Published in final edited form as:

Physiol Behav. 2020 February 01; 214: 112770. doi:10.1016/j.physbeh.2019.112770.

Metabotropic glutamate receptor subtype 5 ($mGlu_5$) is necessary for estradiol mitigation of light-induced anxiety behavior in female rats

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Abstract

Anxiety-related behaviors are influenced by steroid hormones such as 17β-estradiol and environmental stimuli such as acute stressors. For example, rats exhibit increased anxiety-related behaviors in the presence, but not the absence, of light. In females, estradiol potentially mitigates these effects. Experiments across behavioral paradigms and brain regions indicate that estradiol action can be mediated via activation of metabotropic glutamate receptors, including Group I subtype five ($mGlu_5$). $mGlu_5$ has been implicated in mediating estradiol's effects upon psychostimulant-induced behaviors, dopamine release and neuron phenotype in striatal regions. Whether estradiol activation of $mGlu_5$ modulates anxiety or locomotor behavior in the absence of psychostimulants is unknown. Here we test if $mGlu_5$ is necessary for estradiol mitigation of light-induced acute anxiety and locomotor behaviors. Ovariectomized adult female rats were pretreated with either the $mGlu_5$ antagonist MPEP or vehicle before estradiol or oil treatment. Anxiety and locomotor behaviors were assessed in the presence or absence of white light to induce high and low acute anxiety behavior phenotypes, respectively. In the presence of white light, estradiol treatment mitigated light-induced anxiety-related behaviors but not overall locomotor activity. MPEP treatment blocked estradiol effects upon light-induced anxiety-related behaviors but did not affect overall locomotor activity. In the absence of white light, estradiol or MPEP treatment did not influence anxiety-related behaviors or locomotor activity, consistent with a low anxiety phenotype. These novel findings indicate that $mGlu_5$ activation is necessary for estradiol mitigation of anxiety-related behaviors induced by an acute stressor.

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Keywords

Estradiol; Metabotropic glutamate receptor 5; female; anxiety; estrogen

1. Introduction

Sex steroid hormones impact a variety of behaviors in adult humans and rodents, including those related to anxiety and locomotion [1-3]. In humans, one of these hormones, 17β-estradiol (estradiol), has been implicated as a modulator of many behaviors, including those related to anxiety [4, 5].Indeed, women are much more likely to exhibit anxiety and depression-related disorders than men [6] demonstrating the importance of investigating hormone action in this context. Sex steroid hormones in females play an important role in locomotor behaviors and disorders, including estrogens such as 17β-estradiol [7-9]. Estrogen levels fluctuate naturally in females during the menstrual cycle and play differential roles depending on developmental period[10], presenting a potentially complex influence on behavioral phenotype [5, 11]. In adulthood, decreases in estradiol levels usually associate with increased susceptibility to anxiety-related behaviors, including within the context of the menstrual cycle, menopause, and hysterectomy [12]. Female rodents exhibit similar sex steroid hormone influenced anxiety and locomotor behaviors. For example, in female rats, higher levels of estradiol can increase locomotor but decrease anxiety-related behaviors [2, 13-15]. This phenomenon generates opportunity for exploration of the mechanisms by which estradiol influences these behaviors in females.

Estradiol potentially influences anxiety-related behaviors via multiple receptor mechanisms. Estradiol binds to estrogen receptors (ERs) including the classical nuclear localized ERα and ERβ, membrane-associated ERα and ERβ, and the plasma membrane localized receptors GPER-1 and Gq-mER [16-19]. Membrane-associated ERα and ERβ can modulate neuron function and animal behavior via several different mechanisms, including stimulating metabotropic glutamate receptors (mGlu) in the absence of glutamate [20]. Stimulation of mGlu induces intracellular signaling cascades which in turn modulate neuronal electrical, morphological, and molecular phenotypes, including changes in gene expression [21]. One receptor in particular, Group 1 metabotropic glutamate receptor subtype 5 ($mGlu_5$), is a key player in estradiol-modulation of neuron phenotype and psychostimulant-induced behaviors associated with the nucleus accumbens [16, 22-24], a sexually differentiated brain region highly sensitive to stress and estradiol [25-31]. However, the role of $mGlu_5$ alone in estradiol-modulation of anxiety or locomotor behavior has not been investigated in the absence of psychostimulants. It is unknown whether estradiol activates $mGlu_5$ to modulate anxiety and locomotor behaviors both outside the context of psychostimulants and in response to an acute stressor. This is a critical knowledge gap because anxiety disorders are more prevalent in females than males and are not necessarily comorbid with drug addiction.

To address this omission, we tested the hypothesis that $mGlu_5$ is necessary for estradiol mitigation of light-induced acute anxiety and locomotor behaviors. In nocturnal rodents such as rats, the presence of white light induces anxiety-associated behaviors as demonstrated through a variety of experimental methodologies that employed illumination to create

aversive, vulnerable locations [32, 33]. In the context of this acute stressor, we performed two experiments employing different groups of rats. In both experiments, we ovariectomized adult female rats and exposed them to either the $mGlu_5$ antagonist 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) or saline before estradiol or oil treatment for four consecutive days. On day one and day four, anxiety and locomotor behaviors were assessed using the open field test. In experiment one, behavior was assessed in the presence of white light, acting as an acute stressor. In experiment two, behavior was assessed in the absence of white light to control for the influence of an acute stressor.

2. Material 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=64) from Charles River Laboratories and were single-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 [34-36]. 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 hour light:dark cycle with lights turning off at 9:00 am. At P60±1, rats were anesthetized using isoflurane and ovariectomized. Behavioral testing occurred two weeks post-gonadectomy, and rats were handled daily beginning one week before injections and behavioral testing.

2.2 Drug and Hormone Exposure

For each experiment, rats were divided into four treatment groups, adapted from a previously published protocol [23, 37]. These four groups of rats received injections consisting of: saline and sesame oil, saline and 17β-estradiol benzoate (Estradiol; Sigma-Aldrich, St. Louis, MO), the $mGlu_5$ antagonist MPEP (2-Methyl-6-(phenylethynyl)pyridine hydrochloride; Tocris Biosciences, Minneapolis, MN) and oil, or MPEP and estradiol. MPEP concentration was 1mg/kg/ml dissolved in saline and 5% dimethylsulfoxide (DMSO), following previous studies [23]. Estradiol concentration was 5μg/0.1ml dissolved in sesame oil and 5% DMSO [37]. Saline and sesame oil control injections likewise each contained 5% DMSO. Injections were given over 4 consecutive days between 7:30 and 9:00 am (Figure 1). Rats were weighed and then received an i.p. injection of either saline or MPEP. Thirty minutes after this initial injection, rats received a subcutaneous injection of either sesame oil or estradiol. Behavioral testing occurred two hours after the initial injection of saline or MPEP on the first and fourth day of injections.

2.3 Behavioral Testing and Data Analysis

All behavioral testing occurred within the first two hours of the animal's dark cycle. For experiment 1, behavioral testing of 40 rats was conducted under white light (250±10 lux) with equal illumination across the open field arena. For experiment 2 in a separate group of 24 rats, behavioral testing was conducted under red light (0.5±0.5 lux). Rats were

individually placed into an open field arena (60cm X 60cm X 60cm; Cleversys Inc, Reston, VA). Activity was recorded for 30 minutes with a video camera located above the open field. Following each test, the open field was thoroughly cleaned with 70% isopropyl alcohol. Locomotion was determined by measuring the total distance traveled in the open field and anxiety behaviors were evaluated using the time spent in the center of the open field, latency to enter the center, and the number of entries into the center. For statistical analysis, rats that did not enter the center were assigned a latency of 1800 seconds, which is the total duration of the test. Rats exhibiting less than 5000 mm of measured total distance traveled in either behavioral assay were excluded from behavioral analysis (n=10, Saline and Oil n=1, MPEP and Oil n=4, Saline and Estradiol n=1, MPEP and Estradiol n=4). All activities were analyzed blind to treatment using TopScan software version 3.0 (Cleversys Inc., Reston, VA), and compared between Day 1 and 4 of testing. This two test experimental design thus incorporates an important control for the *a priori* expected habituation to the testing environment due to repeated exposure to the open field arena [38], as well as for the complex behavioral effects of novelty to the open field arena [39]. This design also allows for the assessment of treatment effects within an individual subject.

2.4 Statistical Analysis

Experimental data was analyzed in SPSS version 26 (IBM, Armonk, NY), Graphpad Prism version 8 (La Jolla, CA), or GPower version 3.1 (Universitat Kiel, Germany). Following a previous protocol of a similar experimental design [40], behavioral data was first examined using a mixed-design factorial ANOVA assessing the effects of test day (first or second behavioral test), hormone (Estradiol or oil), and drug (MPEP or saline). If no three-way interaction was detected, two-way interactions were further decomposed to test for the effects of treatment at each testing day using a two-way repeated measures and non-repeated measures ANOVA and Holm-Sidak's multiple comparisons test. Weight data was analyzed using a two-way ANOVA. Hedge's g effect size values were also calculated, as was achieved power (1-β error probability). *P* values less than 0.05 were *a priori* considered significant.

3. Results

3.1 Experiment 1: Presence of White Light

3.1.1 Weight Difference—Estradiol exposure inhibits weight gain in ovariectomized female rats [41], which is not attenuated by MPEP exposure [23]. Thus, as a positive control for the efficacy of our injection paradigm, we analyzed differences in weight between days four and one of injections. Rats exposed to estradiol demonstrated attenuated weight gain compared to rats not exposed to estradiol, and this effect was not blocked by MPEP exposure (Figure 2; Interaction: $F_{(1,36)}$=0.166, p=0.686; Hormone $F_{(1,36)}$=67.60, p<0.001; Drug $F_{(1,36)}$=0.243, p=0.625). This finding indicates that estradiol injections were effective.

3.1.2 Anxiety-related Behaviors—Estradiol increased the time spent in the center of the open field arena and exposure to MPEP blocked estradiol's effect (Figure 3A). This was shown through a significant increase in time spent in the center from day one to day four for rats treated with saline and estradiol but not any other group (Mixed-design ANOVA: No Day x Hormone x Drug interaction detected: $F_{(1,30)}$=1.478, p=0.234; Two-way RM

ANOVA: Treatment x Day: $F_{(3,30)}$=3.993, p=0.017, Power=0.9999; Treatment: $F_{(3,30)}$=1.330, p=0.283; Day: $F_{(1,30)}$=0.0002, p=0.988; Subject: $F_{(30,30)}$=3.626, p<0.001; Saline and Estradiol: $t_{(30)}$=3.008, p=0.021, g= −0.579). The increase in time spent in the center by rats treated with saline and estradiol is likewise evident when the data is instead analyzed as the change between the two test days and compared across groups (Figure 3B). This increase in time in the center by rats treated with saline and estradiol reached significance when compared to rats treated with MPEP and oil (Interaction: $F_{(1,30)}$=1.455, p=0.237; Hormone: $F_{(1,30)}$=4.647, p=0.039; Drug: $F_{(1,30)}$=4.502, p=0.042; MPEP and Oil x Saline and Estradiol: $t_{(30)}$=3.121, p=0.024, g=1.511).

Exposure to estradiol decreased the latency to enter the center, and estradiol's effects were attenuated by MPEP (Figure 3C). Latency significantly increased between day one and day four for rats treated with saline and oil, but not for any other group (Mixed-design ANOVA: No Day x Hormone x Drug interaction detected: $F_{(1,30)}$=3.145, p=0.086; Two-way RM ANOVA: Treatment x Day: $F_{(3,30)}$=3.082, p=0.042, Power=0.9998; Treatment: $F_{(3,30)}$=2.758, p=0.06; Day: $F_{(1,30)}$=6.142, p=0.019; Subject: $F_{(30,30)}$=0.995, p=0.505; Saline and Oil: $t_{(30)}$=3.322, p=0.009, g=−1.27). When further analyzing this data as the change between the two test days and compared across groups, similar results were seen (Figure 3D). Interestingly, rats treated with saline and estradiol showed an overall decrease in latency, which differed from rats treated with saline and oil (Interaction: $F_{(1,30)}$=3.145, p=0.086; Hormone: $F_{(1,30)}$=4.99, p=0.033; Drug: $F_{(1,30)}$=0.007, p=0.932; Saline and Oil x Saline and Estradiol: $t_{(30)}$=3.018, p=0.031, g=1.365). Estradiol's action on decreasing latency to enter the center was attenuated by exposure to MPEP, given that rats treated with MPEP and estradiol did not differ from rats treated with saline and oil or saline and estradiol (MPEP and Estradiol x Saline and Oil: $t_{(30)}$=1.475, p=0.48, g=0.764; MPEP and Estradiol x Saline and Estradiol: $t_{(30)}$=1.305, p=0.491, g=0.723).

Rats treated with saline and estradiol exhibited an increased number of entries into the center from day one to day four, and this effect was blocked by exposure to MPEP (Figure 3E). This was demonstrated by a significant decrease for rats treated with MPEP and estradiol between days one and four of treatment which was not exhibited by rats in any other treatment group (Mixed-design ANOVA: No Day x Hormone x Drug interaction detected: $F_{(1,30)}$=3.666, p=0.065; Two-way RM ANOVA: Treatment x Day: $F_{(3,30)}$=4.916, p=0.007, Power=0.9999; Treatment: $F_{(3,30)}$=1.460, p=0.245; Day: $F_{(1,30)}$=7.352, p=0.011; Subject: $F_{(30,30)}$=9.562, p<0.001; MPEP and Estradiol: $t_{(30)}$=2.742, p=0.040, g= 0.762). The effects of MPEP on blocking estradiol's actions were more obvious when the data was analyzed as the change between the two test days and compared across groups (Figure 3F). This analysis found that rats treated with saline and estradiol differed from rats treated with MPEP and estradiol and MPEP and oil (Interaction: $F_{(1,30)}$=3.666, p=0.065; Hormone: $F_{(1,30)}$=2.061, p=0.162; Drug: $F_{(1,30)}$=7.840, p=0.009; Saline and Estradiol x MPEP and Oil: $t_{(30)}$=3.090, p=0.021, g=6.459; Saline and Estradiol x MPEP and Estradiol: $t_{(30)}$=3.311, p=0.015, g=1.395). Overall, these findings indicate that MPEP blocked estradiol's mitigation of anxiety-related behaviors in the presence of an acute stressor, white light.

3.1.3 Overall Locomotor Activity—Total distance traveled in the open field arena was influenced by the day of exposure (Figure 4A). Treatment of either saline and oil or MPEP

and oil resulted in a significant decrease in total distance traveled from day one to day four (Mixed-design ANOVA: No Day x Hormone x Drug interaction detected: $F_{(1,30)}$=0.198, p=0.659; Two-way RM ANOVA: Treatment x Day: $F_{(3,30)}$=1.616, p=0.206, Power=0.9698; Treatment: $F_{(3,30)}$=0.114, p=0.951; Day: $F_{(1,30)}$=15.68, p=0.0004; Subject: $F_{(30,30)}$=6.133, p<0.0001; Saline and Oil: $t_{(30)}$=2.595, p=0.043, g= 0.561; MPEP and Oil: $t_{(30)}$=3.106, p=0.016, g= 0.768). The conclusion that estradiol may be modulating the effects of habituation is not robust, as when the data were analyzed as the change between the two test days and compared across groups, no differences between groups were detected (Figure 4B; Interaction: $F_{(1,30)}$=0.198, p=0.66, Hormone: $F_{(1,30)}$=2.934, p=0.097, Drug: $F_{(1,30)}$=1.251,p=0.272).

3.2 Experiment 2: Absence of White Light

We hypothesized that if the effects of estradiol and MPEP were specific to the acute stress induced by the presence of light, then estradiol and MPEP should have little effect in the absence of white light. To test this hypothesis, which is a critical control for whether light induced a stress-response, we assayed anxiety-related and overall locomotor behaviors in a different cohort of rats under the influence of estradiol and MPEP in the absence of white light.

3.2.1 Weight Difference—Rats exposed to estradiol demonstrated attenuated weight gain compared to rats not exposed to estradiol, and this effect was not blocked by MPEP exposure (Figure 5; Interaction: $F_{(1,15)}$=0.004, p=0.95; Hormone $F_{(1,15)}$=43.51, p<0.001; Drug $F_{(1,15)}$=0.014, p=0.909). This finding again indicates that estradiol injections were effective in this cohort of rats.

3.2.2 Anxiety-related Behaviors—Exposure to estradiol and/or MPEP did not significantly modulate the time spent in the center of the open field arena (Figure 6A). In the absence of white light, no differences in time spent in the center were detected within any group (Mixed-factor ANOVA: No Day x Hormone x Drug interaction detected: $F_{(1,16)}$=1.494, p=0.239; Two-way RM ANOVA: Treatment x Day: $F_{(3,16)}$=1.372, p=0.287, Power=0.934; Treatment: $F_{(3,16)}$=0.386, p=0.765; Day: $F_{(1,16)}$=0.023, p=0.882; Subject: $F_{(16,16)}$=2.857, p=0.022). Likewise, when time spent in the center was analyzed as the change between the two test days and compared across groups, no differences were detected (Figure 6B; Interaction: $F_{(1,16)}$=1.494, p=0.239; Hormone: $F_{(1,16)}$=1.662, p=0.216; Drug: $F_{(1,16)}$=0.943, p=0.346).

Exposure to estradiol and/or MPEP also did not significantly modulate the latency to enter the center of the open field arena (Figure 6C). In the absence of white light, no differences in latency to enter the center were detected within groups (Mixed-factor ANOVA: No Day x Hormone x Drug interaction detected: $F_{(1,16)}$=1.527, p=0.234; Two-way RM ANOVA: Treatment x Day: $F_{(3,16)}$=0.741, p=0.543, Power=0.372; Treatment: $F_{(3,16)}$=0.736, p=0.546; Day: $F_{(1,16)}$=0.916, p=0.353; Subject: $F_{(16,16)}$=0.913, p=0.571). Likewise, when the latency to enter the center was analyzed as the change between the two test days and compared across groups, no differences were detected (Figure 6D; Interaction: $F_{(1,16)}$=1.527, p=0.234; Hormone: $F_{(1,16)}$=0.26, p=0.617; Drug: $F_{(1,16)}$=0.452, p=0.511).

Exposure to estradiol and/or MPEP did not significantly modulate the number of entries into the center of the open field arena (Figure 6E). In the absence of white light, no differences in the number of center entries were detected within groups (Mixed-factor ANOVA: No Day x Hormone x Drug interaction detected: $F_{(1,16)}$=0.319, p=0.580; Two-way RM ANOVA: Treatment x Day: $F_{(3,16)}$=1.124, p=0.369, Power=0.875; Treatment: $F_{(3,16)}$=0.326, p=0.807; Day: $F_{(1,16)}$=2.467, p=0.136; Subject: $F_{(16,16)}$=8.202, p<0.001). Likewise, when the number of center entries was analyzed as the change between the two test days and compared across groups, no differences were detected (Figure 6F; Interaction: $F_{(1,16)}$=0.319, p=0.580; Hormone: $F_{(1,16)}$=2.043, p=0.172; Drug: $F_{(1,16)}$=0.842, p=0.372). Overall, these findings indicate that estradiol or MPEP exposure did not influence anxiety-related behaviors in the absence of white light, consistent with the hypothesis that white light acted as an acute stressor.

3.2.3 Overall Locomotor Activity—Total distance traveled in the open field arena was influenced by the day of exposure (Figure 7A). Treatment of either saline and estradiol or MPEP and estradiol resulted in a significant decrease in total distance traveled from day one to day four (Mixed-design ANOVA: No Day x Hormone x Drug interaction detected: $F_{(1,16)}$=0.238, p=0.632; Two-way RM ANOVA: Treatment x Day: $F_{(3,16)}$=1.385, p=0.283, Power=0.937; Treatment: $F_{(3,16)}$=1.544, p=0.242; Day: $F_{(1,16)}$=27.33, p<0.001; Subject: $F_{(16,16)}$=9.855, p<0.001; Saline and Estradiol: $t_{(16)}$=2.875, p=0.033, g= 2.240; MPEP and Estradiol: $t_{(16)}$=4.179, p=0.003, g= 0.690). However, the conclusion that estradiol is modulating the effects of habituation is not robust, as when the data was analyzed as the change between the two test days and compared across groups, no differences between groups were detected (Figure 7B; Interaction: $F_{(1,16)}$=0.238, p=0.632; Hormone: $F_{(1,16)}$=3.079, p=0.098; Drug: $F_{(1,16)}$=0.644, p=0.434).

4. Discussion

Our findings reveal that $mGlu_5$ is necessary for estradiol mitigation of anxiety-related behaviors in the presence but not absence of white light. In the presence of light, estradiol mitigation of anxiety-related behaviors was blocked by MPEP. In the absence of white light, neither estradiol nor MPEP had an effect on anxiety-related behaviors, revealing that the presence of an acute stressor is required for modulation of anxiety-related behaviors by estradiol and $mGlu_5$. As expected, in the presence and absence of white light, overall locomotion decreased between the first and fourth day of testing due to habituation to the open field arena.

The phenomenon of light as an acute stressor has long been demonstrated in nocturnal rodents such as rats and mice who, in the presence of light, exhibit anxiety-related behaviors [32, 33, 42] and elevation of stress-associated neuromodulators such as corticosterone [43]. Here, we extend this literature by using light as a tool to test the roles of estradiol and $mGlu_5$ in the context of an acute stressor. In the absence of white light, rats exhibited a highly exploratory phenotype and fewer anxiety-associated behaviors. We interpret our current study as indicating that the effects of estradiol and MPEP were muted in rats assayed in the absence of white light due to the low-anxiety environment. In contrast, in the presence of white light which is more aversive, anxiety-related behaviors were more prominent and

clearly modulated by estradiol and MPEP. To our knowledge, no previous study has tested the role of $mGlu_5$ in estradiol-mediated abrogation of light-induced anxiety behaviors. Complementing our work, at least two other studies have examined the role of the estrous cycle and estradiol in the context of white light. In one study of adult female rats, sensitivity to white light, as indicated by anxiety-related behaviors, changed across the estrous cycle in intact rats and in response to estradiol or progesterone treatment in gonadectomized rats [44]. In a second study in adult female mice, a similar finding was made that the sensitivity of locomotor, memory, and anxiety-related behaviors to white light varied across estrous cycle phases [45]. Both of these studies suggest that estradiol and perhaps progesterone are important components of the hormonal mechanism influencing acute anxiety-related behaviors.

Interestingly, when estradiol is provided outside of the context of the estrous cycle we note that there is some divergence in the literature on estradiol's acute effects on female locomotion in the open field [46-49]. Though estradiol is usually characterized to induce an increase in locomotor activity, some of these studies show estradiol inducing a decrease in activity in the open field or detect no change in locomotion. This divergence in results could potentially be due to the impact of environmental variables such as light and novelty, as well as the usual concerns regarding experimental power, dose and route of estradiol exposure, and strain/species differences [46-49]. Possibly explaining this divergent literature, our study tentatively suggests a differential effect of estradiol on open field locomotor activity between the presence and absence of white light. Estradiol treatment in the presence of white light seems to decrease the effects of habituation, while estradiol treatment in the absence of white light seems to enhance the effects of habituation. Detecting the influence of estradiol was dependent on the type of analysis employed, producing another possible avenue for divergence in interpretation. Note that this divergence in the literature regarding estradiol's effects on locomotion in the open field is distinct from estradiol's effects on other forms of locomotion. For example, estradiol exerts a substantial influence on motivated locomotor behaviors such as voluntary wheel running behavior [50, 51].

One potential caveat to this study is the issue of experimental power for detecting changes in the behavior of rats tested in the absence of white light, in that it may be possible that an effect of estradiol in the absence of white light was missed due to an inappropriate sample size. To test whether in the absence of white light the lack of differences in anxiety-related and locomotor behavior between treatments was due to an underpowered study, a power analysis was performed for each metric targeting the main treatment and day interaction within subjects in both the absence and presence of white light. The main effects for anxiety-related and locomotor behaviors were found to be sufficiently powered in both the absence and presence of white light. This indicates that the sample size was sufficient for detecting any notable behavioral differences, although it is always possible than an increased sample size could allow for the detection of more subtle differences.

MPEP is an antagonist of the Group I receptor $mGlu_5$ [52, 53]. Glutamate receptors are highly implicated in anxiety phenotypes [54, 55]. Metabotropic glutamate receptors are extensively implicated in anxiety behaviors, within the context of acute and chronic stressors and disorders such as generalized anxiety disorder, social anxiety, and post-traumatic stress

disorder [56, 57]. Pharmaceuticals targeting mGlu have entered clinical trials [58-60], but with inconclusive clinical efficacy. Unfortunately much of this literature, especially in the preclinical context, does not include female subjects or consider sex as a biological variable, a serious deficiency that has historically been pervasive within the neurosciences community [61-64]. This omission is unfortunate, because women exhibit increased incidence and more robust phenotypes of anxiety-related disorders compared to men [6, 65, 66], and examples of sex-specific differences in receptor signaling have been demonstrated in other contexts [67, 68]. More specific to the present study, previous work has demonstrated that estradiol activates mGlu via membrane estrogen receptors in a variety of brain regions and contexts [16]. Regarding this topic and anxiety-related behaviors, direct infusion of Group 1 mGlu agonists, stimulating both $mGlu_{1a}$ and $mGlu_5$, into the basolateral amygdala (BLA) produces anxiolytic effects only in the presence of estradiol in ovariectomized female rats in a generalized anxiety model conducted in the absence of white light [69]. Similarly, in a conflict-based anxiety model, stimulation of group 1 mGlu in the BLA had sex specific effects, presenting anxiolytic effects in ovariectomized females but anxiogenic effects in males [70]. These manuscripts suggest a significant and widespread sex-sensitive role in ER/mGlu interactions in the context of various types of anxiety. The current experiment further suggests a role of ER/mGlu interactions in female anxiety-related behavior, however further studies assessing whether a similar pathway also influences male anxiety-related behavior will be needed to determine whether this scenario is sex-specific.

The experiment presented here does not delineate the specific neural substrate where estradiol and $mGlu_5$ mediate anxiety-related behaviors. Several brain regions are prominent targets for future research. The amygdala, which expresses both ER and $mGlu_5$ [70, 71], helps regulate anxiety behaviors [72, 73] and is a possible component to the behaviors assessed here. In addition, the nucleus accumbens (NAc) within the striatum has been implicated in various types of anxiety-related behaviors and disorders [74, 75]. However, despite the NAc region's interconnection to the amygdala and other relevant brain regions [76, 77], its role in stress and anxiety behaviors is less well understood [78]. The NAc also expresses both ERs and mGlu, suggesting that this region could be a key player in estradiol's mediation of anxiety-related behaviors through activation of $mGlu_5$ [79, 80]. Estradiol acts in the NAc through the ER/$mGlu_5$ pathway to influence neuron spine density [22, 81], and in the striatal regions to influence neuronal transcription factor phosphorylation and dopamine release [24, 82]. Estradiol through the ER/$mGlu_5$ pathway also mediates psychostimulant-induced behaviors, including increased self-administration of cocaine and cocaine-induced locomotor responses [23, 40, 83]. Collectively, this literature implicates estradiol and the ER/mGlu pathway as a highly relevant neuromodulator of motivated behavior [26], which in turn suggests a role for the NAc in the interactions of estradiol and anxiety-related behaviors. It is not yet known which ER is involved in this specific ER/$mGlu_5$ pathway. Membrane-associated ERα, ERβ, and GPER-1 are all possible targets. In one study, treatment with an ERβ agonist resulted in a decrease in anxiety behaviors in female rats, while treatment with an ERαagonist had anxiogenic effects [46]. These findings suggest that ERβ may be the receptor involved in this specific ER/$mGlu_5$ pathway, and this hypothesis should be addressed in future experiments.

Overall, during periods of female rodent sexual receptivity, estradiol's neural and behavioral modulation is thought to enhance the likelihood of successful reproduction by decreasing anxiety-related behaviors [18, 84-86]. Not surprisingly, estradiol acts to enhance reproductive fitness by simultaneously modulating a wide range of behaviors and neural circuits via divergent molecular mechanisms. Interpreting our results in the context of reproductive fitness, the action of estradiol in mitigating the stressful effects of white-light potentially allows for increased displays of sexual receptivity by utilizing the widespread and highly conserved mGlu mechanism. This decrease in anxiety-related behaviors via activation of $mGlu_5$ is perhaps another mechanism by which estradiol tightly regulates female reproduction across multiple brain regions.

To conclude, this study demonstrates that in females, $mGlu_5$ is required for estradiol to mitigate anxiety-related behaviors in the presence of an acute-stressor. This finding extends our understanding of the mechanisms underlying estradiol action in the context of both mGlu and anxiety. More broadly, this work strongly indicates that biological sex and sex steroid hormone action should be considered when investigating treatments for anxiety-related disorders.

Acknowledgements

We thank Dr. Jinyan Cao and Stephanie Proano for surgical training, and David Dorris with assistance with animal handling.

Funding: This work was supported by the NIH R01 MH109471 (JM) and NIH P30ES025128 (Center for Human Health and the Environment).

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Highlights

- Estradiol decreases female anxiety-related behaviors.
- The $mGlu_5$ inhibitor MPEP blocks the anxiolytic effects of estradiol in females.
- MPEP did not block locomotor behavior in females.

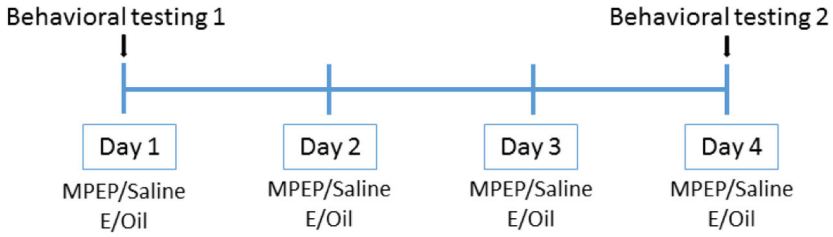

Figure 1.

Schematic depicting experimental paradigm. Behavioral testing was conducted under white light in experiment 1 and under red light in experiment 2.

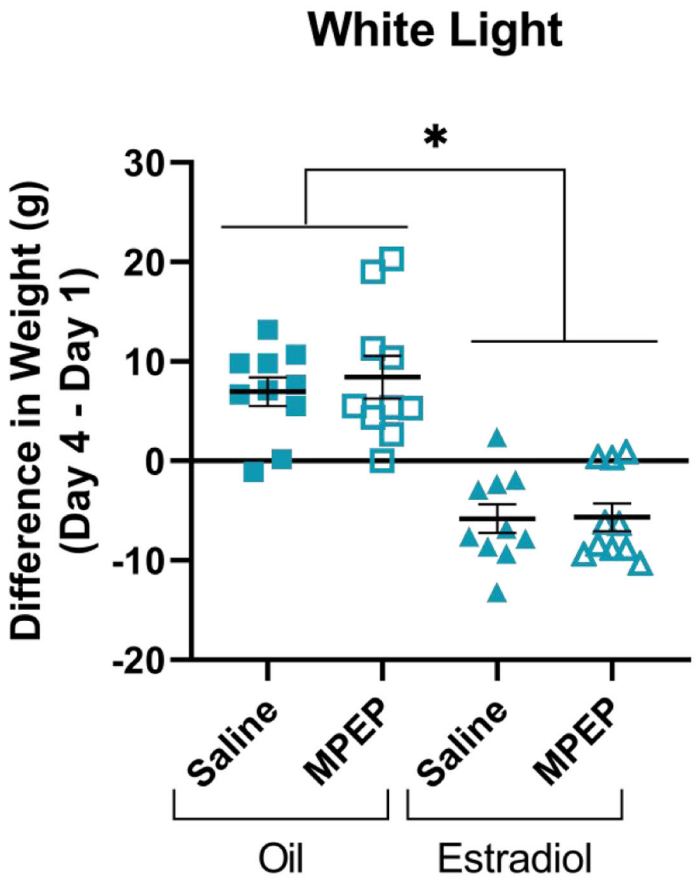

Figure 2.

Estradiol exposure attenuated weight gain for rats tested in the presence of white light. For females injected with estradiol, the overall difference in weight was negative from day 4 to day 1 of estradiol exposure. Exposure to MPEP did not block this effect on weight change. Acronyms: * = $p<0.05$.

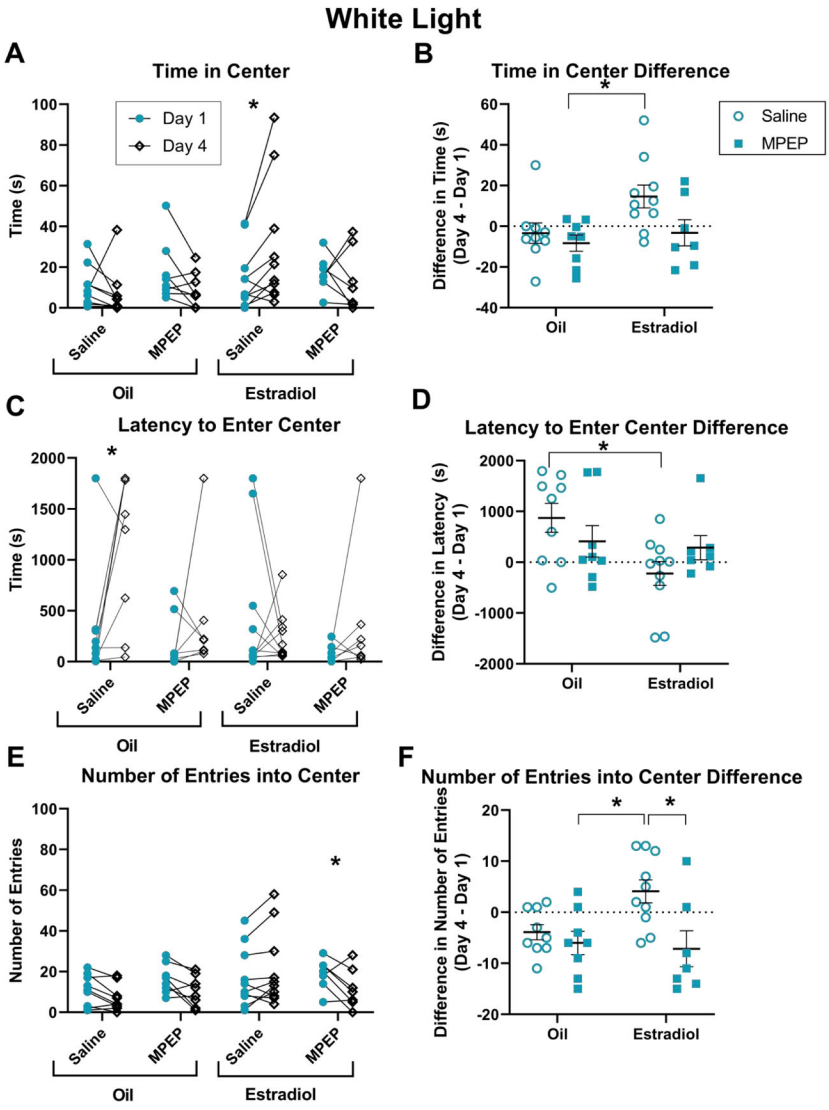

Figure 3.

Estradiol's influence on anxiety-related behavior was blocked by the $mGlu_5$ inhibitor MPEP in the presence of the acute stressor white light. A) Time spent in the center of the open field within subject on testing day 1 and day 4. B) Difference in the time spent in the center of the open field from testing day 4 to day 1. C) Latency to enter the center of the open field within subject on testing day 1 and day 4. D) Difference in the latency to enter the center of the open field from testing day 4 to day 1. E) Number of entries into the center of the open field within subject on testing day 1 and day 4. F) Difference in the number of entries into the center of the open field from testing day 4 to day 1. Acronyms: * = $p<0.05$.

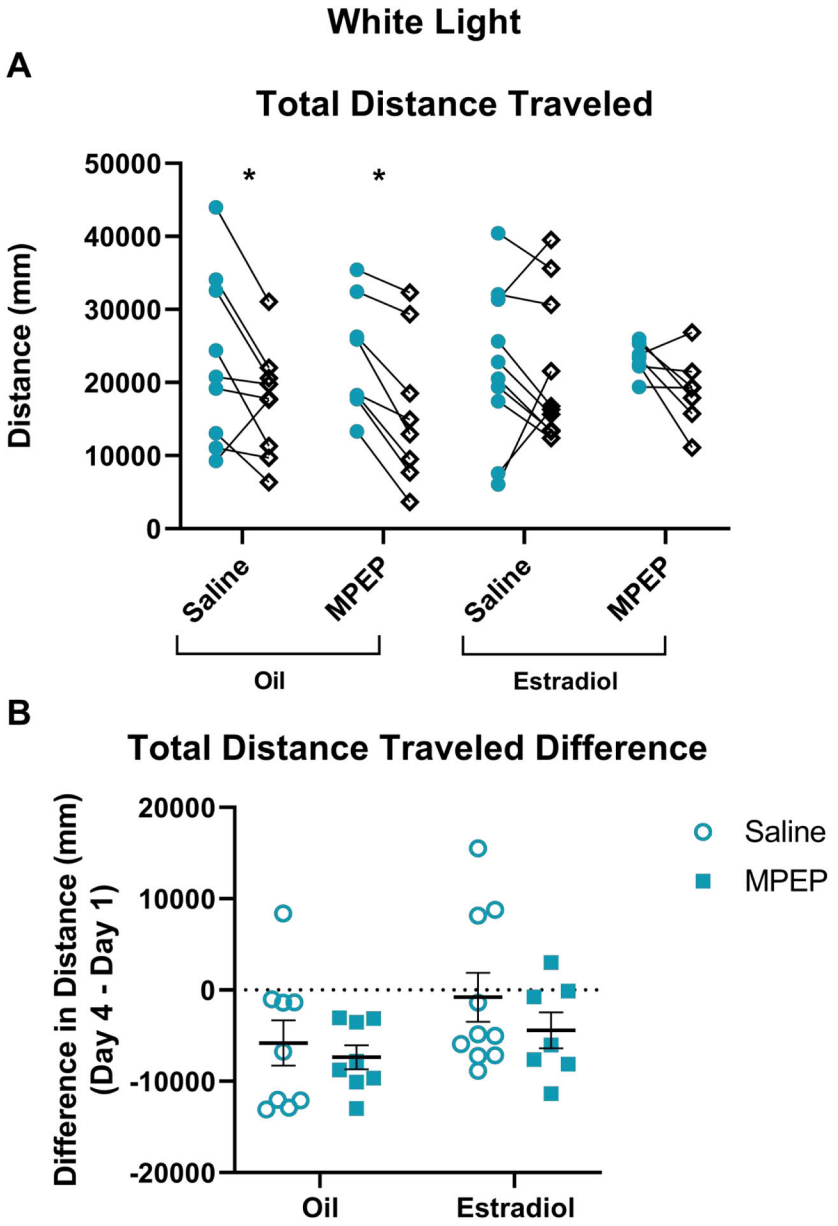

Figure 4.

Locomotor behavior was not influenced by the $mGlu_5$ inhibitor MPEP in the presence of the acute stressor white light. A) Total distance traveled in the open field within subject on testing day 1 and day 4. B) Difference in the total distance traveled from testing day 4 to day 1. Acronyms: * = $p<0.05$.

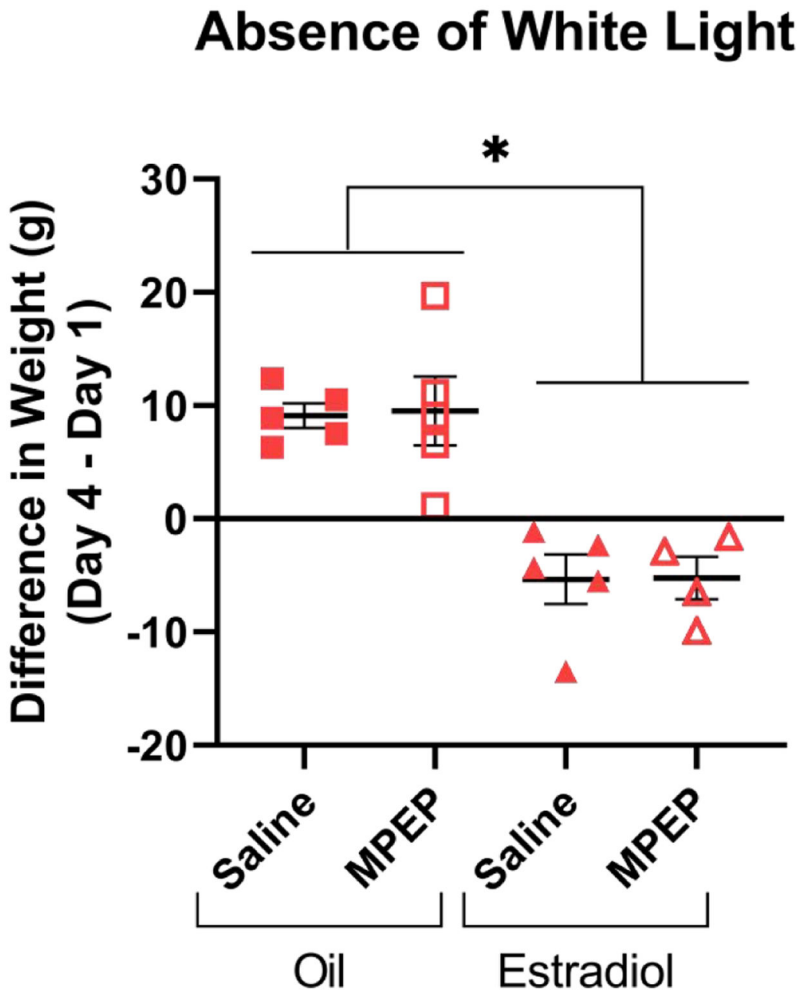

Figure 5.

Estradiol exposure attenuated weight gain for rats tested in the absence of white light. For females injected with estradiol, the overall difference in weight was negative from day 4 to day 1 of estradiol exposure. Exposure to MPEP did not block this effect on weight change. Acronyms: * = $p<0.05$.

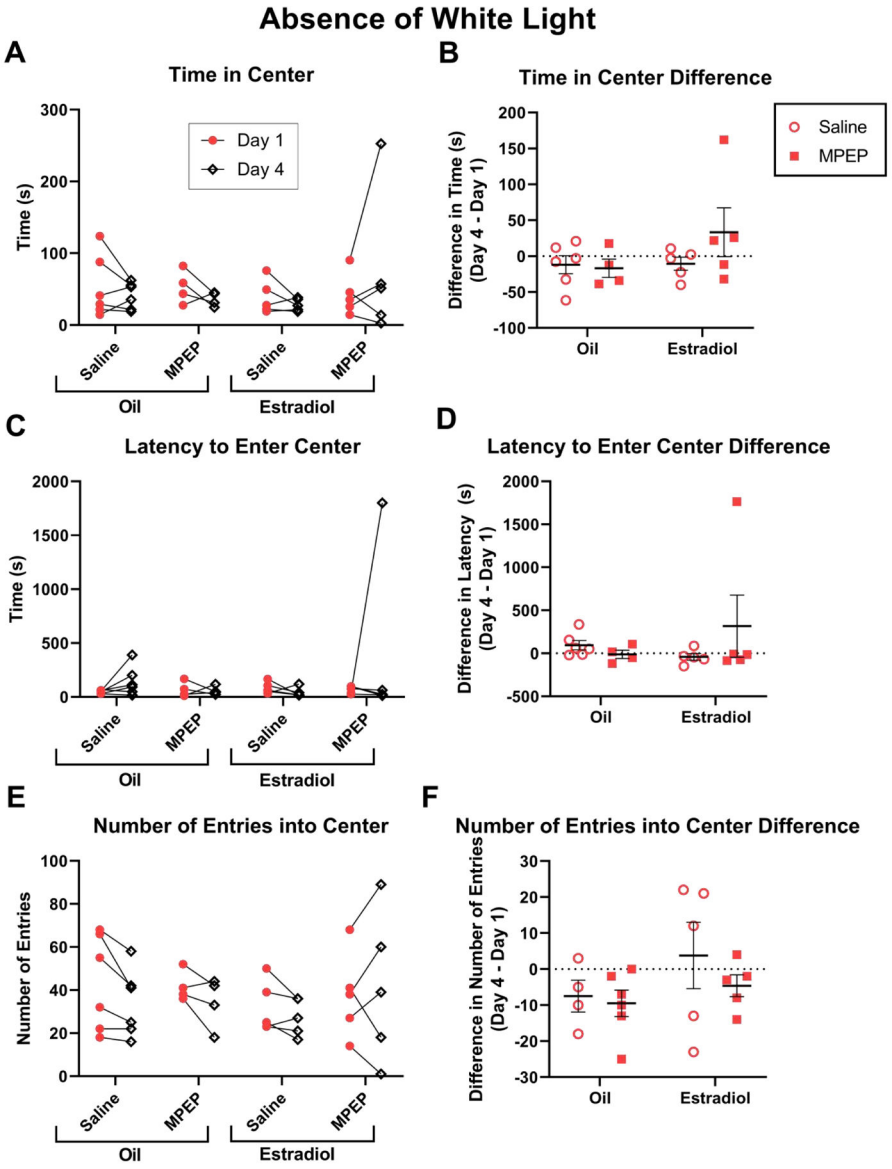

Figure 6.

Exposure to estradiol and/or MPEP did not influence anxiety-related behaviors in the open field in the absence of white light. A) Time spent in the center of the open field within subject on testing day 1 and day 4. B) Difference in the time spent in the center of the open field from testing day 4 to day 1. C) Latency to enter the center of the open field within subject on testing day 1 and day 4. D) Difference in the latency to enter the center of the open field from testing day 4 to day 1. E) Number of entries into the center of the open field within subject on testing day 1 and day 4. F) Difference in the number of entries into the center of the open field from testing day 4 to day 1. Acronyms: * = $p<0.05$.

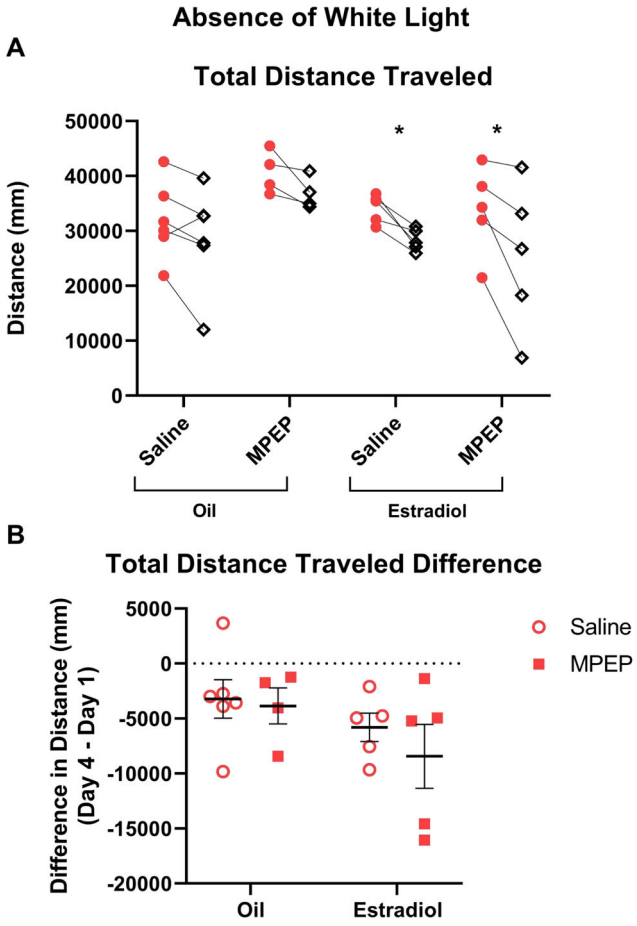

Figure 7.

Locomotor behavior was not influenced by the $mGlu_5$ inhibitor MPEP in the absence of white light. A) Total distance traveled in the open field within subject on testing day 1 and day 4. B) Difference in the total distance traveled from testing day 4 to day 1. Acronyms: * = $p<0.05$.