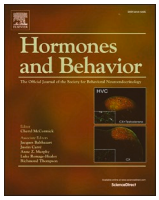
Hormones and Behavior 152 (2023) 105363

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh



No detectable changes in anxiety-related and locomotor behaviors in adult ovariectomized female rats exposed to estradiol, the ERβ agonist DPN or the ERα agonist PPT

Christiana K. Miller [a], John Meitzen [a,b,c,*]

[a] Department of Biological Sciences, North Carolina State University, Raleigh, NC, United States of America
[b] Center for Human Health and the Environment, North Carolina State University, Raleigh, NC, United States of America
[c] Comparative Medicine Institute, North Carolina State University, Raleigh, NC, United States of America

ARTICLE INFO

Keywords:
Estradiol
Sex
Locomotion
Anxiety
Estrogen receptor

ABSTRACT

The sex steroid hormone 17β-estradiol (estradiol) and its Estrogen Receptors (ERs) have been linked to modulation of anxiety-related and locomotor behaviors in female rodents. Research suggests that estradiol mitigates anxiety-related behaviors through activating Estrogen Receptor (ER)β and increases locomotor behaviors through ERα. The influence of ERs on these behaviors cannot always be detected. Here we discuss two experiments in which we tested the hypothesis that anxiety-related behaviors would decrease after ERβ activation and locomotor behaviors would increase after ERα activation, and also assessed the persistence of these behavioral effects by varying the timing of behavioral testing. Two cohorts of adult female ovariectomized rats were exposed to estradiol, the ERβ agonist DPN, the ERα agonist PPT, or oil for four consecutive days. Body mass was assessed throughout as a positive control. In both cohorts, open field behaviors were assessed on the first day of exposure. In one cohort (Experiment 1), open field, light/dark box, and elevated plus maze behaviors were assessed on the final day of injections. In the second cohort (Experiment 2), these behaviors were assessed 24 h after the final exposure. As expected, significant differences in body mass were detected in response to estradiol and PPT exposure, validating the estradiol and ER manipulation. No significant differences were observed in anxiety-related or locomotor behaviors across treatment groups, indicating that the efficacy of these agonists as therapeutic agents may be limited. We review these results in the context of previous literature, emphasizing relevant variables that may obscure ER-related actions on behavior.

1. Introduction

Naturally cycling sex steroid hormones such as 17β-estradiol (estradiol) modulate anxiety-related and locomotor behaviors. In women, an increase in symptoms of anxiety is typically associated with lower estradiol level phases of the menstrual cycle or following menopause (Campbell and Whitehead, 1977; Cameron et al., 1988; Cohen et al., 2003; Yonkers et al., 2008; Pestana et al., 2022). In rodents, anxiety-related behaviors change across the estrous cycle (Mora et al., 1996; Díaz-Véliz et al., 1997; Marcondes et al., 2001; Miller et al., 2020, 2021) but the direct effect of estradiol on these behaviors is not always consistent across the literature. Some studies have found that estradiol treatment is anxiolytic, while others find no difference or in some cases even an increase in anxiety-related behaviors (Mora et al., 1996; Gogos et al., 2018; Graham and Scott, 2018). There are also differences between motivated and non-motivated behaviors. For instance, an increased presence of estradiol in female rodents robustly increases voluntary wheel running related behaviors (Krentzel et al., 2020), however an increase in activity is not always seen in other non-motivated behavioral tasks as assessed using an open field apparatus (Rodier, 1971; Palermo-Neto and Dorce, 1990; Morgan and Pfaff, 2001; Lund et al., 2005; Bowen et al., 2012). Due to the variation concerning estradiol's modulation of both anxiety-related and locomotor behaviors, it is likely that the exact behavioral effects of estradiol depend on an array of complex and intersecting factors of differing levels of visibility and influence, as well the roles of multiple types of estrogen receptors

* Corresponding author at: Dept. of Biological Sciences, NC State University, 144 David Clark Labs, Campus Box 7617, Raleigh, NC 27695-7617, United States of America.
E-mail address: jemeitze@ncsu.edu (J. Meitzen).

https://doi.org/10.1016/j.yhbeh.2023.105363
Received 29 December 2022; Received in revised form 7 April 2023; Accepted 10 April 2023
Available online 21 April 2023

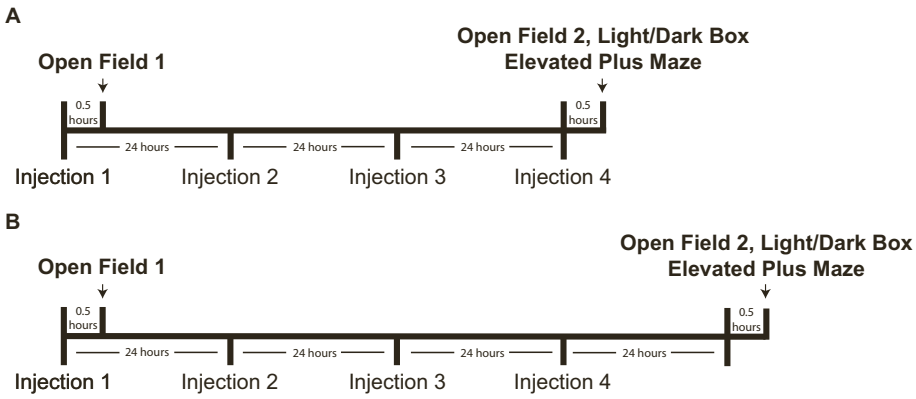


Fig. 1. Schematic depicting experimental paradigms. In both experiments, rats were given four consecutive injections 24 h apart. (A) In experiment 1, behavioral testing was performed 0.5 h after the first and last injection. (B) In experiment 2, behavioral testing was performed 0.5 h after the first and 24.5 h after the last injection.

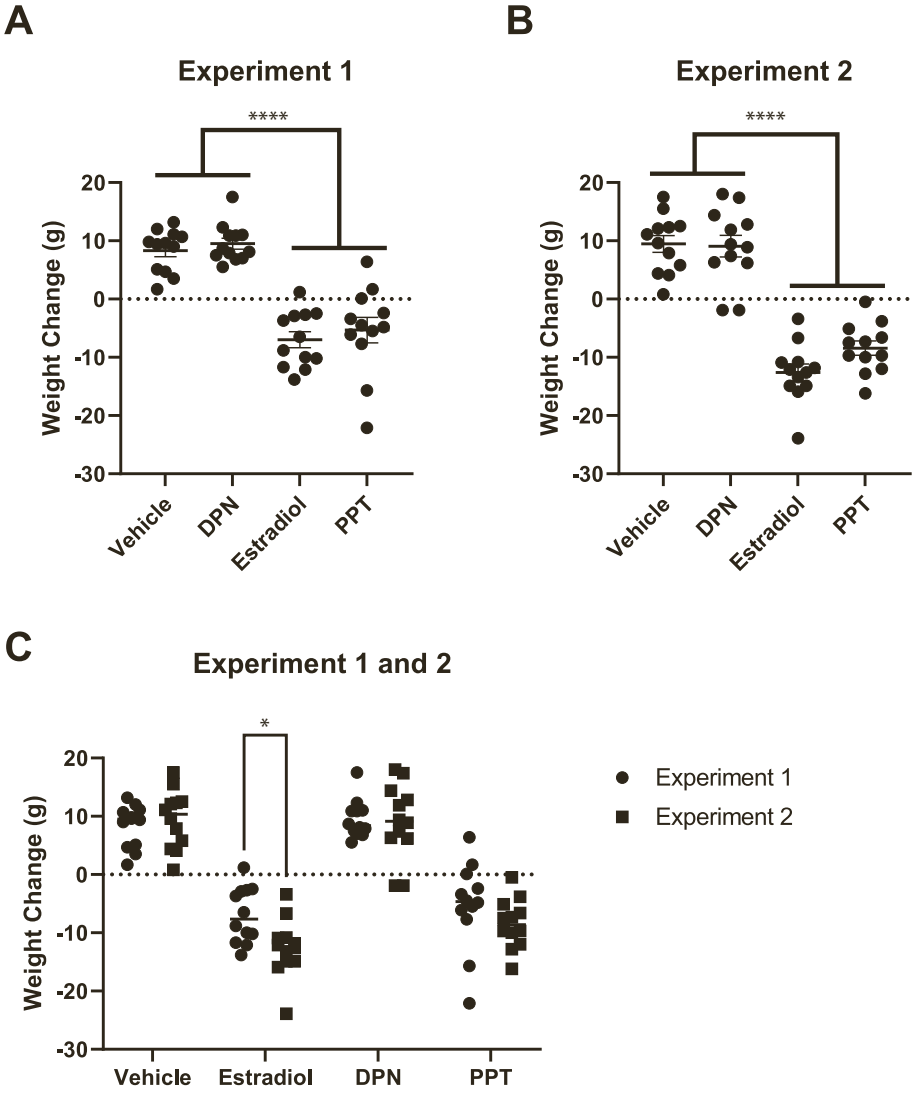


Fig. 2. Estradiol or PPT treatment attenuated weight gain for rats as measured from the first day of injections to the day of behavior. (A) Experiment 1: Overall weight change between the first and last day of injections was negative for rats treated with estradiol or PPT, and positive for rats treated with vehicle or DPN. (B) Experiment 2: Overall weight change between the first day of injections and 24 h after the last day of injections was negative for rats treated with estradiol or PPT, and positive for rats treated with vehicle or DPN. (C) Comparisons between experiments 1 and 2. Rats treated with estradiol experienced a more significant weight decrease from the first day of injections when weight was measured 24 h following the last injection as opposed to the last day of injections. Acronyms: * = $p < 0.05$.

(ERs).

Estradiol action on behavior is dependent on the presence and type of receptor to which estradiol binds, including ERα, ERβ, GPER-1 and/or Gq-coupled membrane ER (Gq-mER). Some of the divergent behavioral effects of estradiol are attributed to distinct action through these receptors. For example, activation or deactivation of either ERα or ERβ can sometimes exert anxiogenic or anxiolytic effects (Imwalle et al., 2005; Lund et al., 2005; Walf and Frye, 2005a,b; Walf et al., 2008; Spiteri et al., 2010; Byrnes et al., 2012; Oyola et al., 2012; Byrnes et al., 2013), and these actions are not necessarily estrogen receptor element

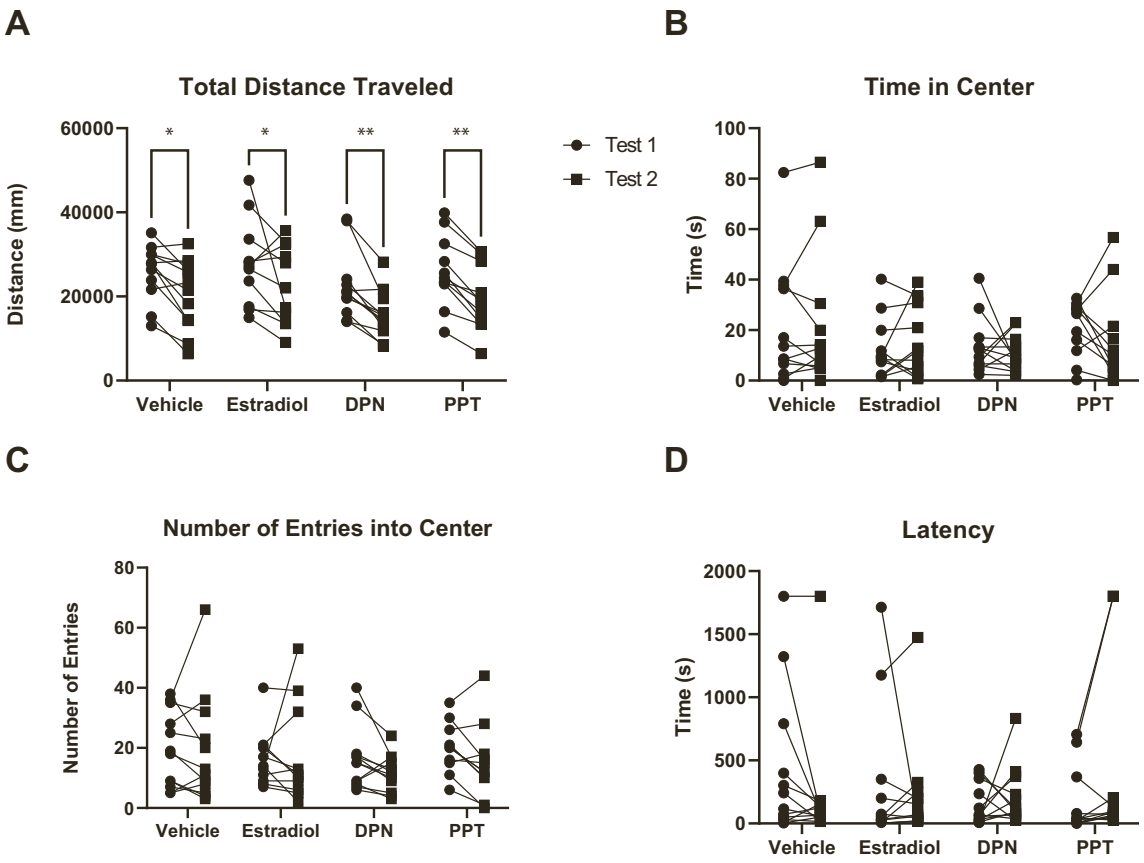


Fig. 3. Experiment 1 analysis of vehicle, estradiol, DPN, and PPT treatments on locomotor and anxiety-related behaviors in the open field under white light. Test 1 was performed on the first day of injections and test 2 was performed on the last day of injections. (A) Total distance traveled in the open field within subject between test 1 and test 2. (B) Time spent in the center of the open field within subject between test 1 and test 2. (C) Number of entries into the center of the open field within subject between test 1 and test 2. (D) Latency to enter the center of the open field within subject between test 1 and test 2.

(ERE)-mediated (Wiersielis et al., 2021). This finding and others mean that estradiol action on behavior may be mediated either through nuclear ER signaling via ERE action or via membrane ER signaling via non-ERE mechanisms. Likewise, ERα but not ERβ is typically the receptor through which estradiol acts to increase activity and regulate metabolism (Ogawa et al., 2003; Bryzgalova et al., 2006; Hertrampf et al., 2008; Spiteri et al., 2012; Xu et al., 2015). The divergent actions of estradiol through ERα and ERβ likely work in concert to ensure both successful reproduction and appropriate behavioral responses to environmental conditions, and to fully understand each receptor's contribution it is important to design experiments that investigate separate ER action.

Here we focused on the role of ERα and ERβ in anxiety-related and non-motivated locomotor behaviors. We tested the hypothesis that anxiety-related behaviors would decrease after ERβ activation and locomotor behaviors would increase after ERα activation, and also assessed the persistence of these behavioral effects by varying the timing of behavioral testing. We conducted two experiments, each with a separate cohort of adult female ovariectomized rats. In both experiments, rats were exposed to estradiol, the ERβ agonist DPN, the ERα agonist PPT, or oil for four consecutive days. Body mass was assessed throughout as a positive control. In both experiments, open field behaviors were assessed on the first day of exposure. In Experiment 1, open field, light/dark box, and elevated plus maze behaviors were assessed on the final day of exposure, 30 min after the last injection. In Experiment 2, these behaviors were assessed 24 h after the final exposure. This differential assessment was performed to observe potential rapid versus long-term behavioral effects of estradiol and ER agonists.

2. Materials and methods

2.1. Animals

All animal protocols were approved by the Institutional Animal Care and Use Committees (IACUC) at North Carolina State University. Female Sprague-Dawley rats were purchased at P50 ($n = 96$) from Charles River Laboratories and were housed at the Biological Resource Facility at North Carolina State University. Cages were BPA free and filled with bedding manufactured from virgin hardwood chips (Beta Chip; NEPCO, Warrensburg, NY) to avoid endocrine disruptors present in corncob bedding (Markaverich et al., 2002; Mani et al., 2005; Villalon Landeros et al., 2012). Soy protein-free rodent chow (2020X; Teklad, Madison, WI) and glass water bottles were provided ad libitum. Rats were housed in a temperature (23 °C, 40 % humidity) and light controlled room on a 12:12 h light:dark cycle with lights turning off at 9:00 am. At P60 ± 1, rats were anesthetized using isoflurane and ovariectomized. Rats were single housed following ovariectomy. After a one-week recovery period, rats were handled daily for one week before injections and behavioral testing. Injections and behavioral testing began two weeks post-gonadectomy.

2.2. Drug and hormone exposure

For each experiment, rats were divided into four treatment groups, adapted from a previously published protocol (Lund et al., 2005). These four groups of rats received injections consisting of: cottonseed oil, 17β-estradiol benzoate (Estradiol; Sigma-Aldrich, St. Louis, MO), the ERα agonist PPT (4,4′,4″-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol;

Table 1
Experiment 1 open field test: analysis of the first 5 and 10 min in the arena.

Property	Vehicle	Estradiol	DPN	PPT	Statistics
First 5 min					
Total distance traveled (mm)	Test 1: 9042 ± 387 Test 2: 7679 ± 631	Test 1: 8703 ± 686 Test 2: 7407 ± 758	Test 1: 8350 ± 565 Test 2: 6319 ± 710	Test 1: 7547 ± 424 Test 2: 6627 ± 737	Treatment × Test: 0.74, 0.01, 0.53 Treatment: 1.11, 0.05, 0.35 *Test: 27.31, 0.10, <0.0001* *Subject: 4.45, 0.69, <0.0001*
Time in center (s)	Test 1: 4.7 ± 1.8 Test 2: 6.9 ± 3.0	Test 1: 5.2 ± 1.7 Test 2: 4.0 ± 1.2	Test 1: 5.1 ± 1.7 Test 2: 5.7 ± 2.3	Test 1: 5.2 ± 1.7 Test 2: 3.5 ± 1.9	Treatment × Test: 0.50, 0.01, 0.69 Treatment: 0.21, 0.01, 0.89 Test: 0.00, 0.00, 0.99 Subject: 1.35, 0.56, 0.16
Number of entries into center	Test 1: 6.3 ± 1.0 Test 2: 5.5 ± 1.0	Test 1: 5.8 ± 1.4 Test 2: 4.4 ± 0.9	Test 1: 5.3 ± 1.2 Test 2: 4.9 ± 1.4	Test 1: 5.1 ± 0.7 Test 2: 4.1 ± 0.9	Treatment × Test: 0.09, 0.00, 0.96 Treatment: 0.38, 0.02, 0.77 Test: 2.04, 0.16, 0.16 *Subject: 2.02, 0.64, 0.011*
First 10 min					
Total distance traveled (mm)	Test 1: 15418 ± 822 Test 2: 13133 ± 831	Test 1: 15370 ± 969 Test 2: 13170 ± 941	Test 1: 14559 ± 921 Test 2: 10371 ± 915	Test 1: 13335 ± 823 Test 2: 10713 ± 1013	Treatment × Test: 1.50, 0.01, 0.23 Treatment: 2.07, 0.10, 0.12 *Test: 56.71, 0.18, <0.0001* *Subject: 4.88, 0.58, <0.0001*
Time in center (s)	Test 1: 11.0 ± 4.1 Test 2: 12.1 ± 4.3	Test 1: 6.8 ± 1.9 Test 2: 6.8 ± 2.5	Test 1: 9.3 ± 2.4 Test 2: 8.2 ± 2.2	Test 1: 10.2 ± 3.1 Test 2: 6.4 ± 2.3	Treatment × Test: 0.51, 0.01, 0.68 Treatment: 0.61, 0.03, 0.61 Test: 0.44, 0.02, 0.51 *Subject: 3.00, 0.71, 0.0002*
Number of entries into center	Test 1: 11.3 ± 2.2 Test 2: 9.3 ± 1.7	Test 1: 8.7 ± 1.9 Test 2: 7.3 ± 1.7	Test 1: 9.9 ± 1.7 Test 2: 7.6 ± 1.4	Test 1: 9.3 ± 1.4 Test 2: 6.6 ± 1.4	Treatment × Test: 0.13, 0.00, 0.94 Treatment: 0.55, 0.03, 0.65 *Test: 7.2, 0.03, 0.010* *Subject: 3.60, 0.73, <0.0001*

Experiment 1 open field data analyzed across the first 5 min, and the first 10 min in the arena. Data from the entire 30 min test period are depicted in Fig. 3. Test 1 was performed on the first day of injections and test 2 was performed on the last day of injections. Latency to enter center is not reported as that attribute does not differ by analysis time and is depicted in Fig. 3. Data are reported as mean ± standard error of the mean. Statistical analysis was 2-way repeated measures ANOVA. F, η^2p, and *p* values are presented sequentially in the "Statistics" column. Degrees of freedom for Treatment × Test, Treatment, and Test analyses are 3, 44. Degrees of freedom for subject analysis is 44, 44. Italics indicate statistically significant differences.

Tocris Biosciences, Minneapolis, MN), or the ERβ agonist DPN (2,3-*bis*(4-Hydroxyphenyl)-propionitrile; Tocris Biosciences, Minneapolis, MN). PPT and DPN concentrations were 1 mg/kg dissolved in cottonseed oil and 5 % dimethylsulfoxide (DMSO), modeled from a previous study (Lund et al., 2005). These doses of DPN and PPT are typical of most literature, although doses widely range (Borrow and Handa, 2017). Estradiol concentration for each rat in the estradiol treatment groups (body weight = 314 ± 32 g) was 10 μg/0.1 ml dissolved in cottonseed oil and 5 % DMSO, following previous studies (Walf and Frye, 2005a,b; Peterson et al., 2015). This dose of estradiol produces physiological levels of estradiol typically observed during proestrus in rats (Viau and Meaney, 1991; Gibbs, 1997; Proano et al., 2020). Cottonseed oil control injections likewise contained 5 % DMSO. Each day of injections, rats were weighed and then received one subcutaneous injection of either cottonseed oil and DMSO (referred to as oil), estradiol, PPT, or DPN. Injections were given blind over 4 consecutive days between 9:00 and 10:30 am (Fig. 1).

2.3. Behavioral testing and data analysis: experiment 1 and 2

All behavioral testing occurred within the first 3 h of the animal's dark cycle, between 10:00 am and 12:00 pm. In experiment 1 ($n = 48$), behavioral testing began 30 min after the injection on the first and fourth day of injections (Fig. 1). In experiment 2 (n = 48), behavioral testing began 30 min after the first injection and 24.5 h after the fourth injection. Following each behavioral test, all equipment was thoroughly cleaned with 70 % isopropyl alcohol.

2.3.1. Open field test

For both experiments, the open field test was conducted twice. In experiment 1, testing occurred on the first and fourth day of injections under white light (175 ± 10 lx). In experiment 2, testing occurred on the first day of injections and again the day after the final injection under white light (250 ± 10 lx). This two test experimental design addresses the complex behavioral effects of novelty and habituation to the open field arena and allows for the assessment of treatment effects within an individual subject (Montgomery, 1955; Thompson and Spencer, 1966; Miller et al., 2020). Rats were individually placed into the center of the open field arena (60 cm × 60 cm × 60 cm; Cleversys Inc., Reston, VA). Following a previously documented protocol (Miller et al., 2020), activity was recorded for 30 min with a video camera located above the open field. In all experiments, locomotion was determined by measuring the total distance traveled in the open field and anxiety-related behaviors were evaluated using the time spent in the center of the open field, number of entries into the center, and latency to enter the center. Data were analyzed across the entire 30 min, the first 5 min, and the first 10 min. For statistical analysis, rats that did not enter the center were assigned a latency of 1800 s, which is the total duration of the test. All activities were analyzed blind to treatment using TopScan software version 3.0 (Cleversys Inc., Reston, VA). Rats exhibiting <5000 mm of measured total distance traveled in either behavioral assay were excluded from behavioral analysis ($n = 4$; Experiment 1: Estradiol = 1, PPT = 1; Experiment 2: Vehicle =1, Estradiol = 1).

2.3.2. Light/dark box

Immediately following the second open field test in both experiments, anxiety-related behavior was further assessed using a light/dark (LD) box under white light (250 ± 10 lx). The LD box contained two chambers; one open, clear chamber that allowed light and one black chamber that obstructed light each measuring 40 cm long, 30 cm wide, and 30 cm tall. Activity was recorded for 5 min with a video camera facing the LD box. Behavior was evaluated as the number of full-body

Table 2
Experiment 2 open field test: analysis of the first 5 and 10 min in the arena.

Property	Vehicle	Estradiol	DPN	PPT	Statistics
First 5 min					
Total distance traveled (mm)	Test 1: 8601 ± 445 Test 2: 7355 ± 524	Test 1: 7594 ± 406 Test 2: 7055 ± 218	Test 1: 7947 ± 497 Test 2: 6979 ± 453	Test 1: 8191 ± 500 Test 2: 7013 ± 533	Treatment × Test: 0.44, 0.01, 0.73 Treatment: 0.49, 0.02, 0.69 *Test: 17.28, 0.09, 0.0002* *Subject: 2.82, 0.64, 0.0005*
Time in center (s)	Test 1: 5.7 ± 1.9 Test 2: 2.8 ± 0.9	Test 1: 4.5 ± 1.3 Test 2: 1.8 ± 0.8	Test 1: 2.1 ± 0.4 Test 2: 2.4 ± 0.6	Test 1: 4.1 ± 1.2 Test 2: 5.8 ± 1.7	Treatment × Test: 2.67, 0.06, 0.0598 Treatment: 1.57, 0.06, 0.21 Test: 1.78, 0.01, 0.19 *Subject: 1.93, 0.57, 0.018*
Number of entries into center	Test 1: 5.9 ± 1.2 Test 2: 4.5 ± 0.6	Test 1: 4.1 ± 1.0 Test 2: 3.1 ± 0.7	Test 1: 4.1 ± 0.5 Test 2: 3.3 ± 0.7	Test 1: 5.1 ± 0.8 Test 2: 3.6 ± 0.7	Treatment × Test: 0.19, 0.00, 0.90 Treatment: 1.09, 0.05, 0.36 *Test: 8.64, 0.05, 0.0053* *Subject: 2.77, 0.65, 0.0007*
First 10 min					
Total distance traveled (mm)	Test 1: 13774 ± 904 Test 2: 11448 ± 903	Test 1: 12913 ± 754 Test 2: 11726 ± 650	Test 1: 13204 ± 858 Test 2: 10978 ± 908	Test 1: 12930 ± 784 Test 2: 11086 ± 908	Treatment × Test: 0.44, 0.01, 0.72 Treatment: 0.13, 0.01, 0.94 *Test: 24.78, 0.11, <0.0001* *Subject: 3.90, 0.70, <0.0001*
Time in center (s)	Test 1: 10.0 ± 2.7 Test 2: 6.2 ± 2.0	Test 1: 6.9 ± 2.0 Test 2: 5.5 ± 2.1	Test 1: 3.4 ± 0.7 Test 2: 4.8 ± 0.	Test 1: 7.6 ± 1.9 Test 2: 9.1 ± 3.7	Treatment × Test: 1.92, 0.02, 0.14 Treatment: 1.02, 0.06, 0.39 Test: 0.39, 0.00, 0.53 *Subject: 4.79, 0.76, <0.0001*
Number of entries into center	Test 1: 9.4 ± 2.0 Test 2: 6.8 ± 1.0	Test 1: 6.5 ± 1.4 Test 2: 6.0 ± 1.5	Test 1: 6.4 ± 0.9 Test 2: 5.9 ± 1.1	Test 1: 7.3 ± 1.2 Test 2: 6.1 ± 1.4	Treatment × Test: 0.58, 0.01, 0.63 Treatment: 0.57, 0.03, 0.64 Test: 3.5, 0.02, 0.068 *Subject: 3.43, 0.73, <0.0001*

Experiment 2 open field data analyzed across the first 5 min, and the first 10 min in the arena. Data from the entire 30 min test period are depicted in Fig. 6. Test 1 was performed on the first day of injections and test 2 was performed 24 h after the last day of injections. Latency to enter center is not reported as that attribute does not differ by analysis time and is depicted in Fig. 6. Data are reported as mean ± standard error of the mean. Statistical analysis was 2-way repeated measures ANOVA. F, η^2p, and *p* values are presented sequentially in the "Statistics" column. Degrees of freedom for Treatment × Test, Treatment, and Test analyses are 3, 42. Degrees of freedom for subject analysis is 42, 42. Italics indicate statistically significant differences.

entries into the light chamber, latency to enter the light chamber, full-body duration in the light chamber, and number of head pokes (defined as the entry of the nose and both eyes) into the light chamber. Rats that did not enter the clear chamber were assigned a latency of 300 s, which is the total duration of the test. Activity was manually scored by an individual blind to treatment.

2.3.3. Elevated plus maze

Immediately following the LD box test, anxiety-related behaviors were further assessed using an elevated plus maze (EPM) under red light (0.5 ± 0.5 lx). The EPM measured 68.5 cm high with arms 50 cm long and 10 cm wide and the closed arm walls measuring 40 cm tall. Activity was recorded for 5 min with a video camera located above the maze. Behavior was evaluated as the number of entries into the open arms, duration spent in the open arms, and total distance traveled in the EPM. All activities were analyzed blind to treatment using TopScan software version 3.0 (Cleversys Inc., Reston, VA). Rats that slipped or fell off the arena were excluded from analysis ($n = 3$; Experiment 2: Vehicle = 1, Estradiol = 1, DPN = 1).

2.4. Statistical analysis

Experimental data was analyzed in Graphpad Prism version 8 (La Jolla, CA). One-way and two-way repeated and non-repeated measures ANOVAs with Holm-Sidak's multiple comparisons test were used for behavioral data in experiments 1 and 2. Since the experiments were not run simultaneously, each experiment is analyzed individually. Weight data was analyzed using a two-way ANOVA with Holm-Sidak's multiple comparisons test and simple linear regressions. Effect sizes are presented as partial eta-squared (η^2p) for F-tests and Cohen's *d* for *t*-tests. *P* values < 0.05 were a priori considered significant.

3. Results

3.1. Weight difference

Estradiol exposure inhibits weight gain in ovariectomized female rats through estradiol action on ERα but not ERβ (Roesch, 2006). As a positive control for the efficacy of our injection paradigm, we analyzed differences in weight between the fourth and first day of injections for experiment 1 and the day after the fourth injection and first day of injections for experiment 2. In both experiments, rats treated with either estradiol or PPT demonstrated attenuated weight gain compared to rats treated with vehicle or DPN (Fig. 2A; One-way ANOVA: $F_{(3,44)} = 35.40$, $\eta^2p = 0.707$, $p < 0.0001$, Fig. 2B; One-way ANOVA: $F_{(3,44)} = 58.38$, $\eta^2p = 0.7992$, $p < 0.0001$). This finding indicates that the estradiol and PPT injections were effective. Additionally, rats treated with estradiol exhibited a more significant weight decrease in experiment 2 compared to experiment 1 when the last weight measurement was taken one day later (Fig. 2C; Two-way ANOVA: Interaction: $F_{(3,88)} = 2.009$, $\eta^2p = 0.0161$, $p = 0.1184$, Treatment: $F_{(3,88)} = 92.44$, $\eta^2p = 0.7396$, $p < 0.0001$, Experiment: $F_{(1,88)} = 3.616$, $\eta^2p = 0.0096$, $p = 0.0605$, Estradiol Experiment 1-Experiment 2: $t_{(88)} = 2.672$, Cohen's $d = 1.151$, $p = 0.0354$).

3.2. Experiment 1

3.2.1. Open field

Open field data were analyzed across the entire 30 min of each test (Fig. 3). Rats in all treatment groups exhibited a decrease in total distance traveled in the open field from the first to second test (Fig. 3A; Two-way RM ANOVA: Treatment × Test: $F_{(3,42)} = 0.2934$, $\eta^2p = 0.00252$, $p = 0.8299$, Treatment: $F_{(3,42)} = 1.452$, $\eta^2p = 0.0709$, $p = 0.2413$, Test: $F_{(3,42)} = 42.85$, $\eta^2p = 0.1225$, $p < 0.0001$, Subject: $F_{(42,42)} = 5.696$, $\eta^2p = 0.6836$, $p < 0.0001$, Vehicle Test 1-Test 2: $t_{(42)} = 2.916$, Cohen's $d = 0.7057$, $p = 0.0113$, Estradiol Test 1-Test 2: $t_{(42)} = 2.712$,

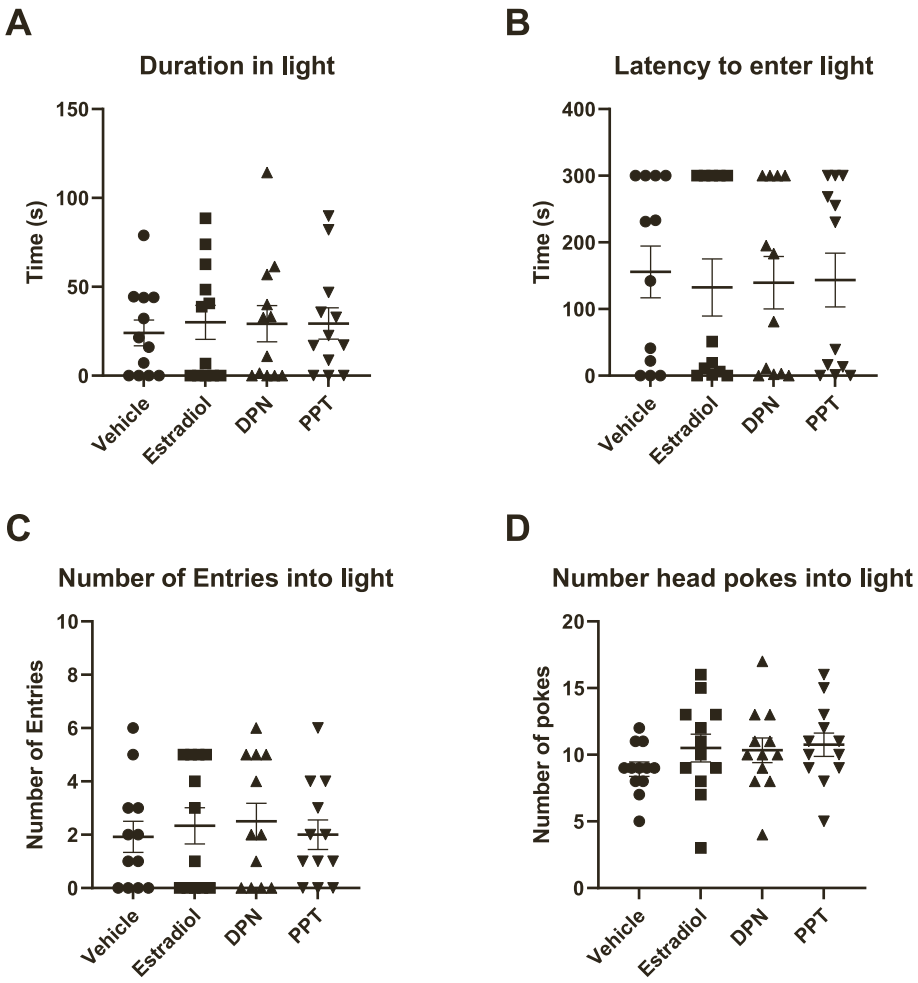


Fig. 4. Experiment 1 analysis of vehicle, estradiol, DPN, and PPT treatments on anxiety-related behaviors in the light dark box. (A) Total full-body duration in the light chamber of the light dark box. (B) Latency to fully enter the light chamber of the light dark box. (C) Number of full entries into the light chamber of the light dark box. (D) Number of head pokes into the light chamber of the light dark box.

Cohen's $d = 0.5236$, p = 0.0113, DPN Test 1-Test 2: $t_{(42)} = 3.915$, Cohen's $d = 1.0225$, $p = 0.0013$, PPT Test 1-Test 2: $t_{(42)} = 3.564$, Cohen's $d = 0.8285$, $p = 0.0028$). No significant differences were detected in the time spent in the center of the open field between tests and treatment groups (Fig. 3B; Two-way RM ANOVA: Treatment × Test: $F_{(3,42)} = 0.6682$, $\eta^2p = 0.00687$, $p = 0.5763$, Treatment: $F_{(3,42)} = 0.8621$, $\eta^2p = 0.0492$, $p = 0.4682$, Test: $F_{(3,42)} = 0.3268$, $\eta^2p = 0.00112$, $p = 0.5706$, Subject: $F_{(42,42)} = 5.550$, $\eta^2p = 0.7989$, $p < 0.0001$). No significant differences were detected in the number of entries into the center of the open field between tests and treatment groups (Fig. 3C; Two-way RM ANOVA: Treatment × Test: $F_{(3,42)} = 0.9166$, $\eta^2p = 0.0117$, $p = 0.4411$, Treatment: $F_{(3,42)} = 0.4837$, $\eta^2p = 0.0268$, $p = 0.6954$, Test: $F_{(3,42)} = 1.949$, $\eta^2p = 0.0083$, $p = 0.1700$, Subject: $F_{(42,42)} = 4.33$, $\eta^2p = 0.7743$, $p < 0.0001$). No significant differences were detected in the latency to enter the center of the open field between tests and treatment groups (Fig. 3D; Two-way RM ANOVA: Treatment × Test: $F_{(3,42)} = 2.024$, $\eta^2p = 0.0305$, $p = 0.1251$, Treatment: $F_{(3,42)} = 0.2869$, $\eta^2p = 0.0152$, $p = 0.8346$, Test: $F_{(3,42)} = 0.0001$, $\eta^2p < 0.0001$, $p = 0.9920$, Subject: $F_{(42,42)} = 3.529$, $\eta^2p = 0.7436$, $p < 0.0001$). Similar findings are made if data are instead analyzed over the first 5 min in the open field (Table 1), or the first 10 min (Table 2).

3.2.2. Light dark box

No significant differences were detected between treatments in the duration of time spent in the light chamber (Fig. 4A; One-way ANOVA: $F_{(3,44)} = 0.0955$, $\eta^2p = 0.006466$, $p = 0.9621$). No significant differences were detected between treatments in the latency to enter the light chamber (Fig. 4B; One-way ANOVA: $F_{(3,44)} = 0.0589$, $\eta^2p = 0.004$, $p = 0.9810$). No significant differences were detected between treatments in the number of entries into the light chamber (Fig. 4C; One-way ANOVA: $F_{(3,44)} = 0.1938$, $\eta^2p = 0.01304$, $p = 0.9001$). No significant differences were detected between treatments in the number of head pokes into the light chamber (Fig. 4D; One-way ANOVA: $F_{(3,44)} = 0.9024$, $\eta^2p = 0.05796$, $p = 0.4476$).

3.2.3. Elevated plus maze

No significant differences were detected between treatments in the time spent in the open arms (Fig. 5A; One-way ANOVA: $F_{(3,44)} = 0.6729$, $\eta^2p = 0.04387$, $p = 0.5733$). No significant differences were detected between treatments in the number of entries into the open arms (Fig. 5B; One-way ANOVA: $F_{(3,44)} = 0.5780$, $\eta^2p = 0.03792$, $p = 0.6325$). No significant differences were detected between treatments in the total distance traveled in the EPM (Fig. 5C; One-way ANOVA: $F_{(3,44)} = 0.4952$, $\eta^2p = 0.03266$, $p = 0.6875$).

3.3. Experiment 2

3.3.1. Open field

Open field data were analyzed across the entire 30 min of each test (Fig. 6). Rats treated with vehicle, estradiol, and DPN but not PPT exhibited a decrease in total distance traveled in the open field from the first to second test (Fig. 6A; Two-way RM ANOVA: Treatment × Test:

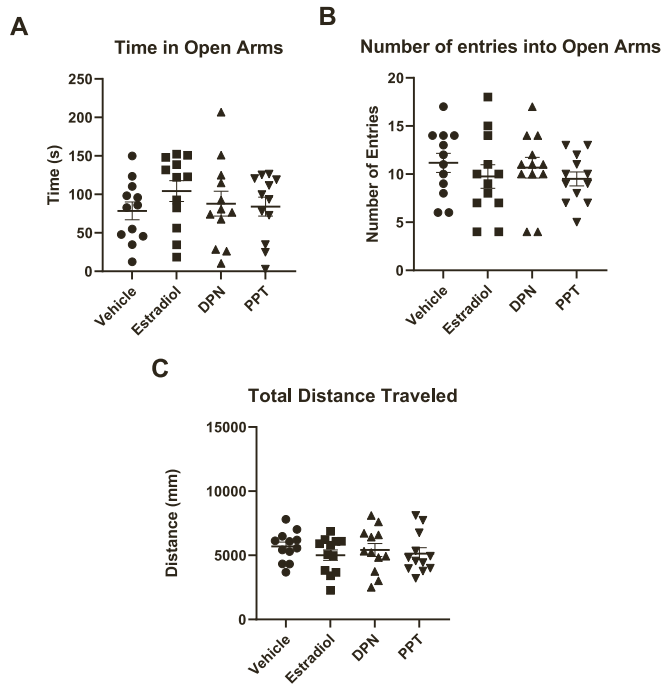


Fig. 5. Experiment 1 analysis of vehicle, estradiol, DPN, and PPT treatments on anxiety-related behaviors in the elevated plus maze under red light. (A) Total duration in the open arms of the elevated plus maze. (B) Number of entries into the open arms of the elevated plus maze. (C) Total distance traveled in the elevated plus maze. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$F_{(3,42)} = 0.9699$, $\eta^2p = 0.006042$, $p = 0.4160$, Treatment: $F_{(3,42)} = 0.5373$, $\eta^2p = 0.03138$, $p = 0.6593$, Test: $F_{(3,42)} = 27.90$, $\eta^2p = 0.05793$, $p < 0.0001$, Subject: $F_{(42,42)} = 9.376$, $\eta^2p = 0.8178$, $p < 0.0001$, Vehicle Test 1-Test 2: $t_{(42)} = 2.973$, Cohen's $d = 0.494$, $p = 0.0145$, Estradiol Test 1-Test 2: $t_{(42)} = 2.686$, Cohen's $d = 0.6714$, $p = 0.0205$, DPN Test 1-Test 2: $t_{(42)} = 3.590$, Cohen's $d = 0.5424$, $p = 0.0034$, PPT Test 1-Test 2: $t_{(42)} = 1.301$, Cohen's $d = 0.2696$, $p = 0.2004$). No significant differences were detected in the time spent in the center of the open field between tests and treatment groups (Fig. 6B; Two-way RM ANOVA: Treatment × Test: $F_{(3,42)} = 2.274$, $\eta^2p = 0.02564$, $p = 0.0939$, Treatment: $F_{(3,42)} = 0.8573$, $\eta^2p = 0.04707$, $p = 0.4707$, Test: $F_{(3,42)} = 0.3403$, $\eta^2p = 0.001279$, $p = 0.5628$, Subject: $F_{(42,42)} = 4.869$, $\eta^2p = 0.7686$, $p < 0.0001$). No significant differences were detected in the number of entries into the center of the open field between tests and treatment groups (Fig. 6C; Two-way RM ANOVA: Treatment × Test: $F_{(3,42)} = 0.2946$, $\eta^2p = 0.003673$, $p = 0.8291$, Treatment: $F_{(3,42)} = 0.7652$, $\eta^2p = 0.04179$, $p = 0.5200$, Test: $F_{(3,42)} = 3.821$, $\eta^2p = 0.01588$, $p = 0.0573$, Subject: $F_{(42,42)} = 4.380$, $\eta^2p = 0.7646$, $p < 0.0001$). No significant differences were detected in the latency to enter the center of the open field between treatment groups, but a significant increase in latency during test 2 was detected in rats treated with estradiol (Fig. 6D; Two-way RM ANOVA: Treatment × Test: $F_{(3,42)} = 1.686$, $\eta^2p = 0.01551$, $p = 0.1844$, Treatment: $F_{(3,42)} = 0.0464$, $\eta^2p = 0.002788$, $p = 0.9866$, Test: $F_{(3,42)} = 3.937$, $\eta^2p = 0.01207$, $p = 0.0538$, Subject: $F_{(42,42)} = 6.536$, $\eta^2p = 0.8416$, $p < 0.0001$, Estradiol Test 1-Test 2: $t_{(42)} = 2.831$, Cohen's $d = -0.5577$, $p = 0.0280$). Similar findings are made if data are instead analyzed over the first 5 min in the open field (Table 1), or the first 10 min (Table 2).

3.3.2. Light dark box

No significant differences were detected between treatments in the duration of time spent in the light chamber (Fig. 7A; One-way ANOVA: $F_{(3,44)} = 0.4706$, $\eta^2p = 0.03109$, $p = 0.7043$). No significant differences were detected between treatments in the latency to enter the light chamber (Fig. 7B; One-way ANOVA: $F_{(3,44)} = 1.571$, $\eta^2p = 0.09676$, $p = 0.2098$). No significant differences were detected between treatments in the number of entries into the light chamber (Fig. 7C; One-way ANOVA: $F_{(3,44)} = 0.6514$, $\eta^2p = 0.04253$, $p = 0.5863$). No significant differences were detected between treatments in the number of head pokes into the light chamber (Fig. 7D; One-way ANOVA: $F_{(3,44)} = 1.793$, $\eta^2p = 0.1089$, $p = 0.1624$).

3.3.3. Elevated plus maze

No significant differences were detected between treatments in the time spent in the open arms (Fig. 8A; One-way ANOVA: $F_{(3,44)} = 1.317$, $\eta^2p = 0.08792$, $p = 0.2817$). No significant differences were detected between treatments in the number of entries into the open arms (Fig. 8B; One-way ANOVA: $F_{(3,44)} = 1.192$, $\eta^2p = 0.08019$, $p = 0.3249$). No significant differences were detected between treatments in the total distance traveled in the EPM (Fig. 8C; One-way ANOVA: $F_{(3,44)} = 1.836$, $\eta^2p = 0.1185$, $p = 0.1556$).

4. Discussion

Our initial hypothesis for this study was that activation of ERβ would decrease anxiety-related behavior, that activation of ERα would increase locomotor behavior, and that this modulation of anxiety-related and locomotor behaviors would be present on the last day of exposure as well as 24 h after the last day of treatment. This hypothesis was clearly not supported, with the most obvious cause, that the estradiol or ER agonist exposures were somehow compromised, invalidated due to the success of the positive control regarding changes in body weight by estradiol and PPT. It is important to note that DPN is not known to attenuate weight gain and thus has no similar positive control to that of estradiol and PPT. However, because the DPN injections were prepared and given in the same way as those containing PPT, modeled after a previous study using the same concentrations that indicated changes in anxiety-related behaviors including in the open field (Lund et al., 2005) as well as a study that did not (Patisaul et al., 2009), it is unlikely that the DPN injections were prepared incorrectly. Overall, these results are similar to those of at least two other studies that also failed to detect an influence of DPN on anxiety-related behaviors (Patisaul et al., 2009; Jacome et al., 2010), suggesting that the utility of DPN as an inhibitor for anxiety may be situational.

This study's hypothesis and the overall experimental design were primarily based on previous work by our lab where estradiol treatment decreased anxiety-related behavior in ovariectomized adult rats (Miller et al., 2020), in conjunction with a widely cited study showing decreases in anxiety-related behaviors in response to exposure to the ERβ agonist DPN (Lund et al., 2005). These previous studies are consistent with select other literature which provides a body of evidence that ERβ modulates anxiety-related behaviors (Ogawa et al., 2003; Imwalle et al., 2005; Lund et al., 2005; Walf and Frye, 2005a,b; Spiteri et al., 2012; Borrow and Handa, 2017). There is also a body of evidence of studies that have failed to detect impacts on anxiety-like behaviors by ER agonists, including DPN (Patisaul et al., 2009; Jacome et al., 2010), a club which our study now joins. Considering these differing findings, it appears that DPN and estradiol sometimes but not always modulate anxiety-related behaviors. Thus, after firmly establishing the integrity of the data to the best of our abilities, the question in our laboratory became: "Why this result?" While we cannot definitively answer this question, we offer the following discussion in the spirit of assisting our field in assessing estrogen modulated behaviors, especially since our previous experiments indicate that variables such as the presence of light as well as acute stress can obscure estrogen-induced effects in common behavioral assessments (Miller et al., 2020, 2021).

Our laboratory's analysis of "why this result" began by recognizing that estradiol exposure in addition to PPT and DPN exposure did not significantly differ from vehicle treatment in any behavioral test. Estradiol treatment was intended to be a positive control regarding the

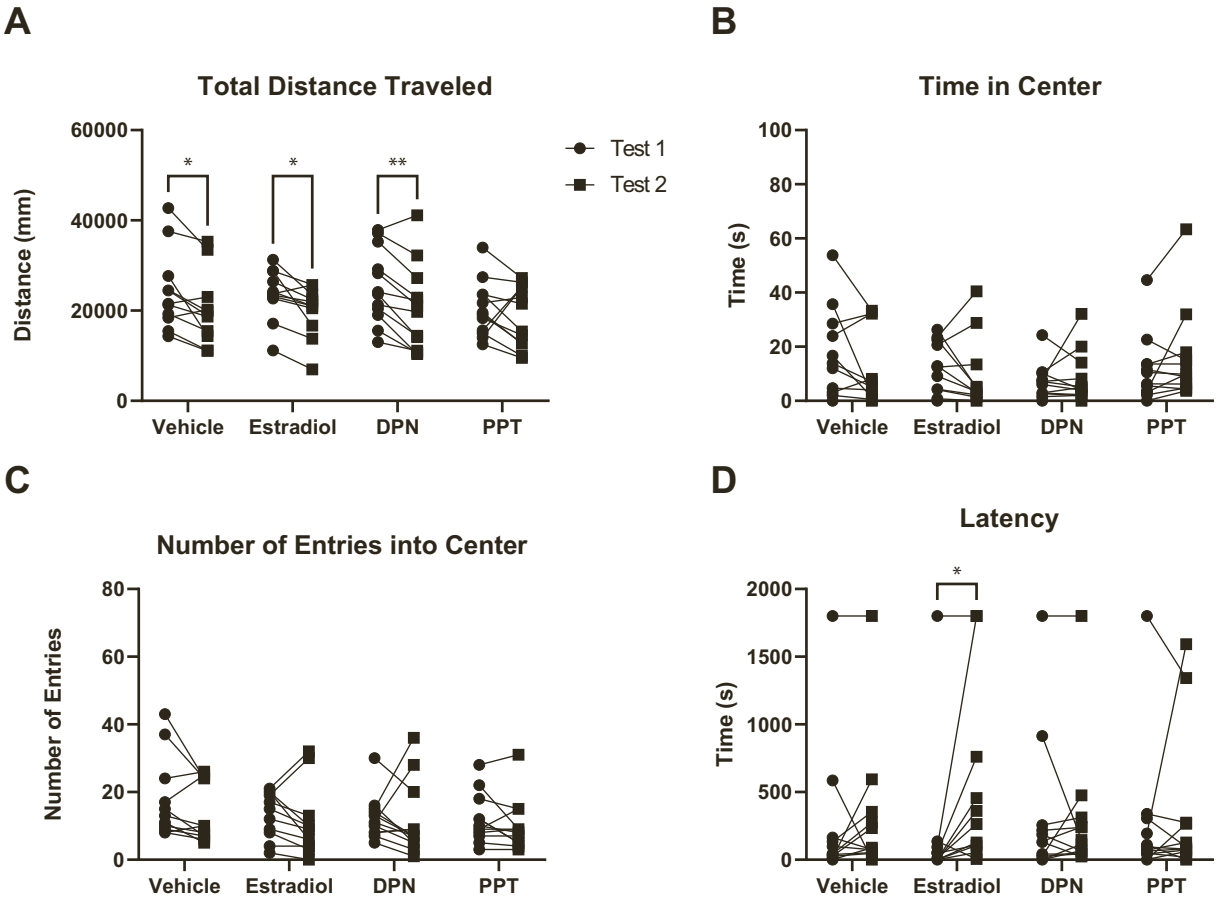


Fig. 6. Experiment 2 analysis of vehicle, estradiol, DPN, and PPT treatments on locomotor and anxiety-related behaviors in the open field under white light. Test 1 was performed on the first day of injections and test 2 was performed 24 h following the last day of injections. (A) Total distance traveled in the open field within subject between test 1 and test 2. (B) Time spent in the center of the open field within subject between test 1 and test 2. (C) Number of entries into the center of the open field within subject between test 1 and test 2. (D) Latency to enter the center of the open field within subject between test 1 and test 2. Acronyms: * = $p < 0.05$.

behavioral results of these experiments in addition to estradiol's actions on weight. A lack of difference between the estradiol and vehicle groups violates the positive control in the sense that an expected change induced by estradiol in behavior was not found. After identifying no significant differences in behavior between the positive (estradiol) and negative (vehicle) controls, we compared these data groups to those of our previous study that tested estradiol modulation of anxiety-related and locomotor behaviors in adult ovariectomized female rats (Miller et al., 2020). When the difference between the time spent in the center of the open field on day 1 and 4 of injections was compared across experiment 1 of the present study and experiment 1 of our previous study using a two way ANOVA, there was a trending interaction between treatment and experiment (Fig. 9; $p = 0.0575$). A significant treatment effect (Fig. 9; $p = 0.0213$) was present only for the estradiol treatment group from our previous study. This comparison revealed that the present study's vehicle group was slightly *elevated* and the estradiol group slightly *decreased* from the previous study's findings, although neither group differed significantly from our previous study (Vehicle: $p = 0.7707$, Estradiol: $p = 0.1450$). However, this combination of changes in both groups is a clue that *something* is different between these cohorts of animals, perhaps indicating that a differential variable is present between the previous study and the current study. This clue, on top of an exhaustive search to make sure that the actual experimental details were correct including properly mixing the drugs, proper behavioral analysis, etc., indicates the presence of a variable or variables that could be impacting how the animals respond to estradiol and its receptor agonists or differences in experimental methodology.

There are many potential variables that could potentially obscure an effect of estradiol and its receptor agonists. One potential explanation for differences in estradiol sensitivity is contamination due to unintended exposure to estradiol or endocrine disruptors. Regarding estradiol contamination, estradiol and vehicle injections were prepared in the same laboratory, thus one possibility is that estradiol somehow contaminated the vehicle group. However, this source of contamination is unlikely as the vehicle treated group did not display the same changes in weight as the estradiol and PPT groups, that the estradiol and oil injections were always separated, vehicle injections were mixed before handling any estradiol, the use of separate instruments and equipment, and extensive cleaning with ethanol. Endocrine disruptors are another potential source of the violation of positive and negative controls as they are found in many materials, especially plastics. It is possible that exposure to an endocrine disruptor during gestation could alter the assessed behaviors (Patisaul et al., 2009; Rebuli et al., 2015; Gillera et al., 2020). It is unlikely that endocrine disruption occurred after arrival in our animal care facility, due to the implementation of strict diet, bedding, and glass water bottle protocols as described in the methods. Other than endocrine disruption or contamination, perhaps the most salient potential variable is a stressor on the animals (Holder and Blaustein, 2014; Maeng and Milad, 2015), as there are large sex differences and estradiol-interactions with stress behavior (Maeng and Milad, 2015; Rainville et al., 2022). There are at least two recent studies that show that estradiol-related modulation only appeared in the context of a stressor, including one from our own laboratory (Miller et al., 2020; Gargiulo et al., 2022). There are also other studies that indicate that earlier stress can compromise later effects of estradiol (Walf and Frye, 2007). For instance, Blaustein and colleagues and others have

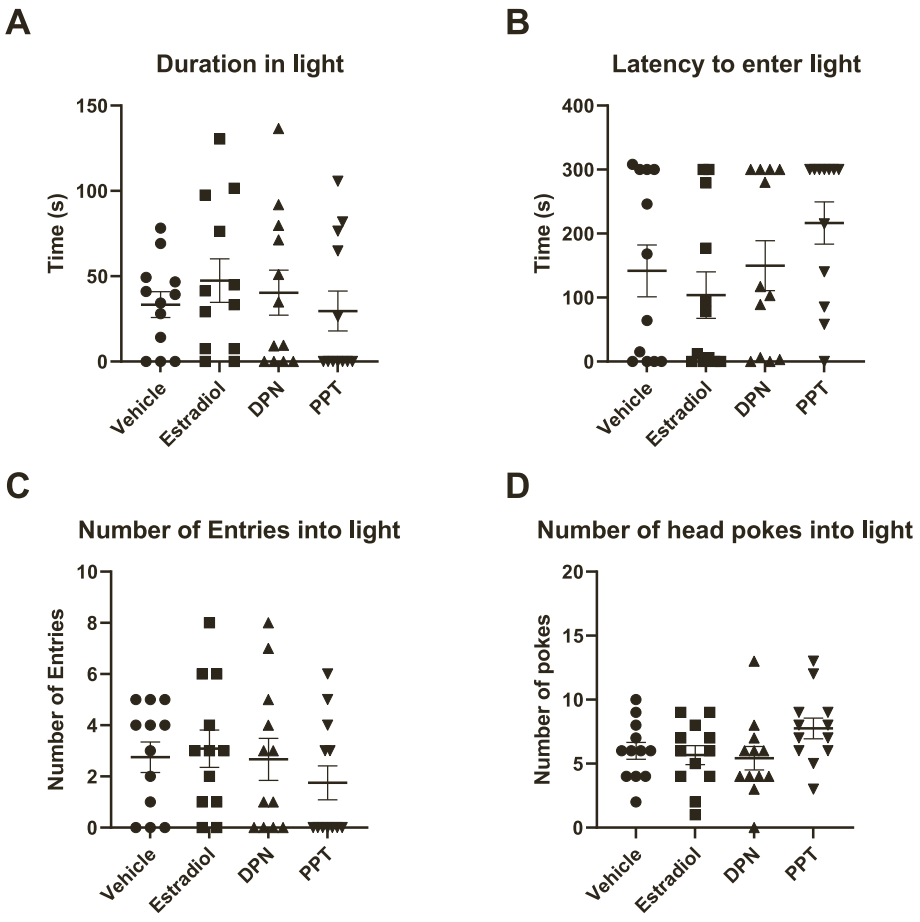


Fig. 7. Experiment 2 analysis of vehicle, estradiol, DPN, and PPT treatments on anxiety-related behaviors in the light dark box. (A) Total full-body duration in the light chamber of the light dark box. (B) Latency to fully enter the light chamber of the light dark box. (C) Number of full entries into the light chamber of the light dark box. (D) Number of head pokes into the light chamber of the light dark box.

documented that shipping stress during critical periods such as gestation and puberty can compromise later effects of estradiol (Holder and Blaustein, 2014). The rats in this study were shipped from a facility in North Carolina around P50 to help avoid this possibility, since P50 is after pubertal initiation. However it is possible that stress still occurred before arrival in our housing facility. What is less clear is why such a stress would matter in the two cohorts of animals used for this study and not in our previous study (Miller et al., 2020). It is also not clear why this particular potential stressor may matter when the original manuscript showing an effect of DPN on mitigating anxiety-like behaviors also had animals shipped during the same general developmental period (Lund et al., 2005).

Our study is not unique in not detecting an estradiol or estrogen receptor effect on non-motivated anxiety-related and locomotor behaviors in various behavioral assessments, and there is considerable variability between ER agonist, dose, and assessment test (Palermo-Neto and Dorce, 1990; Morgan and Pfaff, 2001; Lund et al., 2005; Borrow and Handa, 2017; Gogos et al., 2018). Even experiments investigating rodent behavior across the natural estrous cycle do not always detect changes in anxiety-related behaviors. In the open field, it is sometimes challenging to detect any differences in non-motivated anxiety-related behaviors between different estrous cycle phases (Gogos et al., 2018; Datta et al., 2019; Scholl et al., 2019; Levy et al., 2023). In the elevated plus maze, rodents in the estrus and proestrus phases typically display reduced anxiety-related behaviors compared to diestrus (Mora et al., 1996; Díaz-Véliz et al., 1997; Marcondes et al., 2001; Maeng and Milad, 2015), but even using this test an estrous cycle effect is not always detected (Scholl et al., 2019). Of course, ovariectomy itself can potentially alter anxiety-like behavior and the sensitivity to estradiol and its agonists (Zimmerberg and Farley, 1993). It is important to note that throughout the literature of estradiol and ER modulation of rodent behaviors, experimental parameters can differ significantly between studies. For instance, light cycle phase, the presence or absence of light during behavioral tests, length and type of test, diet, age, and dose of hormone or ER agonist treatment and more can all be sources of variability during testing (Patisaul et al., 2009; Diz-Chaves et al., 2012; Sestakova et al., 2013; Jin et al., 2021). Many publications do not include details about some or all of these variables or behavioral protocols. Indeed, the manuscript upon which our study is based does not describe in great detail how behavioral testing in the open field, elevated plus maze, and light/dark box was conducted and it is possible that our methods differed (Lund et al., 2005). To be fair, this situation is not unique to this particular study, or field and well-meaning scientists struggle on how much detail to report in methods sections. Furthermore, as pointed out in a recent review, “hidden variables” such as cage ventilation systems, social dominance structures, circadian rhythms, and transport stress (discussed further below) of which the working scientists may be unaware could directly impact behavioral studies (Butler-Struben et al., 2022). Overall, the discrepancies in results due to variations in testing methods demonstrate that consistency is extremely important when assessing estradiol's modulation of rodent behavior.

Two examples of inconsistency in the literature are how variables relating to light and novelty are handled (Miller et al., 2020, 2021). It is feasible that these and other variables indirectly or directly modulate behavior in a way that conceals estradiol and ER agonist effects in ovariectomized females. The presence of light is a known stressor to

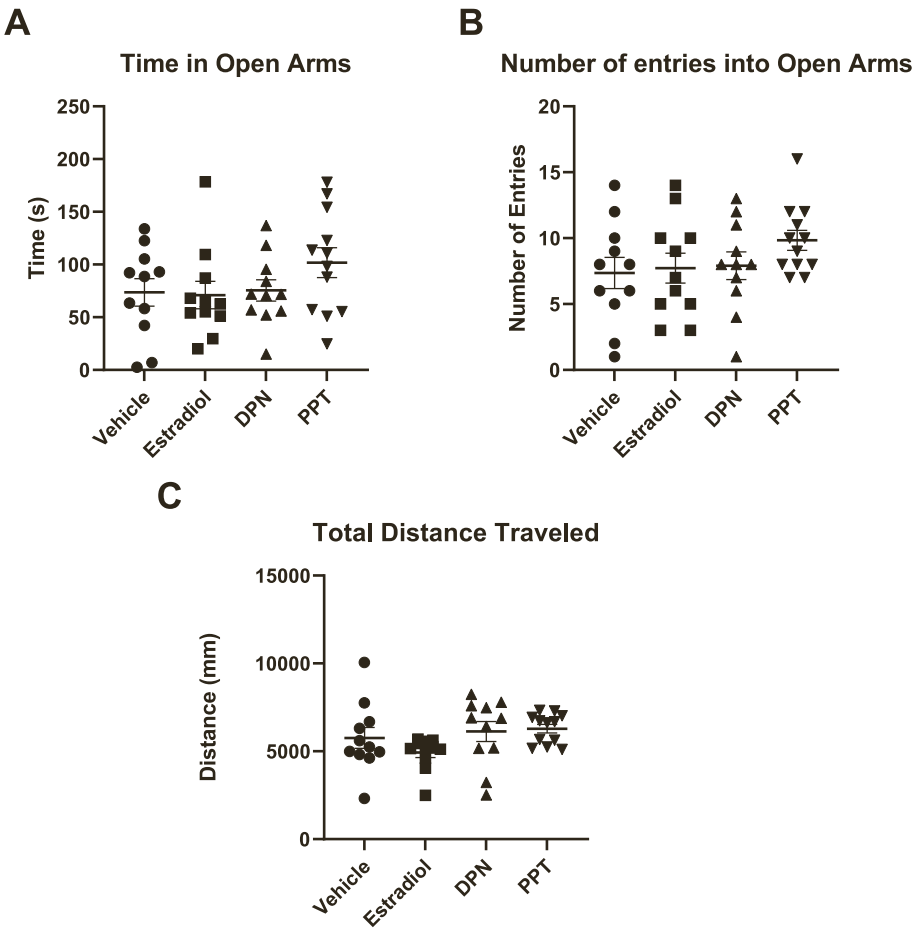


Fig. 8. Experiment 2 analysis of vehicle, estradiol, DPN, and PPT treatments on anxiety-related behaviors in the elevated plus maze under red light. (A) Total duration in the open arms of the elevated plus maze. (B) Number of entries into the open arms of the elevated plus maze. (C) Total distance traveled in the elevated plus maze. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rodents, and the sensitivity of anxiety-related and locomotor behaviors in response to light changes across the estrous cycle and with estradiol treatment (Crawley and Goodwin, 1980; Mora et al., 1996; Claustrat et al., 2008; Sestakova et al., 2013; Datta et al., 2019). For example, some behavioral tests are performed during the light phase while some are performed during the dark phase, and lighting conditions during testing can range from dim white light, intense white light, or dim red light. In our previous study investigating mechanisms of estradiol modulation of behavior in the presence or absence of white light, estradiol effects on anxiety-related behaviors in the open field test were only detected in the presence of white light, suggesting that exogenous estradiol's influence is evident in the presence of an acute stressor but muted in a low-anxiety environment (Miller et al., 2020). This finding of estrous cycle effects in the open field in white light is similar to that in studies of mice (Jaric et al., 2019; Rocks et al., 2022). It is possible that estradiol-effects on anxiety-like behaviors may vary by light intensity, although it is unclear whether there is a minimal threshold of a light stimulus that is sufficient for producing a maximal anxiety-like effect.

Another consideration is novelty of the open field as a significant factor when testing anxiety-related and locomotor behaviors (Miller et al., 2020). Animals might be tested either once or twice in each test, may undergo several different tests during the same day (Datta et al., 2019; Scholl et al., 2019), and testing times within the open field can range from 5, 10, 20 or 30 min (Handa et al., 1993; Prut and Belzung, 2003; Gogos et al., 2018; Levy et al., 2023). To help mitigate this variability we analyzed the first 5 min, the first 10 min, and the entire 30 min spent in the open field. Novelty greatly obscures the effects of the estrous cycle on anxiety-related behaviors, and a shorter length of time in the open field limits the detection of estrous cycle-induced effects (Miller et al., 2021). In our previous study (Miller et al., 2021), the presence of light during the test so robustly increased anxiety-related behaviors that estrous cycle effects and behavioral responses to novelty were reduced. The present experiment accounted for novelty and individual variation in the open field test by performing the test twice to allow for individual comparisons. Novelty was not controlled for when performing the LD box and EPM behavioral tests, which is a generally standard protocol for most laboratories. It is also possible that subjecting rats to the open field immediately before the LD box and EPM complicated results of the final behavioral tests. While studies assessing a battery of behaviors using multiple behavioral tests often perform one test per day (Rock et al., 2019; Gillera et al., 2020), this approach is problematic when assessing a dynamic variable such as estradiol levels or ER activation. A second exposure to the open field followed by subsequent novel tests may have added inadvertent stressors and novelty that hindered the detection of estradiol effects. Additionally, the timing of these behavioral tests following hormone injection differed between our current and previous study. Previously, open field testing occurred 1.5 h following the hormone injection (Miller et al., 2020). To incorporate all behavioral tests within the first 3 h of the dark phase, open field testing in the present study occurred only 30 min after injections. It is possible that behavioral testing occurred too soon after the rats received injections; however, this timing was used successfully in another study (Lund et al., 2005) so it is unlikely that this alone was a substantial issue. It is possible that the combination of using three behavioral tests on each rat and the early initiation of testing after injections introduced unpredictable and extraneous variables that

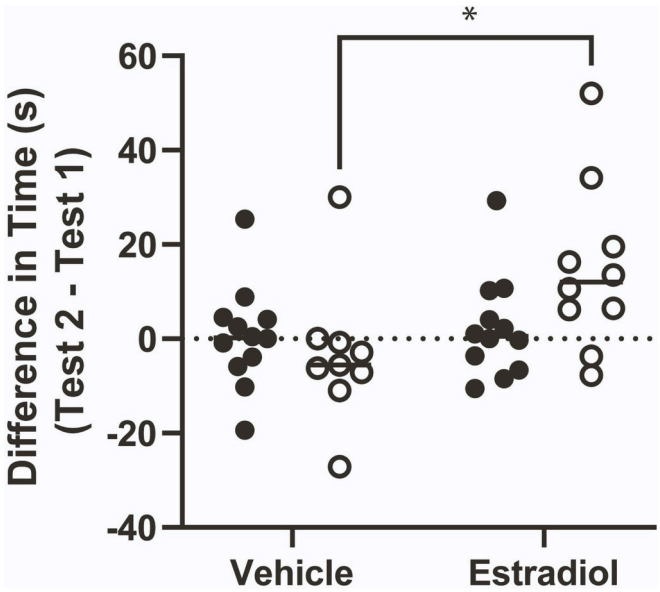


Fig. 9. Data from experiment 1 of the current study compared to the data from experiment 1 of Miller et al. (2020). Black dots represent data from the current study, and white dots represent data re-plotted from Miller et al. (2020). Data is presented as the difference in the time spent in the center of the open field from test 2 to test 1. There was a significant difference between vehicle and estradiol groups from rats in Miller et al. (2020) that were tested in the open field under bright light. However, in experiment 1 of the current study, rats treated with estradiol did not show any differences compared to rats treated with vehicle. There were no significant differences between vehicle-treated or estradiol-treated rats when comparing between experiments. Acronyms: * = $p < 0.05$.

participated in the violation of our controls. However, the original manuscript demonstrating that DPN mitigated anxiety-like behaviors performed all three of these tests sequentially as well (Lund et al., 2005). Overall, tests that differ in novelty, length, and lighting will produce varying results that may make reproducibility and comparisons between labs difficult, but on the other hand, differences in methods could also indicate the robustness of an experimental effect. Of course, these are not the only factors to likely produce variability across experimental studies, and further research is needed to understand other factors that contribute to behavioral differences such as light cycle phase, habituation times, test type, species/strain, and housing conditions, among others [8]. We note that our current study employed the same strain of rat as the original study demonstrating that DPN mitigated anxiety-like behaviors (Lund et al., 2005). The current study also employed the same dose of DPN as Lund and colleagues, although it is possible that higher or lower doses of DPN/PPT and estradiol could exert differential effects.

We advise that laboratories take several factors in consideration when designing future experiments. First, to avoid estradiol or endocrine disruptor contamination if possible and avoid possible shipping stress by breeding the rats in house. Second, that the presence, absence, and intensity of light is consistent and documented in the experimental methods. Third, to consider assessing behavior using only one behavioral test per rodent per day to prevent unnecessary interactions of stress and novelty. For the current experiment, a different design incorporating three cohorts of animals to assess behavior in specific apparatus could have been employed, with the caveat that this approach vastly increases experimental expense and logistics. Fourth, it is important when publishing to write detailed methods that report as many protocols and experimental characteristics as possible to allow for reproducibility and accurate expansion of the work, and at this point there are multiple guidelines to assist with documentation, including the ARRIVE system (Percie du Sert et al., 2020a,b). Although we cannot realistically consider every possible factor, perhaps reporting more methodology can lead to new perspectives on our discoveries. In reality, animal behavior is not influenced by one controlled variable at a time but rather an integration of countless variables, making the application of experiments involving hormone action on anxiety-related behaviors challenging and sometimes unpredictable. We view this complex arena as an exciting and worthy challenge for future experiments in which behavioral neuroendocrinologists can make important contributions to understanding how internal and external variables interact to influence animal behavior, taking us back to the foundational experiment of our field. Most behavioral experiments, including our own, that test relevant behaviors aim to assess only one or two specific variables, as incorporating multiple factors within an experimental design becomes complicated. However, in doing so there are often missed opportunities to address the importance of varying environmental interactions, leading to an expanse of divergent literature in which individual studies cannot represent a complete picture. Future experiments, perhaps using artificial intelligence and machine learning assistants, will hopefully augment our ability to examine how multiple factors participate together to help the animal evaluate its internal and external environment and to respond appropriately, demonstrating the importance of considering interactions of external and internal factors to fully understand the complexities of animal behavior. We believe that investigations of the neuroendocrine mechanisms behind behavior are only beginning, as the context by which hormones influence behavior varies significantly according to the environment. If nothing else, the experiments presented here taught our laboratory to recognize that behavioral modulation is a product of an animal's individual circumstance. We look forward to the future experiments conducted by behavioral neuroendocrinologists, allowing the completion of comprehensive models integrating the internal and external factors responsible for animal behavior.

Data availability

Data will be made available on request.

Acknowledgements

C.K.M. designed the experiments, collected data, analyzed data, and contributed to drafts the manuscript. J.M. designed the experiments, analyzed data, and contributed to drafts of the manuscript. We would like to thank the Biological Resources Facilities of North Carolina State University for assistance and animal care resources, David Dorris for technical assistance, Dr. Heather Patisaul for advice regarding behavior experiments and analysis, and Drs. Kurt Marsden, Russell Borski, Miles Engell, and Sabrina Robertson for encouraging publication.

Funding statement

This work was supported by the following funding sources: National Institutes of Health (NIH) Grants R01 MH-109471 (J.M.) and P30 ES-025128 (Center for Human Health and the Environment).

References

Borrow, A.P., Handa, R.J., 2017. Estrogen receptors modulation of anxiety-like behavior. Vitam. Horm. 103, 27–52.

Bowen, R.S., Knab, A.M., Hamilton, A.T., McCall, J.R., Moore-Harrison, T.L., Lightfoot, J. T., 2012. Effects of supraphysiological doses of sex steroids on wheel running activity in mice. J. Steroids Horm Sci. 3 (2), 110.

Bryzgalova, G., Gao, H., Ahren, B., Zierath, J.R., Galuska, D., Steiler, T.L., Dahlman-Wright, K., Nilsson, S., Gustafsson, J.A., Efendic, S., Khan, A., 2006. Evidence that oestrogen receptor-alpha plays an important role in the regulation of glucose homeostasis in mice: insulin sensitivity in the liver. Diabetologia 49 (3), 588–597.

Butler-Struben, H.M., Kentner, A.C., Trainor, B.C., 2022. What's wrong with my experiment?: the impact of hidden variables on neuropsychopharmacology research. Neuropsychopharmacology 47 (7), 1285–1291.

Byrnes, E.M., Casey, K., Bridges, R.S., 2012. Reproductive experience modifies the effects of estrogen receptor alpha activity on anxiety-like behavior and corticotropin releasing hormone mRNA expression. Horm. Behav. 61 (1), 44–49.
Byrnes, E.M., Casey, K., Carini, L.M., Bridges, R.S., 2013. Reproductive experience alters neural and behavioural responses to acute oestrogen receptor alpha activation. J. Neuroendocrinol. 25 (12), 1280–1289.
Cameron, O.G., Kuttesch, D., McPhee, K., Curtis, G.C., 1988. Menstrual fluctuation in the symptoms of panic anxiety. J. Affect. Disord. 15 (2), 169–174.
Campbell, S., Whitehead, M., 1977. Oestrogen therapy and the menopausal syndrome. Clin. Obstet. Gynaecol. 4 (1), 31–47.
Claustrat, B., Valatx, J.L., Harthe, C., Brun, J., 2008. Effect of constant light on prolactin and corticosterone rhythms evaluated using a noninvasive urine sampling protocol in the rat. Horm. Metab. Res. 40 (6), 398–403.
Cohen, L.S., Soares, C.N., Poitras, J.R., Prouty, J., Alexander, A.B., Shifren, J.L., 2003. Short-term use of estradiol for depression in perimenopausal and postmenopausal women: a preliminary report. Am. J. Psychiatry 160 (8), 1519–1522.
Crawley, J., Goodwin, F.K., 1980. Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. Pharmacol. Biochem. Behav. 13 (2), 167–170.
Datta, S., Samanta, D., Tiwary, B., Chaudhuri, A.G., Chakrabarti, N., 2019. Sex and estrous cycle dependent changes in locomotor activity, anxiety and memory performance in aged mice after exposure of light at night. Behav. Brain Res. 365, 198–209.
Díaz-Véliz, G., Alarcón, T., Espinoza, C., Dussaubat, N., Mora, S., 1997. Ketanserin and anxiety levels: influence of gender, estrous cycle, ovariectomy and ovarian hormones in female rats. Pharmacol. Biochem. Behav. 58 (3), 637–642.
Diz-Chaves, Y., Kwiatkowska-Naqvi, A., Von Hülst, H., Pernía, O., Carrero, P., Garcia-Segura, L.M., 2012. Behavioral effects of estradiol therapy in ovariectomized rats depend on the age when the treatment is initiated. Exp. Gerontol. 47 (1), 93–99.
Gargiulo, A.T., Hu, J., Ravaglia, I.C., Hawks, A., Li, X., Sweasy, K., Grafe, L., 2022. Sex differences in cognitive flexibility are driven by the estrous cycle and stress-dependent. Front. Behav. Neurosci. 16, 958301.
Gibbs, R.B., 1997. Effects of estrogen on basal forebrain cholinergic neurons vary as a function of dose and duration of treatment. Brain Res. 757 (1), 10–16.
Gillera, S.E.A., Marinello, W.P., Horman, B.M., Phillips, A.L., Ruis, M.T., Stapleton, H.M., Reif, D.M., Patisaul, H.B., 2020. Sex-specific effects of perinatal FireMaster(R) 550 (FM 550) exposure on socioemotional behavior in prairie voles. Neurotoxicol. Teratol. 79, 106840.
Gogos, A., McCarthy, M., Walker, A.J., Udawela, M., Gibbons, A., Dean, B., Kusljic, S., 2018. Differential effects of chronic 17beta-oestradiol treatment on rat behaviours relevant to depression. J. Neuroendocrinol. 30 (11), e12652.
Graham, B.M., Scott, E., 2018. Effects of systemic estradiol on fear extinction in female rats are dependent on interactions between dose, estrous phase, and endogenous estradiol levels. Horm. Behav. 97, 67–74.
Handa, R.J., Cross, M.K., George, M., Gordon, B.H., Burgess, L.H., Cabrera, T.M., Hata, N., Campbell, D.B., Lorens, S.A., 1993. Neuroendocrine and neurochemical responses to novelty stress in young and old male F344 rats: effects of d-fenfluramine treatment. Pharmacol. Biochem. Behav. 46 (1), 101–109.
Hertrampf, T., Seibel, J., Laudenbach, U., Fritzemeier, K.H., Diel, P., 2008. Analysis of the effects of oestrogen receptor alpha (ERalpha)- and ERbeta-selective ligands given in combination to ovariectomized rats. Br. J. Pharmacol. 153 (7), 1432–1437.
Holder, M.K., Blaustein, J.D., 2014. Puberty and adolescence as a time of vulnerability to stressors that alter neurobehavioral processes. Front. Neuroendocrinol. 35 (1), 89–110.
Imwalle, D.B., Gustafsson, J.A., Rissman, E.F., 2005. Lack of functional estrogen receptor beta influences anxiety behavior and serotonin content in female mice. Physiol. Behav. 84 (1), 157–163.
Jacome, L.F., Gautreaux, C., Inagaki, T., Mohan, G., Alves, S., Lubbers, L.S., Luine, V., 2010. Estradiol and ERβ agonists enhance recognition memory, and DPN, an ERβ agonist, alters brain monoamines. Neurobiol. Learn. Mem. 94 (4), 488–498.
Jaric, I., Rocks, D., Greally, J.M., Suzuki, M., Kundakovic, M., 2019. Chromatin organization in the female mouse brain fluctuates across the oestrous cycle. Nat. Commun. 10 (1), 2851.
Jin, X., Zhang, Y., Celniker, S.E., Xia, Y., Mao, J.H., Snijders, A.M., Chang, H., 2021. Gut microbiome partially mediates and coordinates the effects of genetics on anxiety-like behavior in collaborative cross mice. Sci. Rep. 11 (1), 270.
Krentzel, A.A., Proano, S., Patisaul, H.B., Meitzen, J., 2020. Temporal and bidirectional influences of estradiol on voluntary wheel running in adult female and male rats. Horm. Behav. 120, 104694.
Levy, D.R., Hunter, N., Lin, S., Robinson, E.M., Gillis, W., Conlin, E.B., Anyoha, R., Shansky, R.M., Datta, S.R., 2023. Mouse spontaneous behavior reflects individual variation rather than estrous state. Curr. Biol. 33 (7), 1358–1364.e4.
Lund, T.D., Rovis, T., Chung, W.C., Handa, R.J., 2005. Novel actions of estrogen receptor-beta on anxiety-related behaviors. Endocrinology 146 (2), 797–807.
Maeng, L.Y., Milad, M.R., 2015. Sex differences in anxiety disorders: interactions between fear, stress, and gonadal hormones. Horm. Behav. 76, 106–117.
Mani, S.K., Reyna, A.M., Alejandro, M.A., Crowley, J., Markaverich, B.M., 2005. Disruption of male sexual behavior in rats by tetrahydrofurandiols (THF-diols). Steroids 70 (11), 750–754.
Marcondes, F.K., Miguel, K.J., Melo, L.L., Spadari-Bratfisch, R.C., 2001. Estrous cycle influences the response of female rats in the elevated plus-maze test. Physiol. Behav. 74 (4–5), 435–440.
Markaverich, B., Mani, S., Alejandro, M.A., Mitchell, A., Markaverich, D., Brown, T., Velez-Trippe, C., Murchison, C., O'Malley, B., Faith, R., 2002. A novel endocrine-disrupting agent in corn with mitogenic activity in human breast and prostatic cancer cells. Environ. Health Perspect. 110 (2), 169–177.
Miller, C.K., Halbing, A.A., Patisaul, H.B., Meitzen, J., 2021. Interactions of the estrous cycle, novelty, and light on female and male rat open field locomotor and anxiety-related behaviors. Physiol. Behav. 228, 113203.
Miller, C.K., Krentzel, A.A., Patisaul, H.B., Meitzen, J., 2020. Metabotropic glutamate receptor subtype 5 (mGlu5) is necessary for estradiol mitigation of light-induced anxiety behavior in female rats. Physiol. Behav. 214, 112770.
Montgomery, K.C., 1955. The relation between fear induced by novel stimulation and exploratory behavior. J. Comp. Physiol. Psychol. 48 (4), 254–260.
Mora, S., Dussaubat, N., Díaz-Véliz, G., 1996. Effects of the estrous cycle and ovarian hormones on behavioral indices of anxiety in female rats. Psychoneuroendocrinology 21 (7), 609–620.
Morgan, M.A., Pfaff, D.W., 2001. Effects of estrogen on activity and fear-related behaviors in mice. Horm. Behav. 40 (4), 472–482.
Ogawa, S., Chan, J., Gustafsson, J.A., Korach, K.S., Pfaff, D.W., 2003. Estrogen increases locomotor activity in mice through estrogen receptor alpha: specificity for the type of activity. Endocrinology 144 (1), 230–239.
Oyola, M.G., Portillo, W., Reyna, A., Foradori, C.D., Kudwa, A., Hinds, L., Handa, R.J., Mani, S.K., 2012. Anxiolytic effects and neuroanatomical targets of estrogen receptor-beta (ERbeta) activation by a selective ERbeta agonist in female mice. Endocrinology 153 (2), 837–846.
Palermo-Neto, J., Dorce, V.A., 1990. Influences of estrogen and/or progesterone on some dopamine related behavior in rats. Gen. Pharmacol. 21 (1), 83–87.
Patisaul, H.B., Burke, K.T., Hinkle, R.E., Adewale, H.B., Shea, D., 2009. Systemic administration of diarylpropionitrile (DPN) or phytoestrogens does not affect anxiety-related behaviors in gonadally intact male rats. Horm. Behav. 55 (2), 319–328.
Percie du Sert, N., Ahluwalia, A., Alam, S., Avey, M.T., Baker, M., Browne, W.J., Clark, A., Cuthill, I.C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S.T., Howells, D.W., Hurst, V., Karp, N.A., Lazic, S.E., Lidster, K., MacCallum, C.J., Macleod, M., Pearl, E.J., Petersen, O.H., Rawle, F., Reynolds, P., Rooney, K., Sena, E. S., Silberberg, S.D., Steckler, T., Würbel, H., 2020. Reporting animal research: explanation and elaboration for the ARRIVE guidelines 2.0. PLoS Biol. 18 (7), e3000411.
Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M.T., Baker, M., Browne, W. J., Clark, A., Cuthill, I.C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S.T., Howells, D.W., Karp, N.A., Lazic, S.E., Lidster, K., MacCallum, C.J., Macleod, M., Pearl, E.J., Petersen, O.H., Rawle, F., Reynolds, P., Rooney, K., Sena, E.S., Silberberg, S.D., Steckler, T., Würbel, H., 2020. The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. PLoS Biol. 18 (7), e3000410.
Pestana, J.E., Islam, N., Van der Eyk, N.L., Graham, B.M., 2022. What pre-clinical rat models can tell us about anxiety across the menstrual cycle in healthy and clinically anxious humans. Curr. Psychiatry Rep. 24 (11), 697–707.
Peterson, B.M., Mermelstein, P.G., Meisel, R.L., 2015. Estradiol mediates dendritic spine plasticity in the nucleus accumbens core through activation of mGluR5. Brain Struct. Funct. 220 (4), 2415–2422.
Proano, S.B., Krentzel, A.A., Meitzen, J., 2020. Differential and synergistic roles of 17β-estradiol and progesterone in modulating adult female rat nucleus accumbens core medium spiny neuron electrophysiology. J. Neurophysiol. 123 (6), 2390–2405.
Prut, L., Belzung, C., 2003. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur. J. Pharmacol. 463 (1–3), 3–33.
Rainville, J.R., Lipuma, T., Hodes, G.E., 2022. Translating the transcriptome: sex differences in the mechanisms of depression and stress, revisited. Biol. Psychiatry 91 (1), 25–35.
Rebuli, M.E., Camacho, L., Adonay, M.E., Reif, D.M., Aylor, D.L., Patisaul, H.B., 2015. Impact of low-dose Oral exposure to bisphenol a (BPA) on juvenile and adult rat exploratory and anxiety behavior: a CLARITY-BPA consortium study. Toxicol. Sci. 148 (2), 341–354.
Rock, K.D., Gillera, S.E.A., Devarasetty, P., Horman, B., Knudsen, G., Birnbaum, L.S., Fenton, S.E., Patisaul, H.B., 2019. Sex-specific behavioral effects following developmental exposure to tetrabromobisphenol a (TBBPA) in wistar rats. Neurotoxicology 75, 136–147.
Rocks, D., Cham, H., Kundakovic, M., 2022. Why the estrous cycle matters for neuroscience. Biol. Sex Differ. 13 (1), 62.
Rodier III, W.I., 1971. Progesterone-estrogen interactions in the control of activity-wheel running in the female rat. J. Comp. Physiol. Psychol. 74 (3), 365–373.
Roesch, D.M., 2006. Effects of selective estrogen receptor agonists on food intake and body weight gain in rats. Physiol. Behav. 87 (1), 39–44.
Scholl, J.L., Afzal, A., Fox, L.C., Watt, M.J., Forster, G.L., 2019. Sex differences in anxiety-like behaviors in rats. Physiol. Behav. 211, 112670.
Sestakova, N., Puzserova, A., Kluknavsky, M., Bernatova, I., 2013. Determination of motor activity and anxiety-related behaviour in rodents: methodological aspects and role of nitric oxide. Interdiscip. Toxicol. 6 (3), 126–135.
Spiteri, T., Musatov, S., Ogawa, S., Ribeiro, A., Pfaff, D.W., Agmo, A., 2010. The role of the estrogen receptor alpha in the medial amygdala and ventromedial nucleus of the hypothalamus in social recognition, anxiety and aggression. Behav. Brain Res. 210 (2), 211–220.
Spiteri, T., Ogawa, S., Musatov, S., Pfaff, D.W., Agmo, A., 2012. The role of the estrogen receptor alpha in the medial preoptic area in sexual incentive motivation, proceptivity and receptivity, anxiety, and wheel running in female rats. Behav. Brain Res. 230 (1), 11–20.
Thompson, R.F., Spencer, W.A., 1966. Habituation: a model phenomenon for the study of neuronal substrates of behavior. Psychol. Rev. 73 (1), 16–43.
Viau, V., Meaney, M.J., 1991. Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. Endocrinology 129 (5), 2503–2511.
Villalon Landeros, R., Morisseau, C., Yoo, H.J., Fu, S.H., Hammock, B.D., Trainor, B.C., 2012. Corncob bedding alters the effects of estrogens on aggressive behavior and

reduces estrogen receptor-alpha expression in the brain. Endocrinology 153 (2), 949–953.
Walf, A.A., Frye, C.A., 2005. Antianxiety and antidepressive behavior produced by physiological estradiol regimen may be modulated by hypothalamic-pituitary-adrenal axis activity. Neuropsychopharmacology 30 (7), 1288–1301.
Walf, A.A., Frye, C.A., 2005. ERbeta-selective estrogen receptor modulators produce antianxiety behavior when administered systemically to ovariectomized rats. Neuropsychopharmacology 30 (9), 1598–1609.
Walf, A.A., Frye, C.A., 2007. Estradiol decreases anxiety behavior and enhances inhibitory avoidance and gestational stress produces opposite effects. Stress 10 (3), 251–260.
Walf, A.A., Koonce, C.J., Frye, C.A., 2008. Estradiol or diarylpropionitrile decrease anxiety-like behavior of wildtype, but not estrogen receptor beta knockout, mice. Behav. Neurosci. 122 (5), 974–981.
Wiersielis, K., Yasrebi, A., Ramirez, P., Verpeut, J., Regan, D., Roepke, T.A., 2021. The influence of estrogen receptor α signaling independent of the estrogen response element on avoidance behavior, social interactions, and palatable ingestive behavior in female mice. Horm. Behav. 136, 105084.
Xu, P., Cao, X., He, Y., Zhu, L., Yang, Y., Saito, K., Wang, C., Yan, X., Hinton Jr., A.O., Zou, F., Ding, H., Xia, Y., Yan, C., Shu, G., Wu, S.P., Yang, B., Feng, Y., Clegg, D.J., DeMarchi, R., Khan, S.A., Tsai, S.Y., DeMayo, F.J., Wu, Q., Tong, Q., Xu, Y., 2015. Estrogen receptor-alpha in medial amygdala neurons regulates body weight. J. Clin. Invest. 125 (7), 2861–2876.
Yonkers, K.A., O'Brien, P.M., Eriksson, E., 2008. Premenstrual syndrome. Lancet 371 (9619), 1200–1210.
Zimmerberg, B., Farley, M.J., 1993. Sex differences in anxiety behavior in rats: role of gonadal hormones. Physiol. Behav. 54 (6), 1119–1124.