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

Kazuya Tomihara^{1,2}, Tomoko Soga², Masayoshi Nomura², Kenneth S. Korach³, Jan-Åke Gustafsson⁴, Donald W. Pfaff², and Sonoko Ogawa²

¹ Department of Psychology, Faculty of Law, Economics, and Humanities, Kagoshima University, Kagoshima, Kagoshima 890-0065, Japan

² Laboratory of Neurobiology and Behavior, The Rockefeller University, New York, NY 10021, USA

³ Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA

⁴ Department of Biosciences and Nutrition, Karolinska Institute, Huddinge, S-141 86, Sweden

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 (β ERKO) female mice and their wild type (β WT) littermates were implanted several different doses (experiment 1: 2.0 μ g/day, experiment 2: 1.0, 0.4, 0.2 or 0.1 μ g/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 β WT and β ERKO 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 β WT mice than β ERKO 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.

Keywords

fear; emotion; social anxiety; behavioral tests; knockout mouse

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].

Correspondence Kazuya Tomihara, Department of Psychology, Faculty of Law, Economics, and Humanities, Kagoshima University, 1-21-30 Korimoto, Kagoshima 890-0065, Japan, Phone/Fax: 81-99-285-7536, E-mail: E-mail: tomihara@leh.kagoshima-u.ac.jp.

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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 hours 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 48h 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 human. Postmenopausal women chronically treated with 17 β -estradiol on 3 month exhibited a significant increase in negative mood and anxiety [10], and 10 years treatment with oestradiol implants in healthy postmenopausal women impaired their cognitive 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 chronically 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 (β ERKO) 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 β ERKO 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.

METHODS

Mice

β ERKO female mice and their wild type (β WT) littermates were used. They were obtained from the β ERKO 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 ten days before the first behavioral tests, subjects were housed with same-sex littermates in plastic cages (30 $\times$ 20 $\times$ 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 (β WT: n=11, β ERKO: n=12) and (2) EB 2.0 μ g/day pellet (β WT: n=12, β ERKO: n=13). In experiment 2, mice were treated with five different doses of EB, (1) EB 0 μ g/day (placebo, β WT: n=10, β ERKO: n=11), (2) EB 0.1 μ g/day (β WT: n=10, β ERKO: n=9), (3) EB 0.2 μ g/day (β WT: n=7, β ERKO: n=7), (4) EB 0.4 μ g/day (β WT: n=10, β ERKO: n=9), or (5) EB 1.0 μ g/day (β WT: n=9, β ERKO: n=11). After ovariectomy and pellet implants, all mice were individually housed in plastic cages (30 $\times$ 20 $\times$ 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*.

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 hours after lights off. (1) Light-Dark Transition (LDT) Test

The test apparatus consisted of a clear plastic box (50 $\times$ 50 $\times$ 35 cm) with a black (light opaque) covered-plastic box (50 $\times$ 25 $\times$ 25 cm) in one side (the dark side). The black box had an open doorway (2 $\times$ 5 cm) that led to the light side of the apparatus, which was illuminated by a 40-

W white bulb (about 420 lux on the floor). Mice were moved from the living room to the testing room at least 1 hour 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 10min: 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.

(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 40cm 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 5min tests.

(3) Social Investigation Test (SIT)

One day before the test, mice were transferred to larger housing cages ($45 \times 23 \times 15.5$ cm). They were tested against gonadally intact male mice of C57BL/6J strain for 10min. Male stimulus mice were placed in a clear perforated (28 small holes with a 5mm diameter on the bottom 3 cm) plexiglass cylinder (7cm diameter $\times$ 12cm height) at least 10min 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.

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.

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 β ERKO-placebo, β WT-placebo and β WT-0.1 μ g/day) from the analysis due to an equipment failure.

RESULTS

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 2.0µg/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 [$F(1, 44) = 2.27, P = 0.14$].

Experiment 2

LDT—In contrast to anxiogenic effects of EB at a higher dose (2.0µg/day) in Experiment 1, EB treatment at lower-doses reduced the levels of anxiety. Overall, the mice implanted with a 0.2µg/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 0.2µg/day EB pellet compared to those in the other treatment groups, although the interaction between genotype and EB treatment was not statistically significant [$F(4, 83) = 0.90, P = 0.47$].

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).

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 0.2µg/day EB pellet [$P < 0.05$ vs placebo group] and lowest in those treated with a 1.0µg/day EB pellet [$P < 0.05$ vs placebo or 0.2µg/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.

Plasma Levels of Estradiol

The plasma levels of estradiol of the animals treated with 1.0µg/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 0.4µg/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 $\mu\text{g/day}$ EB), the plasma concentrations were not at a detectable level. There were no genotype differences at any dose.

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 the Experiment 1, we tested animals in the LDT apparatus and found that EB treatment at the dose of 2.0 $\mu\text{g/day}$ decreased the time spent in the light side and the number of transitions between dark and light side. 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 the Experiment 1 (data not shown). Although we did not conduct EPM nor SIT in the 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 $\mu\text{g/capsule}$) 7 days before behavioral tests [8,9]. Since both βWT and βERKO mice responded similarly to EB treatment in the 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 same distribution of ER- α as βWT [22].

In contrast to the results in the Experiment 1, the mice implanted with a 0.2 $\mu\text{g/day}$ 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 $\mu\text{g/day}$ 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 $\mu\text{g/day}$ 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 seem 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 were enhanced compare 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, were decreased [9]. These results suggest that the moving distance in an anxious situation is not reflects 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 can not 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 the Experiment 2, we found the effects of gene disruption of ER- β on anxiolytic action of estrogen, as the β WT mice treated with a 0.2 μ g/day EB pellet showed increased moving distance, but not β ERKO 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. β ERKO mice showed higher anxiety-related behavior than β WT 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 β ERKO and β WT mice in the emergence latency to the light side of LDT, and the result also suggested overall higher anxiety of β ERKO than β WT 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 progesterin 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 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 μ g/day, plasma levels of estradiol were not at a detectable level in the present study. Estradiol levels in mice treated with 0.4 μ g/day of EB pellet were 31 (+24) pg/ml for β ERKO and 11 (+35) pg/ml for β WT 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 were approximately 30pg/ml [37, 38]. The estradiol level of pregnant female is lower than that of mice treated with 1.0 μ g/day of EB pellet in our study, rather similar to that of mice treated with 0.4 μ g/day of EB pellet. On the other hand, those in the mice treated with 1.0 μ g/day EB were much higher than physiological levels, i.e., 76 (+99) pg/ml and 114 (+124) pg/ml in β ERKO and β WT mice, respectively. Although we did not measure the plasma levels of estradiol in the mice which were treated with 2.0 μ g/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 the 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 has multidimensional components and situation-dependant aspects. Especially, since ER- β is considered to play 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 compare 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 the Experiment 2, as indicated that the animals treated with 1.0 μ g/day EB pellet showed the highest anxiety level among the treatment groups in the 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 β ERKO 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 β WT. The exact role of ER- α in the regulation of anxiety levels needs to be determined in further studies.

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Figure 1.

The results (Mean+SEM) of the Light/Dark Transition (LDT) test in the Experiment 1. Regardless of genotype, mice treated with EB at the dose of 2.0 μ g/day (β WT: n=12, β ERKO: n=13) showed significantly higher levels of anxiety compared to the placebo treated mice (β WT: n=11, β ERKO: 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 dark and light side (b). The latency to the first emergence from the dark to the light compartment also tended to increase in the EB treated groups. (* $P < 0.05$)

Figure 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 the 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 groups were 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), EB 1.0 μg/day (βWT: n=9, βERKO: n=11)

Figure 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 μ g/day group of the same genotype). placebo (β WT: $n=9$, β ERKO: $n=10$), EB 0.1 μ g/day (β WT: $n=9$, β ERKO: $n=9$), EB 0.2 μ g/day (β WT: $n=7$, β ERKO: $n=7$), EB 0.4 μ g/day (β WT: $n=10$, β ERKO: $n=9$), EB 1.0 μ g/day (β WT: $n=9$, β ERKO: $n=11$)