Sex-Specific Involvement of Estrogen Receptors in Behavioral Responses to Stress and Psychomotor Activation

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Fluctuating hormone levels, such as estradiol might underlie the difference in the prevalence of psychiatric disorders observed in women vs. men. Estradiol exerts its effects primarily through binding on the two classical estrogen receptor subtypes, alpha (ERα) and beta (ERβ). Both receptors have been suggested to have a role in the development of psychiatric disorders, however, most of the current literature is limited to their role in females. We investigated the role of estrogen receptors on cognition (novel-object recognition), anxiety (open-field test, elevated-plus maze, and light/dark box), stress-responsive behaviors (forced-swim test, learned helplessness following inescapable shock, and sucrose preference), pre-pulse inhibition (PPI) and amphetamine-induced hyperlocomotion in both male and female mice either lacking the ERα or ERβ receptor. We found that female Esr1−/− mice have attenuated pre-pulse inhibition, whereas female Esr2−/− mice manifested enhanced pre-pulse inhibition. No pre-pulse inhibition difference was observed in male Esr1−/− and Esr2−/− mice. Moreover, amphetamine-induced hyperlocomotion was decreased in male Esr1−/−, but not Esr2−/− mice, while female Esr1−/− and Esr2−/− mice showed an enhanced response. Genetic absence of ERα did not alter the escape capability or sucrose preference following inescapable shock in both male and female mice. In contrast, female, but not male Esr2−/− mice, manifested decreased escape failures compared with controls. Lack of Esr2 gene in male mice was associated with decreased sucrose preference following inescapable shock, suggesting susceptibility for development of anhedonia following stress. No sucrose preference differences were found in female Esr2−/− mice following inescapable shock stress. Lastly, we demonstrated that lack of Esr1 or Esr2 genes had no effect on memory and anxiety-like behaviors in both male and female mice. Our findings indicate a differential sex-specific involvement of estrogen receptors in the development of stress-mediated maladaptive behaviors as well as psychomotor activation responses suggesting that these receptors might act as potential treatment targets in a sex-specific manner.

Keywords: estrogen receptor alpha, estrogen receptor beta, depressive-like behaviors, mood disorders, sex differences, stress, amphetamine, psychomotor activation
INTRODUCTION

Mental disorders are extremely common, affecting approximately 18.3% of the U.S. adult population (see 2016 National Survey on Drug Use and Health). The prevalence of many mental disorders, including anxiety and depressive disorders, are higher among women than men (see 2016 National Survey on Drug Use and Health). These gender differences have been attributed, at least partly, to fluctuations of the ovarian hormone estradiol [see (1)]. Specifically, an increase in estradiol occurring during female puberty has been associated with increased prevalence of mood disorders [see (2, 3)]. Additionally, several studies showed that the incidence of depression (4–6) and anxiety (7) increases in women during the menopausal transition, a period that is characterized by robust fluctuations in estrogen levels, before overall levels drop to approximately 10% of estrogen levels during the pre-menopausal period. Although estradiol treatment was shown to alleviate depressive symptoms in women (8, 9), the mechanistic relationship between estrogen and depression remains unclear.

Estradiol exerts its effects through binding to two classical estrogen receptor subtypes, the estrogen receptor alpha (ERα) and beta (ERβ) and the non-classical G-protein coupled estrogen receptor, GPR30 (10). These receptors have been suggested to play a role in the pathophysiology of mood disorders. Specifically, Esr1 gene variants, which code for ERα, have been associated with increased risk of developing depression in women (11–15). In rats, estradiol, via acting at the ERα, normalized postpartum-induced anxiety- and depressive-like behavior, measured in the elevated-plus maze (EPM) and the forced-swim tests (FST), respectively (16). Moreover, knockdown of the ERα selectively in the posterior-dorsal amygdala of female mice decreased anxiety-like behavior as demonstrated by the increase time spend in the light compartment of the light/dark box (L/D box) (17), suggesting a possible role of ERα in regulating anxiety behaviors. Although, the role of ERα has been extensively investigated in women and female animals, its role in male depression and anxiety has received limited attention. However, a genetic association study identified a possible link between Esr1 polymorphisms and depression in men (18).

Polymorphisms in Esr2, which codes for ERβ, have been associated with moderate depressive symptoms in women (18), whereas there are no studies investigating the role of Esr2 polymorphisms in male depression. In rodent studies, selective ERβ ligands (19), as well as estradiol (20) decrease immobility time in the FST in wild-type, but not Esr2 knockout female mice. In addition, ERβ, but not ERα agonists decreased immobility time in the FST in ovariectomized rats (21), suggesting that activation of ERβ induces antidepressant effects in female rodents. There is a report suggesting antidepressant efficacy of an ERγ agonist in the tail-suspension test in male mice (22). Administration of the ERβ agonist diarylpropionitrile decreased anxiety-like behaviors in female wild-type mice but not in mice lacking the ERβ receptor gene (23).

Overall, most of the existing studies that have been published and investigated the role of the estrogen receptors in anxiety and depressive behaviors mainly concentrate on a single sex, as estradiol is considered a “female” hormone. Furthermore, limited studies assessed for the involvement of either ERβ or ERα in behavioral responses to stress. Therefore, in the present study, we sought to understand the role of estrogen receptors in anxiety, as well as in depressive-related behavioral responses following stress in both female and male mice.

METHODS

Mice

Esr1 and Esr2 breeding pairs were obtained from Jackson laboratories. Wild-type, heterozygous and homozygous Esr1 mice were bred in-house by breeding heterozygous males and females. Heterozygous and homozygous Esr2 mice were bred in-house by breeding heterozygous females and homozygous males. Both Esr1 and Esr2 mice were bred on a C57BL/6J background. At the time of behavioral testing, the age of the animals was 8–12 weeks. Mice were grouped-housed and maintained under a 12 h light–dark cycle (lights on at 7:00 a.m.). Water and food was available ad libitum. All mice were housed in the same room in individually ventilated cages. All experimental procedures were approved by the University of Maryland Animal Care and Use Committee and were conducted in full accordance with the NIH Guide for the Care and Use of Laboratory Animals. Tail samples were obtained prior to weaning and genotyped by TransnetYX, Inc. (Cordova, TN, USA). The primer sequences are as follows:

Esr1 genotype: Wild-type-Forward: TCGGGCATCGCTTACG; Reverse: GGCGACACGCCTGTGAG. Esr1-Forward: CATTTC TCAGATTTGTTGGCAAGTCTC; Reverse: GGCGACACGC TGTTGAG

Esr2 genotype: Wild-type-Forward: CCAAGAGGATGCTGACTCT. ACTTCT: Reverse: CAGACACGTAATGACCCAGATG. Esr2-Forward: GCCAAAGGATGCTCCTTC; Reverse: TC CATCACAGGACTGACTTAGACT.

Behavioral Characterization

All behavioral experiments were performed during the light phase of the light/dark cycle between 10:00 a.m. and 3:00 p.m. The order of testing within animals (Figure 1A) was determined by the degree of stressfulness of each test with the least stressful conducted first and the most potentially distressing test last (24–26). The numbers of animals used for the open-field test, light/dark box, elevated-plus maze, novel-object recognition, forced-swim test, learned helplessness, shock sensitivity, and sucrose preference are as follows—Esr1+/+, Esr1+/-, Esr1−/−. Females: n = 12, 20, 11; Males: n = 8, 12, 9 and Esr2+/+, Esr2−/− Females: n = 9, 8; Males: n = 13, 11. The numbers of animals used for the pre-pulse inhibition and amphetamine-induced hyperlocomotion are as follows—Esr1+/+, Esr1+/-, Esr1−/−. Females: n = 11, 11, 10; Males: n = 10, 10, 8 and Esr2+/+, Esr2−/−. Females: n = 11, 9; Males: n = 7, 8.

Open-Field Test (OFT)

The OFT was performed under 300 Lux white lighting. Mice were individually placed into open-field arenas (100 × 100 × 38 cm; San Diego Instruments, San Diego, CA) for a 10-min period. The sessions were recorded using an overhead, digital video-camera.
Distanced traveled and time spent in the center of the arena was analyzed using TopScan v2.0 (CleverSys, Inc., Reston VA).

**Light/Dark Box (L/D box)**
The L/D box was used as previously described (26), with minor modifications. Briefly, mice were placed in the illuminated compartment of the L/D box (35 × 35 cm), facing the wall opposite to the dark compartment, and allowed to explore the whole apparatus for 5 min. The sessions were recorded using a video-camera and the time spent in the illuminated and dark compartment was scored using TopScan v2.0 (CleverSys, Inc., Reston VA).

**Elevated-Plus Maze (EPM)**
The EPM was carried in dim white lighting conditions (∼5 lux). The apparatus consisted of 2 closed arms and 2 open arms (39 × 5 cm each) and was elevated 50 cm above the floor (Stoelting, Woodale, IL). The experiment was carried out as previously described (27). The time spent in the open and closed arms of the EPM during the 5-min test was recorded by an overhead digital video-camera and scored using TopScan v2.0 automated scoring software (CleverSys, Inc., Reston VA). Amount of time spent in the open arms was used as the primary outcome for the anxiety behavioral assessment.

**Novel-Object Recognition (NOR)**
Short-term recognition memory was assessed using the novel object recognition task protocol, as previously described (28, 29). The NOR was carried in dim white lighting conditions (∼10–15 lux). The apparatus and objects used here has been previously described by Zanos et al. (28). The test was conducted over two days. On the first day, the habituation phase, the animals were allowed to explore an empty novel object recognition apparatus (40 × 9 × 23 cm) for 30 min and then returned to their home cages. On the second day, the mice were re-introduced into the same apparatus, but this time containing two identical objects fixed on the floor, which they were allowed to explore for 30 min. After this familiarization phase, mice were immediately returned to their home-cages for 30 min. The mice were then placed back into the novel object recognition apparatus, in which one of the “familiar” objects was replaced by a “novel” object (retention phase) for 4 min. All three phases of the novel object recognition test were recorded via an overhead video-camera and analyzed using TopScan v2.0 automated scoring software (CleverSys, Inc., Reston VA). The time spent interacting with the familiar and novel objects during the retention phase was measured. A discrimination ratio was calculated by dividing the time of interaction with the novel object by the total time of interaction with both objects during the retention phase.

**Forced-Swim Test (FST)**
The FST was performed in normal white light conditions (∼300 lux) and was performed as previously described (30). Briefly, mice were subjected to a 6-min swim session in clear Plexiglas cylinders (30-cm height × 20-cm diameter) filled with 15 cm of water (23 ± 1°C). Sessions were recorded using a digital video camera. Immobility time, defined as passive floating with no additional activity other than that necessary to keep the animal's head above water, was scored for the last 4 min of the 6-min test by a trained experimenter blind to the genotypes.

**Learned Helplessness**
The learned helplessness paradigm was performed in accordance with Dao et al. (31) and was separated in two phases: training and test. On Day 1 mice received inescapable shock training (0.3 mA, 2 s shock duration 120 trials, inter-trial interval 15 s) in one compartment of the two-compartment Coulbourn Mouse Shuttle Cage (Coulbourn Instruments, Whitehall, PA). On day 2, learned helplessness test consisted of 45 escapable shock trials (0.3 mA, duration of open door: 15 s). The average inter-trial interval was 20 s. In trials 1–5, the gate opened concomitantly with the shock initiation and stayed open for the duration of the shock. In trials 6–45, the gate with a 3 s delay after initiation of the shock. In all trials, shock was terminated if mice passed through the gate to the other compartment. Number of escape failures and escape latency was automatically measured by GraphicState 3.01 (Coulbourn Instruments, Whitehall, PA).

**Shock Sensitivity Test**
Mice were tested for their sensitivity to shock as previously described with minor modifications (32). Briefly, mice were subjected to increment shock intensities (0.02–0.50 mA; 0.02 mA increments from 0.02 to 0.2 mA and 0.5 mA increments
from 0.2 to 0.5 mA) and testing for their flinch response. The increments occurred every 30 s and the shock delivery were automatically controlled by GraphicState 3.01 (Coulbourn Instruments, Whitehall, PA). Scoring was performed live by an experienced experimenter blind to the genotypes.

Sucrose Preference Test
For assessing the baseline sucrose preference, mice were singly housed for 72 h and presented with two identical bottles containing either tap water or 1% sucrose solution. After baseline sucrose measurement, mice were re-group housed. Following the learned helplessness testing, similar to the baseline measurements, mice were singly housed for 72 h and presented with two identical bottles containing either tap water or 1% sucrose solution. The location of the sucrose and tap water bottle was changed every day to avoid the development of side preference.

Pre-pulse Inhibition (PPI)
The pre-pulse inhibition (PPI) paradigm was performed as previously described (33), with minor modifications. Mice were individually tested in acoustic startle boxes (SR-LAB; San Diego Instruments, San Diego, CA). The animals were placed in the startle chamber for a 30-min habituation period. The experiment started with a further 5-min adaptation period during which the mice were exposed to a constant background noise (67 db), followed by five initial startle stimuli (120 db, 40-ms duration each). Subsequently, animals were exposed to five different trial types: pulse alone trials (120 db, 40-ms duration), three prepulse trials of 76, 81, and 86 db of white noise bursts (20-ms duration) preceding a 120-db pulse by 100 ms, and background (67 db) no-stimuli trials. Each of these trials was randomly presented five times. The percentage PPI was calculated using the following formula: [(magnitude on pulse alone trial—magnitude on prepulse + pulse trial)/magnitude on pulse alone trial] × 100.

Amphetamine-Induced Hyperlocomotion
The amphetamine-induced hyperlocomotion experiment was performed under white lighting conditions of ∼80 lux. Mice were placed into the open-field arenas (50 × 50 × 38 cm; San Diego Instruments) for a 30-min habituation period, as described in the OFT protocol above. Following the habituation period, the locomotion response to a saline injection (5 ml/kg, i.p.) was assessed for 30 min. After that, mice were administered d-amphetamine (2 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO) and placed back to the arena for 60 min to assess their locomotion response. Distanced traveled and time spent in the center of the arena was analyzed using TopScan v2.0 (CleverSys, Inc., Reston VA).

All the behavioral assessments were performed by an experimenter blind to the genotype of animals. The OFT, novel object recognition, L/D box, EPM, FST, learned helplessness, sucrose preference and shock sensitivity were performed on the same animals starting from the least to the most stressful test (for timeline see Figure 1A). There was a 7-day gap period between the FST and learned helplessness and a 7-day gap between sucrose preference post-stress and shock sensitivity, when mice remained undisturbed in their home cages. The PPI and amphetamine-induced hyperlocomotion was performed on the same animals with at least a 7-day gap between these tests (for timeline see Figure 1B).

Statistical Analysis
The OFT, L/D box, EPM, novel object recognition, FST, and acoustic startle as well as, the total escape failures in the learned helplessness test with the wild-type, heterozygous and homozygous Esr1 mice were analyzed using one-way ANOVA. The OFT, L/D box, EPM, novel object recognition, FST, and acoustic startle as well as, the total escape failures in the learned helplessness test with the heterozygous and homozygous Esr2 mice were analyzed with an unpaired Student’s t-test. The average escape latency, escape failures, sucrose preference, pre-pulse inhibition and amphetamine-induced hyperlocomotion for both Esr1 and Esr2 were analyzed with a repeated measure two-way ANOVA. Datasets that fail to pass normality test as tested by Shapiro-Wilk test, were analyzed by Kruskal-Wallis and Mann-Whitney test. Fisher’s exact test was used as an additional analysis for % pre-pulse inhibition to test whether the proportion of mice decreased their PPI was significantly different between genotypes. The male and female mice experiments were performed in separate cohorts, and thus are not combined on the same graphs and statistical analyses. Holm-Sidak post-hoc test was performed when a significant interaction effect was observed in the ANOVAs. Statistical significance was set at p < 0.05. All analyses were performed using GraphPad Prism v 6.01. All values are expressed as mean ± SEM. Statistical details are summarized in Table 1.

RESULTS
Effects of Esr1 and Esr2 Genes on Anxiety-Related Behaviors in Male and Female Mice
Open-Field Test
We first assessed OFT behavior in male and female heterozygous (Esr1+/−) and homozygous (Esr1−/−) Esr1 mice, as well as their littersmate wild-type controls (Esr1+/+). No difference was observed in the total distance traveled between the groups in both male (Figure 2A) and female mice (Figure 2K). Similarly, no difference was observed in the time-spent in the center of the open field arena in male (Figure 2B) and female mice (Figure 2L).

Moreover, OFT behavior was assessed in male and female heterozygous (Esr2+/−) and homozygous (Esr2−/−) Esr2 mice. No difference was observed in the total distance traveled between the genotype groups in both male (Figure 4A) and female mice (Figure 4K). Similarly, no difference was observed in the time-spent in the center of the open field arena in male (Figure 4B) and female mice (Figure 4L).
TABLE 1 | Details of statistical analyses.

OVERALL EFFECTS FOR FIGURE 2

| Genotype | Intensity | Interaction |
|----------|-----------|-------------|
| Males    |           |             |
| Distance traveled | $F_{(2, 26)} = 1.703$ | $P = 0.202$ |
| Time in center | $F_{(2, 26)} = 0.308$ | $P = 0.738$ |
| Light/Dark Box | $F_{(2, 24)} = 0.991$ | $P = 0.386$ |
| Elevated-plus maze | $F_{(2, 25)} = 1.697$ | $P = 0.204$ |
| Novel-object recognition | $F_{(2, 24)} = 0.352$ | $P = 0.707$ |
| Forced-swim test | $F_{(2, 26)} = 0.810$ | $P = 0.456$ |
| Females   |           |             |
| Distance traveled | $F_{(2, 40)} = 3.047$ | $P = 0.059$ |
| Time in center | $F_{(2, 40)} = 0.385$ | $P = 0.683$ |
| Light/Dark Box | $F_{(2, 40)} = 0.057$ | $P = 0.945$ |
| Elevated-plus maze | $H = 0.696$, $Df = 2$ | $P = 0.706$ |
| Novel-object recognition | $F_{(2, 39)} = 0.201$ | $P = 0.819$ |
| Forced-swim test | $H = 1.253$, $Df = 2$ | $P = 0.535$ |
| Pre-pulse inhibition | $H = 6.646$, $Df = 8$ | $P = 0.575$ |

OVERALL EFFECTS FOR FIGURE 3

| Genotype | Time | Interaction |
|----------|------|-------------|
| Males    |      |             |
| Escape latency | $F_{(2, 26)} = 0.698$ | $P = 0.507$ |
| Escape failures | $F_{(2, 26)} = 0.774$ | $P = 0.472$ |
| Total escape failures | $F_{(2, 26)} = 0.774$ | $P = 0.472$ |
| Sucrose preference | $H = 0.213$, $Df = 2$ | $P = 0.213$ |
| Females   |      |             |
| Escape latency | $H = 39.41$, $Df = 26$ | $P = 0.046$ |
| Escape failures | $H = 40.06$, $Df = 26$ | $P = 0.039$ |
| Total escape failures | $H = 7.167$, $Df = 2$ | $P = 0.028$ |
| Sucrose preference | $F_{(2, 26)} = 0.669$ | $P = 0.431$ |

OVERALL EFFECTS FOR FIGURE 4

| Genotype | Intensity | Interaction |
|----------|-----------|-------------|
| Males    |           |             |
| Distance traveled | $t_{(22)} = 0.170$ | $P = 0.867$ |
| Time in center | $t_{(22)} = 0.273$ | $P = 0.787$ |
| Light/Dark Box | $t_{(22)} = 0.960$ | $P = 0.347$ |
| Elevated-plus maze | $t_{(22)} = 0.433$ | $P = 0.689$ |
| Novel-object recognition | $t_{(22)} = 0.145$ | $P = 0.886$ |
| Forced-swim test | $t_{(22)} = 0.510$ | $P = 0.615$ |
| Females   |           |             |
| Pre-pulse inhibition | $F_{(1, 12)} = 1.329$ | $P = 0.271$ |
| Amphetamine-induced hyperlocomotion | $F_{(2, 24)} = 19.58$ | $P < 0.001$ |
| Timeline | $F_{(1, 13)} = 0.035$ | $P = 0.854$ |
| Total distance traveled | $F_{(1, 13)} = 0.035$ | $P = 0.854$ |

(Continued)
TABLE 1 | Continued

| Females | Intensity | Interaction |
|---------|----------|-------------|
| Genotype | Time | P | F | df | P |
| Escape latency | $F_{(1,17)} = 6.373$ | $P = 0.022$ | $F_{(2,34)} = 5.197$ | $P = 0.011$ | $F_{(2,34)} = 2.892$ | $P = 0.069$ |
| Pre-pulse inhibition | | | |
| Amphetamine-induced hyperlocomotion | | | |
| Genotype | Time | P | F | df | P |
| Total distance traveled | $F_{(1,13)} = 2.037$ | $P = 0.177$ | $F_{(23,299)} = 149.8$ | $P < 0.001$ | $F_{(23,299)} = 2.367$ | $P < 0.001$ |
| | | | $F_{(2,26)} = 659.3$ | $P < 0.001$ | $F_{(2,26)} = 4.787$ | $P = 0.017$ |

OVERALL EFFECTS FOR FIGURE 5

| Males | Genotype | Time | Interaction |
|-------|----------|------|-------------|
| Escape latency | | | $F_{(1,17)} = 5.644$ | $P = 0.027$ | $F_{(1,17)} = 17.63$ | $P = 0.000$ |
| Escape failures | | | $F_{(1,17)} = 5.644$ | $P = 0.027$ | $F_{(1,17)} = 17.63$ | $P = 0.000$ |
| Total escape failures | $F_{(1,17)} = 51.50$ | $P = 0.000$ | $F_{(1,17)} = 51.50$ | $P = 0.000$ | $F_{(1,17)} = 51.50$ | $P = 0.000$ |
| Sucrose preference | $F_{(1,22)} = 6.001$ | $P = 0.023$ | $F_{(1,22)} = 5.644$ | $P = 0.027$ | $F_{(1,22)} = 17.63$ | $P = 0.000$ |
| Females | Genotype | Time | Interaction |
| Escape latency | $F_{(1,14)} = 3.489$ | $P = 0.083$ | $F_{(8,112)} = 1.674$ | $P = 0.113$ | $F_{(8,112)} = 2.116$ | $P = 0.040$ |
| Escape failures | $F_{(1,14)} = 0.947$ | $P = 0.347$ | $F_{(8,112)} = 3.068$ | $P = 0.004$ | $F_{(8,112)} = 1.219$ | $P = 0.294$ |
| Total escape failures | $f_{(14)} = 2.274$ | $P = 0.039$ | $F_{(8,112)} = 2.116$ | $P = 0.040$ | $F_{(8,112)} = 1.219$ | $P = 0.294$ |
| Sucrose preference | $H = 6.863$, df = 3 | $P = 0.076$ | $F_{(1,14)} = 3.489$ | $P = 0.083$ | $F_{(8,112)} = 1.674$ | $P = 0.113$ |

Light/Dark Box
We measured the time that mice choose to spend in the brightly illuminated area of the L/D box. No effect of Esr1 knocked-down (neither Esr1$^{+/−}$ or Esr1$^{−/−}$) was observed in the in male (Figure 2C) or female (Figure 2M) mice compared with their wild-type littermates.

In addition, both male (Figure 4C) and female (Figure 4M) Esr2$^{−/−}$ and Esr2$^{+/−}$ mice spent similar time in the illuminated area of the L/D box.

Elevated-Plus Maze
We measured the time mice choose to spend in the open arms of the EPM. No difference was observed between the different genotypes (Esr1$^{+/+}$, Esr1$^{+/−}$, or Esr1$^{−/−}$) in male (Figure 2D) and female (Figure 2N) mice.

Also, no difference was observed between the different genotypes in male (Figure 4D) and female (Figure 4N) Esr2 mice.

Effects of Esr1 and Esr2 Deletion on Novel Object Memory in Male and Female Mice
Short-term recognition memory was assessed using the novel object recognition test. Neither male (Figure 2E) or female (Figure 2O) Esr1$^{−/−}$ mice manifest object recognition impairment, since there was no difference in the novel object recognition discrimination ratio compared with the wild-type controls.

Moreover, both male (Figure 4E) and female (Figure 4O) Esr2 mice did not show any genotype dependent object recognition impairment as assessed with a novel object recognition discrimination ratio.

Effect of Esr1 and Esr2 Deletion on Sensorimotor Gating in Male and Female Mice
Sensorimotor gating deficits were assessed using the PPI paradigm. No statistically significant difference was observed in either male (Figures 2G,H) or female (Figures 2Q,R) Esr1$^{−/−}$ mice in the % PPI. However, Fisher’s exact test revealed a near significant difference between female Esr1$^{+/+}$ and Esr1$^{−/−}$ (Figure 2R).

No difference was observed between the genotypes in the startle amplitude (Male mice-Esr1$^{+/+}$: 250.8 ± 53.14, Esr1$^{+/−}$: 235.9 ± 74.03, Esr1$^{−/−}$: 270.5 ± 59.86; Female mice-Esr1$^{+/+}$: 156.0 ± 30.29, Esr1$^{+/−}$: 148.9 ± 32.97, Esr1$^{−/−}$: 265.2 ± 63.61).

No difference was observed in male Esr2$^{−/−}$ mice in the % PPI (Figures 4G,H) and in the startle amplitude (Esr2$^{+/−}$: 231.1 ± 36.26, Esr2$^{−/−}$: 205.3 ± 26.94) compared with their littermate controls. However, an increase in the % PPI was observed in female Esr2$^{−/−}$ compared with Esr2$^{+/−}$ mice (Figure 4Q). No difference was observed in the contingency analysis (Figure 4R) and startle response (Esr2$^{+/−}$: 197.3 ± 30.16, Esr2$^{−/−}$: 166.6 ± 34.57).
Effect of Esr1 and Esr2 Deletion on \(d\)-Amphetamine-Induced Hyperlocomotion in Male and Female Mice

An increase in locomotor activity was observed in both male \([F(46, 667) = 2.866, p < 0.001; \text{Figure } 2I, F(4, 58) = 5.628, p < 0.001; \text{Figure } 2J]\) and female mice \([F(46, 667) = 2.552, p < 0.001; \text{Figure } 2K, L]\) from all the genotypes. However, male \(Esr1^{+/−}\) mice showed a lower response to \(d\)-amphetamine as indicated by the lower distance traveled compared with \(Esr1^{+/+}\) \((p < 0.001)\) and \(Esr1^{+/−}\) \((p < 0.01)\) mice \((\text{Figures } 2I, J)\). In contrast, female \(Esr1^{+/−}\) mice had a greater response to the \(d\)-amphetamine compared with \(Esr1^{+/+}\) and \(Esr1^{+/−}\) mice \((\text{Figures } 2S, T)\).

Following administration of \(d\)-amphetamine, an increase in locomotor activity was observed in both male \((\text{Figures } 4I, J)\) and female mice \((\text{Figures } 4S, T)\) from all the genotypes. No difference was observed in the \(d\)-amphetamine-induced hyperlocomotion between male \(Esr2^{+/−}\) and \(Esr2^{−/−}\) mice \((\text{Figures } 4I, J)\). In contrast, female \(Esr2^{−/−}\) mice had a greater locomotor response to \(d\)-amphetamine as shown by the higher distance traveled compared with \(Esr2^{+/-}\) mice \([F(23, 299) = 2.367, p < 0.001; \text{Figure } 4G, I, J]\). In female mice, \(Esr2^{−/−}\) mice had a greater response to \(d\)-amphetamine compared with \(Esr2^{+/-}\) mice \([F(2, 26) = 4.787, p = 0.017; \text{Figure } 4T]\).

Effects of Esr1 and Esr2 Deletion on Behavioral Despair and Anhedonia in Male and Female Mice

Forced-Swim Test

Behavioral despair was assessed using the FST in male and female \(Esr1^{+/−}\) and \(Esr1^{−/−}\) mice, as well as their littermate wild-type controls. Under baseline conditions, no effect of the deletion of...
Esr1 was observed in the FST in either male (Figure 2E) or female mice (Figure 2P).

No difference was also observed between Esr2+/− and Esr2−/− in the immobility time in male (Figure 4F) and female mice (Figure 4P).

Learned Helplessness
Development of helpless behavior was tested following inescapable shock. No difference in the escape latency (Figure 3A), and escape failures (Figures 3B,C) was identified between male Esr1+/−, or Esr1−/− mice compared with their littermate wild-type controls. In agreement, there was no difference in escape latency (Figure 3E) and escape failures (Figures 3E,G) between Esr1−/−, Esr1+/− and Esr1+/+ mice. The learned helplessness response was not affected by differences in shock perception since no differences were observed in the flinch response in the shock sensitivity test by either male (Esr1+/+: 0.045 ± 0.005, Esr1+/−: 0.037 ± 0.028, Esr1−/−: 0.055 ± 0.027) or female (Esr1+/+: 0.047 ± 0.006, Esr1+/−: 0.054 ± 0.026, Esr1−/−: 0.038 ± 0.023) mice.

No difference in the escape latency (Figure 5A), and escape failures (Figures 5B,C) between the male Esr2+/− and Esr2−/−; while female Esr2−/− mice had lower escape latency [F(8,112) = 2.116, p = 0.04; Figure 5E] and lower total escape failures [F(14) = 2.274, p = 0.039; Figure 5G] compared with Esr2+/− mice. Although, there was a statistical significance difference in the average escape latency and total escape failures, the breakdown of escape failures does not reach statistical significance (Figure 5F).

The learned helplessness response was not affected by any differences in shock perception since no differences were observed in the flinch response in the shock sensitivity test by both male (Esr2+/−: 0.057 ± 0.007, Esr2−/−: 0.062 ± 0.007) and female (Esr2+/−: 0.091 ± 0.005, Esr2−/−: 0.090 ± 0.007) mice.

Sucrose Preference
The development of anhedonia was tested at baseline (stress-naïve) conditions and following footshock stress (as described in the learned helplessness experiment) in male and female Esr1+/− and Esr1−/− mice, as well as wild-type control mice. No differences were observed between the different genotypes at baseline sucrose preference following stress in either male (Figure 3D) or female mice (Figure 3H).

In addition, the development of anhedonia was tested at baseline and following the learned helplessness procedure in male and female Esr2+/− and Esr2−/− mice. Although no difference was observed in sucrose preference prior to learned helplessness, following stress, male Esr2−/− mice decreased their sucrose preference compared with controls as well as compared with their pre-stress sucrose preference [F(1,22) = 17.63, p < 0.001; Figure 3D]. No difference was observed in female Esr2−/− and Esr2+/− mice was observed both before and after stress (Figure 3H).

DISCUSSION
In the present study, we demonstrate that the lack of ERα and ERβ are differentially involved in the development of helplessness and anhedonia in male and female mice following stress. To our knowledge, this is the first study to investigate the effects ERα and ERβ using knockout mice in the development of helplessness, i.e.,
FIGURE 4 | Baseline behavioral characterization of male and female Esr2<sup>+/−</sup> and Esr2<sup>−/−</sup> mice. No difference was observed between the different genotypes in male mice in (A,B) the open-field test, (C) light/dark box, (D) elevated-plus maze, (E) novel-object recognition, (F) forced-swim test, (G) pre-pulse inhibition, (H) contingency representation of pre-pulse inhibition and (I,J) amphetamine-induced hyperlocomotion. No difference was observed between the different genotypes in female mice in (K,L) the open-field test, (M) light/dark box, (N) elevated-plus maze, (O) novel-object recognition and (P) forced-swim test. (Q) Female Esr2<sup>−/−</sup> mice showed higher pre-pulse inhibition compared with Esr2<sup>+/−</sup> mice. (R) No difference was observed in contingency representation of pre-pulse inhibition. (S,T) An enhanced amphetamine response was observed in the male Esr2<sup>−/−</sup> mice compared with Esr2<sup>+/−</sup> mice. *p < 0.05, **p < 0.01; n = 13, 11 for (A–F), n = 7, 8 for (G–J), n = 9, 8 for (K–P), n = 11, 9 for (Q–T).

increase in escape failures following inescapable shock training, as well as the development of anhedonia following stress in both male and female mice. We demonstrate that female Esr2<sup>−/−</sup> mice manifested significantly lower escape failures in the learned helplessness test, as well as an overall higher sucrose preference following stress compared with the heterozygous controls suggesting that deletion of Esr2 gene might be beneficial against the development of maladaptive behaviors following stress. Our finding is not in line with previous findings that administration of ERβ agonists in ovariectomized female rats decrease immobility time in the FST (21, 34). The different approaches used to investigate the role of ERβ in responses to stress between the aforementioned and present study might account for these differences. For example, here we investigated the development of helplessness following the exposure of mice to stress (inescapable shock), whereas the previously published reports used the FST in stress-naïve rats as a measure of antidepressant efficacy of ERβ agonists. Moreover, our use of intact mice vs. the use of ovariectomized rats in this earlier study might also contribute to these differences.

While we demonstrate that deletion of Esr2 gene in female mice has a protective effect against the development of helplessness, this is not the case in male Esr2<sup>−/−</sup> mice, which had similar escape failures compared to the Esr2<sup>+/−</sup> mice. However, we observed a decrease in sucrose preference following inescapable shock stress in male Esr2<sup>−/−</sup> compared with Esr2<sup>+/−</sup> control mice, suggesting that Esr2<sup>−/−</sup> male mice are more susceptible to stress-induced anhedonia. To our knowledge this is the first study to demonstrate a role of Esr2 gene in male mice in the development of stress-induced anhedonia, a core symptom of depression in humans, and further investigation is warranted to identify the specific role of this receptor in male depression.

In contrast with the effects of Esr2 deletion, following inescapable shock, we did not observe any statistically significant differences in either male or female Esr1<sup>−/−</sup> mice compared with either wild-type and heterozygous littermates. Moreover,
neither male nor female \textit{Esr1}\(^{-/-}\) mice showed any anhedonia symptoms as measured by sucrose preference prior or after the inescapable shock stress suggesting that \textit{Esr1} is not substantially involved in the development of these behavioral responses. These conclusions are also in agreement with our finding that both male and female \textit{Esr1}\(^{-/-}\) mice do not manifest any differences in the FST compared with their wild-type controls. It has been recently reported that ER\(\alpha\) in the nucleus accumbens drives a pro-resilient phenotype in both male and female mice (35). This is in apparent contrast with our findings demonstrating that lack of \textit{Esr1}\(^{-/-}\) does not induce susceptibility to develop learned helplessness or result in changes in sucrose preference following stress in either sex. It may be that while increased ER\(\alpha\) driven transcription mediates resilience, lack of the gene does not modulate susceptibility.

Although we demonstrated differential effects of both estrogen receptors in male and female mice in response to stress, we did not observe any effects of genetic deletion of estrogen receptors in anxiety-related tests. In line with our findings, Krezel et al. (36) demonstrated that both male and female \textit{Esr1}\(^{-/-}\) mice had similar thigmotaxis and spent similar time in open arms of the EPM compared with wild-type mice. While, our findings in male \textit{Esr2}\(^{-/-}\) mice are also in agreement with other reports (36, 37), their finding that female \textit{Esr2}\(^{-/-}\) mice have higher anxiety-like behaviors compared with wild-type mice, as measured by the EPM and OFT, are in contrast with the results presented here. In line with our findings, other reports demonstrated that female \textit{Esr2}\(^{-/-}\) have similar performance on anxiety behavioral tests as wild-type mice (23, 38). Interestingly, Walf et al. (38) also demonstrated that female wild-type as well as in \textit{Esr2}\(^{-/-}\) mice during proestrus had higher open field-central entries, which was interpreted as an anxiolytic effect. Therefore, future studies should address this limitation and further investigate the effects of the estrous cycle and estrogen receptors in anxiety-like behaviors.

Moreover, another limitation of the current study is the use of heterozygous mice for \textit{Esr2} instead of wild-type as controls. This might be particularly important when negative results are obtained, such as for the anxiety-related behaviors, since heterozygous mice might have different phenotypes than wild-types. However, it was previously shown that \textit{Esr2}\(^{+/+}\) behave in a similar manner as wild-type mice in the elevated plus-mice (39), a test assessing anxiety-like behaviors. Considering this, and the fact that our findings are also in agreement with other studies (20, 36, 37, 40) provides confidence to our results. However, for this reason the negative results comparing \textit{Esr2}\(^{+/+}\) to \textit{Esr2}\(^{-/-}\) should be interpreted cautiously as a full wild-type control was not included in the experimental design. In addition, since we are using conventional knockout mice, the absence of differences in these behaviors could be due to compensatory mechanisms.

Sex differences have been reported in patients with major depression, with male, but not female, patients demonstrating decreased PPI compared with healthy controls (41, 42). In order to test if estrogen receptors might be implicated in these sex differences, we assessed male and female \textit{Esr1} and \textit{Esr2} knockout mice in the PPI paradigm. Although we observed no significant difference with the male \textit{Esr1}\(^{-/-}\) and \textit{Esr2}\(^{-/-}\) mice, deletion

![Figure 5](image-url)
of estrogen receptors in female mice exerted differential effects. Specifically, contingency analysis demonstrated a near significant decrease in % PPI in Esr1−/− compared with Esr1+/+. This may be related to the finding that a decrease in % PPI is observed in rodents following ovariectomy, an effect that is normalized with estradiol replacement (43, 44); thus, considering our results we postulate that this decrease is attributed to the actions of estradiol through ERα. In contrast with the effects of Esr1 deletion, female Esr2−/− mice manifested higher PPI than their littermate controls suggesting an enhanced sensorimotor gating response. In combination with our findings that deletion of Esr2 gene in female mice results in decreased escape failures, and the literature demonstrating stress-induced decreases in PPI (45–47), these results suggest that deletion of Esr2 gene in females might result in stress resilience. Enhanced PPI in this case might be also associated with an improved ability of these mice to deal with information processing. Moreover, considering the literature supporting a protective effect of estradiol in women with schizophrenia [see (48)], our findings cannot preclude that the differential regulation of PPI response in Esr1 and Esr2 knockout female mice might have a functional relevance to schizophrenia. However, further investigation is warranted for better understanding this finding and the possible implications of estrogen receptors in stress resilience and/or schizophrenia.

Furthermore, we observed that male Esr1−/− mice showed attenuated response to amphetamine, as measured by hyperlocomotion, compared with Esr1+/− and Esr1+/+, suggesting a possible interaction between Esr1−/− and the dopaminergic signaling. The possible interaction between Esr1 and the dopaminergic system is also supported by the findings that male mice lacking Esr1 have decreased tyrosine hydroxylase (TH) mRNA and protein levels in the midbrain (49), which might also explain our findings that male Esr1−/− mice manifest attenuated response to amphetamine-induced hyperlocomotion compared with WT mice. A possible interaction between these two systems is further supported by our findings in female mice; however, in this instance lack of Esr1 in females enhanced the amphetamine-induced hyperlocomotion compared with controls. A decrease in TH mRNA and protein levels in the midbrain was also observed in female mice lacking Esr1−/− (49). These differences in the amphetamine-induced locomotion in Esr1−/− mice could be related to this finding, or could be influenced by differences in their hormonal status compared with WT mice. Both male and female Esr1−/− mice display highly atrophied reproductive organs and are infertile (50–53) and female Esr1−/− mice are anovulatory and acyclic (54, 55). Considering that gonadal hormones can affect dopamine response [see (56–58)], the difference in the hormonal status of these mice could contribute to the observed differences in amphetamine-induced hyperlocomotion. An enhancement of amphetamine-induced hyperlocomotion was also observed in female Esr2 knockout mice compared with their respective controls, whereas no difference was observed in male Esr2−/− mice. Female Esr2−/− have been reported to have similar levels of TH immunoreactive cells in the midbrain compared to WT mice (59), though this does not preclude differences in dopamine neuron activity or release. Interestingly, ERα and ERP agonists have been shown to reverse the amphetamine-induced disruption of PPI (60), which further supports a role of these receptors in modulating response to amphetamine. In addition, estradiol is known to modulate several dopamine-related behaviors such as sexual motivation (61), as well as to increase the rewarding effects of d-amphetamine (62, 63); however, the exact role of estrogen receptors needs to be further investigated.

Overall, we demonstrate that deletion of either Esr1 or Esr2 differentially affects the development of stress-related responses as well as psychomotor responses in male and female mice. Specifically, deletion of Esr2 in male mice led to increased susceptibility for the development of stress-related maladaptive behaviors, whereas deletion of Esr2 in female mice resulted in resilience against the development of such behaviors. Also the amphetamine locomotor response was attenuated in male Esr1−/− mice, while female Esr1−/− and Esr2−/− showed enhanced response. The present findings suggest that differential manipulation of Esr1 and Esr2 in males and females might have potential applications for the treatment of mood disorders.

**AUTHOR CONTRIBUTIONS**

PG and TG designed the experiment. PG, PZ, and CJ performed the experiments presented in this manuscript. PG analyzed all the results included in this manuscript. PG and TG drafted the paper. PZ and CJ critically reviewed the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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