22 ± 1 °C with food and water available ad libitum. All procedures were conducted in accordance with the Institutional Animal Care and Use Committee of The Rockefeller University.

2.2. Experiment 1. Effects of pre-exposure to a female odor on the avoidance responses of males to predator odor

2.2.1. Apparatus

Odor (predator, non-predator) responses of individual male mice (αERWT, βERWT, αERKO, and βERKO) were tested during

the mid-dark period in a translucent Plexiglas *Y*-maze apparatus (5 cm diameter) with 30 cm arms. The stimulus compartments at the end of the two arms of the *Y*-maze in which odor cues were placed, and the start box in which a male mouse was placed, were each 14 cm long. A solid Plexiglas barrier restricted the mouse to the start box, while perforated Plexiglas barriers at the ends of the two stimulus arms prevented contact with the odor sources while allowing detection of the odor. Removable solid Plexiglas barriers, present at 'seams' 8 cm into each of the stimulus arms, prevented exposure of the mice to the odor cues until the designated test times.

2.2.2. Procedures

To minimize novelty responses, male mice ($n = 10$, per group) were placed in the apparatus and allowed to explore the various arms (after being held in the start box for 5 min) for 30 min on 2 consecutive days prior to testing. On the test day, a male mouse was placed in the start box of the apparatus for 15 min after which the solid barrier was removed, allowing the mouse access to the two arms of the *Y*-maze. Two minutes later, the Plexiglas barriers in the arms were removed exposing the mouse to the stimulus odors in each arm. During the subsequent 5 min, the duration of the time spent by a mouse in each arm within 8 cm of an odor source was recorded. 'Preference' as used hereafter is defined as the duration of time the mouse spent in the one stimulus arm of interest divided by the total time spent in the two stimulus arms. Preference here implies actively going to and inspecting/approaching the potentially threatening stimulus (cat odor) and as such providing a measure of risk taking and boldness. Although "increased preference" can be interpreted as a reduced "avoidance response", preference rather than avoidance ratios (corrected for total time actively responding to both odors) are considered as the appropriate measure to be used for statistical analyses (Hardy and Field, 1998; Wagner, 1998; Kavaliers et al., 2003, 2006).

The stimulus odor choice conditions were of a predator (cat) odor vs non-predator (control novel odor) odor. Cat odor was provided by a 2 cm^2 strip of cloth collar worn by a cat for 2 weeks, while novel odor was provided by a 2 cm^2 strip of clean collar treated with dilute (10%) natural almond extract. Results of prior studies showed that the almond odor had no evident aversive effects on male mice (Kavaliers et al., 2004).

Testing of male mice in the *Y*-maze was carried out after a 1 min exposure to the odors of either an estrous or non-estrous female. During the odor exposures, male mice ($n = 10$, in each case) were individually placed in a Plexiglas partitioned area ($12.5 \times 15 \times 10\,cm^3$) that was provided with a vented Plexiglas tube, 10 cm in length, 3 cm in diameter and sealed at each end with fine plastic mesh across which a mouse could neither traverse nor reach. The tube contained the urine and associated odorous secretions of either a familiar or a novel estrous or non-estrous female to which the male could come into close olfactory contact. When a male needed to be familiarized with female odor for experimental purposes, he was exposed for 24 h to the soiled bedding of a female collected over 24 h. This included a 1 h exposure to the actual female across a wire mesh through which direct olfactory contact was possible, further ensuring that a male was exposed to non-volatile odor cues that are considered to be important for individual recognition (Cheetham et al., 2007). Testing of males occurred on the following day after the 24 h exposure.

Freshly deposited urine and associated odors were obtained from single females that were placed for 1 h in a clean cage lined with blank filter paper (Whatman No. 4, England). Examination under an ultraviolet light confirmed that the filter papers were marked by the females, who generally urinated within 5 min of arriving into the cage. The filter paper that lined a single cage was cut into strips and inserted into to the exposure tubes. Additional urine-marked filter paper that was collected over several days and frozen was added to the tube to ensure that estrous female odor was present. Each male was presented with the odor of a single female. Isolated females were primed with substrate from the cages of Swiss Webster males to stimulate estrous cycling (Marsden and Bronson, 1965). Non-primed females were used to obtain non-estrous odors. Wet mount vaginal smears were used to determine the estrous state of the females.

2.2.3. Data analyses

All of the preference ratios were transformed to natural log (ln) values prior to analysis by analysis of variance (ANOVA) with Tukey's post hoc tests with a 0.05 significance level.

2.3. Experiment 2. Effects of pre-exposure to female odor on predator-induced analgesia in males

2.3.1. Procedures

Nociceptive responses of the individual WT and KO mice were carried out for a minimum of 4 days after the choice tests. During the mid-to-late light period in a room separate from their holding rooms, male mice (αERWT, βERWT, αERKO, and βERKO) that had been exposed for 24 h to the odors of a familiar estrous female (as described for Experiment 1) were individually placed in clean cages ($25 \times 15 \times 20\,cm^3$) and exposed for 1 min to the urinary odors of either a familiar (female odor that a male was exposed to for 24 h) or novel estrous or non-estrous female using the vented tube described in Experiment 1. The familiar and novel female odors were different from those used in Experiment 1. After the female odor source was removed the males were exposed to a second vented Plexiglas tube (10 cm long, 3 cm diameter and sealed at each end with plastic mesh) containing the cat odor.

Prior to any odor exposure, immediately after exposure to the odor of a female, and after exposure to the predator odor for 1 min, the nociceptive responses of individual mice were determined using the 'hot-plate' test. Animals were placed individually on a warmed surface (analgesiometer, AccuScan Instruments, Columbus, OH, USA) maintained at $50 \pm 0.5\,^{\circ}C$ and the latency of the first foot lift or lick, whichever came first, was recorded. After this response was displayed, or after 60 s, the mouse was quickly removed from the surface and returned to his cage. Pilot and previous investigations showed that repeated handling procedures, including assessments of nociceptive responses, had no significant effects on nociceptive sensitivity (Kavaliers et al., 2001).

2.3.2. Data analyses

Data were analyzed with a mixed-design repeated measures ANOVA with Tukey's post hoc tests. All analyses were performed using SPPS with 0.05 level of significance.

3. Results

3.1. Experiment 1. Predator odor avoidance

All of the male mice displayed a similar marked overall preference for non-predator odor and avoidance of the predator odor when presented with the predator (cat odor) and non-predator (control odor) stimulus odor combination, with only 15–22% of their time being spent in the arm holding the predator odor (Figure 1A, B). This relative preference for non-predator odor and avoidance of predator odor was affected by pre-exposure to estrous female odor with a significant main effect of female odor condition (F (2,108) 50.25, $p<0.001$), genotype (F (3, 108) = 18.08, $p<0.001$) and a significant interaction of male genotype and female pre-exposure (F (6,108) = 7.157, $p<0.001$).

The responses of the WT males were significantly affected by the female odor condition (αERWT, F (2, 27) = 15.93, $p<0.01$); βERWT, F (2, 27) = 40.14, $p<0.001$). Brief (1 min) pre-exposure to the odors of a novel estrous female significantly decreased the avoidance and increased the inspection of and preference for the predator odor by the αERWT and βERWT male mice (all p's <0.001) who now spent 28–35% of their time active in the predator arm. Exposure to the odors of the familiar female had no significant effects on the avoidance of the predator odor.

The responses of the αERKO males were not affected (F (2, 27) = 1.963, $p = 0.180$) by the female odor exposure condition. Neither the familiar nor novel estrous female odor exposure had any significant effect on their responses to, and avoidance of, the predator odor (Figure 1A). In contrast, the βERKO males displayed a significantly reduced avoidance of and greater interest in the predator odor (F (2, 27) = 71.25, $p<0.001$) after pre-exposure to estrous female odor. However, the βERKO males displayed equivalent, significantly reduced avoidance responses to the cat odor after exposure to either the familiar ($p<0.001$) or novel ($p<0.001$) female odor (Figure 1B). In both cases the responses were not significantly different from those of the βERWT males that received a brief exposure to the odor of a novel female.

Brief (1 min) pre-exposure to the odors of either a familiar or novel non-estrous female or an empty tube had no significant effects on the avoidance of the predator odor displayed by any of the WT or KO male mice (not shown). Their avoidance responses were not significantly different from those displayed by the WT and KO males that received no prior exposure to female odor (Figure 1A, B).

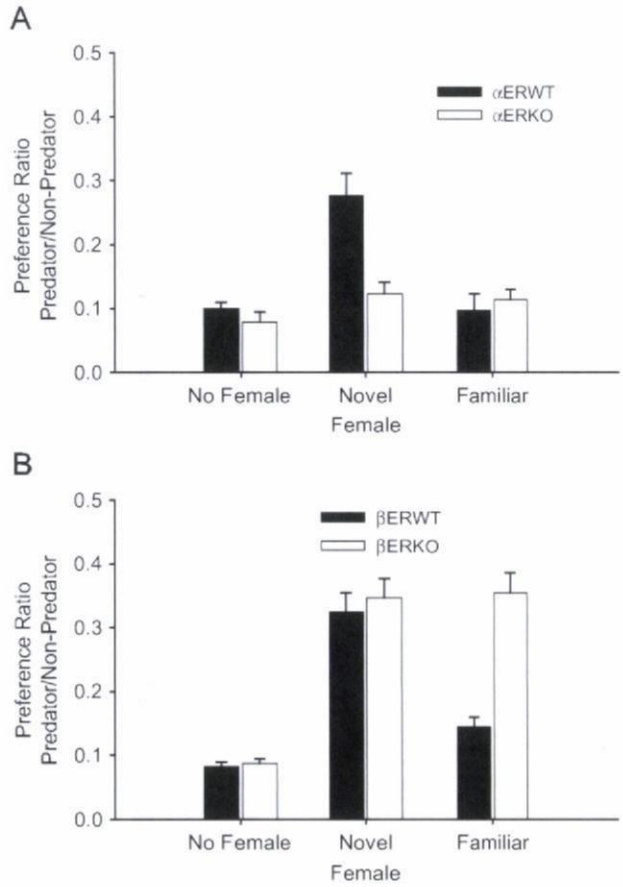

Figure 1 (A–B) Effects of a 1 min pre-exposure to the odors of either a familiar or novel (unfamiliar) estrous female on the subsequent responses of (A) αERWT, αERKO and (B) βERWT, βERKO male mice in a *Y*-maze odor choice apparatus to a predator (cat odor) and non-predator (novel odor, almond), odor combinations. The responses of mice receiving no prior odor exposures (no female) are also shown. Responses are given as preference ratios (e.g. time spent in the vicinity of the predator odor/time spent in the vicinity of the predator odor+time spent in the vicinity of the non-predator odor). Increased preference indicates an augmented interest in, and approach to, the predator odor and is indicative of a reduced avoidance of the predator odor. Preferences were determined over a 5 min period. $N = 10$ in all cases. Vertical lines denote a standard error of the mean.

3.2. Experiment 2. Predator and female-odor-induced analgesia

3.2.1. Effects of exposure to cat odor

All of the WT and KO mice that were exposed to the odor of a predator (cat) for 1 min in the absence of female odor showed increased thermal response latencies (F (1,27) > 4.04; $p<0.03$), indicative of the induction of analgesia (Figure 2A–D). There were no significant differences in the levels of cat-odor-induced analgesia displayed by the four genotypes. Exposure to the novel almond odor (not shown) had no significant effects on nociceptive sensitivity. There were also no significant differences in basal nociceptive sensitivity between the WTs and KOs, consistent with the results of prior investigations (Spooner et al., 2007).

3.2.2. Effects of exposure to female odor

Both the WT and KO males that were exposed to estrous female odors displayed analgesic responses with the magnitude of these responses being dependent on prior

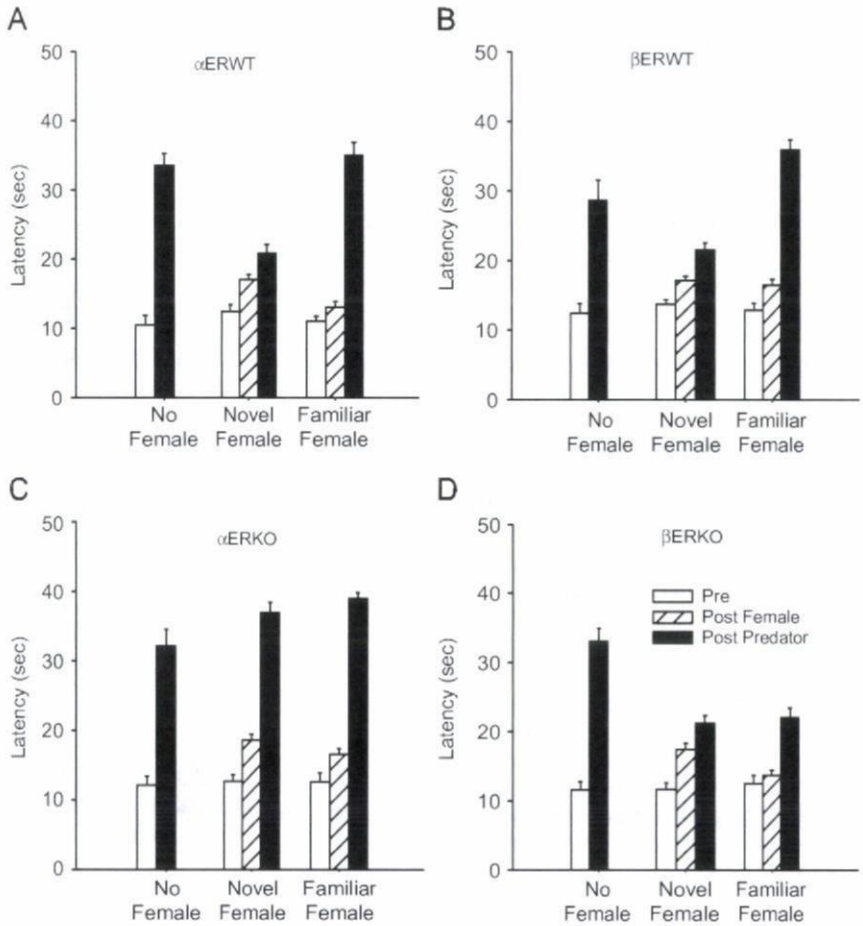

Figure 2 (A–D) Nociceptive responses of: (A) αERWT; (B) βERWT; (C) αERKO; and (D) βERKO male mice that were exposed for either 1 min to the odors of either a familiar or unfamiliar (novel) estrous female and then exposed for 1 min to predator (cat) odor. Responses of mice (control) receiving no female odor exposure are also shown. Nociceptive sensitivity, as measured by the latency of response to a 50 °C thermal surface was determined before any odor exposures (baseline), after exposure a female (post-female) and after exposure to the predator odor (post-predator). $N = 10$, in all cases. Vertical lines denote a standard error of the mean.

familiarity with the female odor (all Fs $(1,18) > 8.77$, p's < 0.03). The odors of novel females elicited significant analgesic responses in all of the genotypes ($p < 0.03$) with the levels of analgesia being markedly lower (all p's < 0.001) than those elicited by predator odor (Figure 2A–D). Exposure to the odors of a familiar female elicited significant analgesic responses in only the αERKO males ($p < 0.01$). Exposure to the odors of non-estrous females had no significant effects on the thermal response latencies of any of the WT or KO males (data not shown). Their response latencies were equivalent to the pre-exposure response latencies shown in Figure 2.

3.2.3. Effects of pre-exposure to female odor on the responses to predator odor

The analgesic responses of the WTs to the predator odor were significantly affected by female odor condition (αERWT, F (2, 36) = 48.542, $p < 0.001$; βERWT, F (2, 36 = 44.85, $p < 0.001$). Pre-exposure of the WT males to the odors of a familiar estrous female had no significant effect on the level of cat-odor-induced analgesia. However, βERWT and αERWT males that were pre-exposed for 1 min to the odor of a novel estrous female and then exposed for 1 min to the odor of a predator displayed significantly (all p's < 0.001) reduced analgesic responses. Their response latencies were significantly (all p's < 0.02) lower than those of the males with either no female odor pre-exposure, or that had been pre-exposed to the odors of a familiar female (αERWT, βERWT, $p < 0.001$) before being presented the predator odor (Figure 2A–C).

The analgesic responses of the αERKO males were not significantly affected by estrous female odor pre-exposure (F (2, 36) = 30.1, $p = 0.09$). Pre-exposure to the odors of either a novel, or a familiar estrous female, had no significant effects on the level of cat-odor-induced analgesia displayed by the αERKO males. Their predator-odor-induced increased response latencies were not significantly different from those seen in the absence of a female. In contrast, the level of cat-odor-induced analgesia displayed by βERKO males was significantly reduced by female odor pre-exposure (F (2, 36) = 40.53, $p = 0.03$). However, the

attenuated responses of the βERKO males were not affected by prior familiarity with the female. The levels of analgesia displayed by βERKO males pre-exposed to either familiar or novel female odors were equivalent and not significantly different from those displayed by βERWT males that were pre-exposed to the odors of a novel female. Pre-exposure to the odors of an unfamiliar non-estrous female had no significant effects on the levels of cat-odor-induced analgesia displayed by any of the males, with the levels of analgesia elicited being equivalent to that seen in the various KOs and WTs following just predator odor exposure (no female, Figure 2).

4. Discussion

Here we show that the genes for ERα and ERβ are differentially involved in mediating the facilitatory effects of female cues on risk taking by males. Male mice are "emboldened" in their responses to a predator after brief exposure to the odors of a novel female with the gene for ERα associated with the sexual mechanisms (response to estrous female) and the genes for ERβ, and likely ERα, with the social (recognition of a novel female) mechanisms of this effect. As "sex-related cues" have a parallel impact on decision making and risk taking in human males (Blanton and Gerrard, 1997; Wilson and Daly, 2004; Ariely and Lowenstein, 2006; Van den Bergh and Dewitte, 2006) our findings suggest that the genes for ERα and ERβ may also have a modulating role on sexually motivated risk taking and decision making in men.

Both WT and KO male mice displayed marked fear, anxiety and stress responses to cat odor. This was assessed in two ways. (i) In an odor choice test where test male mice displayed an intense aversion to, and avoidance of, cat odor (Figure 1). This predator-odor avoidance involves a heightened anxiety that is sensitive to anxiolytic agents (e.g. Blanchard et al., 1993; Dielenberg et al., 1999). (ii) By the assessment of nociceptive sensitivity. Brief exposure of the WT and KO mice to cat-odor-elicited decreases in nociceptive sensitivity (Figure 2), indicative of the induction of analgesia. This analgesia is not simply a reflection of increased fearfulness and "freezing". Rather, it is associated with stress-induced activation of opioid and non-opioid neurochemical mechanism (Kavaliers and Colwell, 1991; Kavaliers and Choleris, 2001). This stress-induced analgesia reflects a motivational shift that facilitates the expression of various active and passive defensive behavioral responses (Fields, 2004), thereby reducing the risk of predation.

Our results show that it is not just the presence of the odors of a female per se that elicits reduced aversive responses to predator odor and a greater risk taking by the WT males. The female has to be both novel and in estrous. Exposure to the odors of a novel non-estrous female had no significant effects on male responses. This supports the presence of both sexual (sexually receptive estrous female) and social (novel estrous female) components in the adaptive expression of this risk facilitatory, emboldening, response. Brief exposure to a novel estrous female may signal the likelihood of an immediate, though temporally limited, availability of a sexually responsive female. The presence of this sexual incentive could facilitate a rapid motivational shift in the males from defensive responses to a search for a sexually receptive female. Human decisions that are made under risk have also been found to be malleable (Fong and McCabe, 1999). In men, sexual cues can affect decision making, facilitating the expression of sexually motivated behaviors (Roney, 2003; Wilson and Daly, 2004; Ariely and Lowenstein, 2006).

Brief exposure to the odors of estrous females also reduced the predator-induced avoidance and analgesic responses of the βERKO males who, however, seemed to be unable to distinguish between the odors of familiar and novel estrous females. Although displaying riskier behaviors, their responses were no longer linked to novel sexually receptive females and increased reproductive chances. In contrast, the αERKO males failed to show any reduction in their aversive responses to predator odor after exposure to either novel or familiar female odors. This indicates that the sexual components of emboldening involve only the gene for ERα while the social (recognition) components involve the gene for ERβ and possibly ERα.

The present findings are in agreement with other studies with ERKO mice showing a differential involvement of the two ERs in sexual and social behavior. ERα and ERβ often have antagonistic actions and transcription effects leading to potentially different behavioral effects (Vasudevan et al., 2001; Lindberg et al., 2003). Loss of ERα is reported to result in a decrease of both male and female sexual behaviors with no evident effects of ERβ deletion (Ogawa et al., 1997, 1999, 2000; Wersinger and Rissman, 2000; Kudwa et al., 2006; Nomura et al., 2006). Although, in both cases the KOs could still distinguish between males and females of various sexual conditions, there are some suggestions that αERKOs may show a decrease in sexual incentive motivation (Wersinger and Rissman, 2000). Regarding the social recognition, female αERKO and βERKO mice have also been shown to differ in the extent of their attenuation of social memory (Choleris et al., 2006). In addition, αERKO males displayed reduced aggressive behavior towards other males while βERKO males displayed enhanced inter-male aggressiveness relative to their WTs (Ogawa et al., 1998, 2000, 2002; Imwalle et al., 2002; Nomura et al., 2002; Rissman et al., 1999; Scordalakes and Rissman, 2003; Dominguez-Salazar et al., 2004). In this regard, it has been suggested that ERβ activation may exert an attenuating effect on male aggression induced by estrogen through ERα-mediated mechanisms (Nomura et al., 2006).

These findings support sexual motivation as a possible underlying factor driving the male behavior in our choice test. The enhanced risk taking elicited by brief exposure to female odors may facilitate mate search and aggressive interactions with other male competitors. The greater risk taking or male boldness may be a "side-effect" of the lower fear and stress responses and greater sexual motivation and "searching" for the briefly available novel female. Augmented sexual motivation is similarly speculated to contribute to the greater risk taking seen in men exposed to sex-related cues of women (Ariely and Lowenstein, 2006; Van den Bergh and Dewitte, 2006).

The responses of the male mice may be elicited by either relatively short-lived highly volatile and/or non-volatile female odor cues that are detected upon close inspection of

the urinary cue (Hurst et al., 2001; Hurst and Beynon, 2004; Cheetham et al., 2007). Volatile and non-volatile odors associated with the major histocompatibility complex and the major urinary proteins provide information about the condition and individual identity of the scent owner (Hurst et al., 2001; Beynon and Hurst, 2003; Hurst and Beynon, 2004). Together these signals provide information about the condition and identity of the female.

Brief pre-exposure to these odor components of a novel female significantly reduced the predator-odor-induced fear and stress responses in the WT males (Figure 1). This reduction is unlikely to be due to changes in male testosterone levels in that in previous studies it was shown that exposure to the odors of a novel female blunted predator-odor-induced rises in corticosterone levels, but was not associated with an immediate increase in testosterone levels (Kavaliers et al., 2001). The impairments of the KO mice also cannot be attributed to differences in basal testosterone levels in that the various WTs and KOs examined here are reported to display similar basal testosterone levels (Ogawa et al., 1998, 2002; Nomura et al., 2002, 2006). Furthermore, increases in testosterone levels in male mice generally occur 15–30 min after exposure to females or their odors (Coquelin and Desjardins, 1982; Smith et al., 1996; Kavaliers et al., 2001) suggesting that the emboldening responses seen 1 min after exposure to female odor are not directly associated with changes in testosterone. The testosterone responses of men to brief interaction with women or their cues are suggested to follow similar temporal patterns (Roney et al., 2003, 2007). Thus, in men, like in male mice, short-term increases in risk taking induced by female cue may occur independent of any rise in testosterone.

While unlikely to be due to changes in testosterone levels, the "emboldening" effects of female odor may involve alterations in the metabolism of testosterone. Increased aromatase activity with subsequent alterations in testosterone metabolism, shifts in central estrogen levels, and possibly ER function, have been proposed to be associated with augmented male sexual interest (Bakker et al., 2002; Balthazar et al., 2005; Taziaux et al., 2007). This is further supported by the findings that non-copulating rats, while having similar testosterone levels as copulating rats, display reduced neuronal aromatase activity and levels of ERα (Portillo et al., 2006, 2007).

These findings are consistent with a proposed "micronet" involving genes for ERα, ERβ, oxytocin (OT), and the OT receptor (OTR) as the regulatory basis for olfactory-mediated social recognition (Choleris et al., 2003, 2004). Oxytocin has been shown to augment "trust" and the use of information provided by others in mice and humans (Kosfeld et al., 2005; Kavaliers et al., 2006). Both of these actions could influence sexually motivated decision making and risk taking. Olfactory signals from the main and accessory olfactory pathways converge at the medial amygdala where the identity of the odor source is most likely determined. In the medial amygdala ERα is needed for the induction of OT receptors and, thus, for the normal action of OT at this level. OT production in the hypothalamus is also under estrogen control, through ERβ. Disruptions at the level of either OT, ER-α, or ER-β genes and their products could lead to impaired processing and/or integration of odor information at the level of the medial amygdale. This could result in impaired discrimination between familiar and unfamiliar female odor, thus, modifying sexual motivation. This is supported by the findings that exposure to the signals of an estrous female results in the activation of brain OT at the level of the paraventricular nucleus of the hypothalamus (Waldherr and Neumann, 2007). Moreover, this OT activation was associated with an anxiolytic response and reduced emotional response to anxiogenic stimuli that is consistent with an enhanced risk taking and boldness.

The use of selective KOs has allowed us to distinguish the sexual and social components of male risk taking from a neurobiological perspective. We have shown that the effects of cues from sexually receptive females on male boldness and risk taking, and likely decision making, involves at the sexual level the gene for ERα and at the social level the gene for ERβ and likely ERα. This raises the possibility that ERα and ERβ may similarly be part of the mechanism(s) whereby sex-related cues impact decision making and risk taking by human males.

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Conflict of interest statement

Jan-Ake Gustafsson is a shareholder, research grant receiver and consultant of KaroBio AB.

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