A mouse model of hormonal contraceptive exposure: depression, motivation, and the stress response

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ABSTRACT

Hormonal contraceptives (HCs) regulate hormonal levels and cycles, and thereby affect many physiological outcomes including stress responsivity and acne. Many individuals also experience overall benefits in regulation of mood, yet up to 10% of HC users experience depression, anxiety, and other adverse events. Despite 60 years of availability, it remains unclear how HCs modulate brain, behavior, and mood. Here, we created and validated a mouse model of HC exposure to answer mechanistic questions arising from studies of human HC use. Young adult female C57Bl/6N mice received either daily oral hormone treatments or a control (10% sucrose) treatment to mimic daily oral contraceptive exposure in humans. Hormone treatments were a combination of ethinyl estradiol (EE) and a progestin—either levonorgestrel (LVNG) or drospirenone (DRSP)—in 10% sucrose. Translationally-relevant doses of EE+LVNG-exposure caused a specific anhedonia-like effect, without indication of broad depression- or anxiety-like presentations. EE+LVNG, but not EE+DRSP, suppressed the acute stress response, an effect consistent with that observed in human HC users. Importantly, the limited changes in behavioral outcomes, together with suppression of regular estrous cycling and suppression of the corticosterone response to acute stress demonstrate the validity and tractability of this model with which to investigate questions difficult to study in human subjects. Thus, this model will be important for identifying mechanisms and risks for adverse effects of HCs on mood that will contribute to a more personalized approach to HC prescribing strategies.

INTRODUCTION

Hormonal contraceptives (HCs) are a critical part of healthcare, providing an unprecedented level of reproductive control that, in turn, has economic and educational benefits for families. In addition, because HCs regulate the menstrual cycle, they also exert health benefits from alleviating menstrual symptoms and treating premenstrual dysphoric disorder, to controlling acne, increasing bone density, and reducing the risk of ovarian, epithelial, and endometrial cancers (Havrilesky et al., 2013; Michels et al., 2018; Murphy et al., 2017; Schindler, 2013). Despite this myriad of benefits for many users, adverse effects on mood remain the most common reason for cessation of use, and at least 4-10% of users experience increased risk for mood disorders (Herzberg et al., 1971; Poromaa & Segebladh, 2012).

While HCs increase depression and anxiety for some users, others experience beneficial effects on mood (Keyes et al., 2013; Porcu et al., 2019; Poromaa & Segebladh, 2012; Tronson & Schuh, 2022). These conflicting findings are likely due to individual differences in mood-related risk factors, differences between HC formulations, and different inferences from analyses that focus on group averages versus those that assess individual risks or changes across time (Beltz, 2022). This raises the question of how to predict who is likely to experience beneficial effects and who is at risk for detrimental effects, but to do that we need to know how HCs affect the brain. After over 60 years of research and use of HCs, it is clear that HCs affect the brain, mood, and stress responses. Here, we propose a new mouse model with which to identify mechanisms by which HCs contribute to regulation of stress and risks for anxiety and depression.

Developing and utilizing animal models is a necessary step towards understanding how HCs modulate the brain and behavior (Hilz, 2022; Tronson & Schuh, 2022). Previous studies have successfully modeled HC exposure in rodents using daily hormone injections, implants, or daily oral gavage protocols to model suppression of ovulation, extensions of fertility, modulation of social and affective behaviors, cognition, and changes in neurochemistry (Allaway et al., 2021; Carrington & Bailey, 1985; Isono et al., 2018; Lacasse et al., 2022; Porcu et al., 2012; Simone et al., 2015). Here we build on these studies to develop and validate a mouse model of HC exposure, specifically targeted to mimic oral contraceptives and the mechanisms by which they regulate the hypothalamic-pituitary-adrenal (HPA) axis, anxiety, and depression-like behaviors.

One reliable physiological effect of HCs is suppression of the acute stress response in many people that use them (Kuhlmann & Wolf, 2005; Mordecai et al., 2017). Specifically, HCs blunt the hypothalamic-pituitary-adrenal (HPA) axis response to acute stress and alter basal cortisol levels (Hertel et al., 2017; Kirschbaum et al., 1995). Given that stress and dysregulated stress-related signaling play a key role in depression and anxiety (Tafet & Nemeroff, 2016), changes in the HPA axis as a consequence of HC use is particularly interesting for identifying how HCs influence risk and resilience to depression (Slattery & Cryan, 2017).

The main goal of this study is to create and validate a mouse model of HC exposure with which to identify how HCs mechanistically affect the brain, mood, motivation, and the stress response, and answer questions that arise from studies of human HC use. We hypothesize that, like in humans, a translationally relevant dose of orally administered hormonal contraceptive will suppress acute stress responses, and trigger only mild changes in anxiety, depression, and anhedonia-like responses. By determining changes in anxiety-, depression-, and anhedonic-like behaviors and identifying interactions between hormonal contraceptives, the HPA-axis, and stress responses, we will be well-placed to answer questions that arise from the human literature and examine detailed mechanisms by which HCs affect depression risk.

METHODS

Animals included young adult female C57Bl6/N mice (9-10 weeks on arrival) from Envigo (Indianapolis, IN). Animals were individually housed in the lab colony room with access to standard chow and water *ad libitum*. The colony room, adjacent to behavioral testing rooms, was maintained at 20 ± 2 °C with a 12h 0700: 1900 light/dark cycle (lights on at 0700 h). Mice were allowed at least 7 days to acclimate to the colony room. All protocols and treatments were approved by the University of Michigan Institutional Animal Care and Use Committee.

Hormone treatments were a combination of ethinyl estradiol (EE, 0.075 µg/mL) and progestin—either the androgenic, third generation levonorgestrel (LVNG, 3 µg/mL) or the fourth generation, anti-androgenic drospirenone (DRSP, 15 µg/mL). These doses were based on prior experiments that showed suppression of ovulation (Isono et al., 2018). EE+LVNG or EE+DRSP was suspended in 10% sucrose. 250µL of 10% sucrose with hormone, or 10% sucrose control was delivered daily in small cell culture dishes in the animals' home cage prior to lights out

(1700-1900h). HC and control treatments were given for at least 2 weeks prior to behavioral tests. Animals were weighed weekly throughout this experiment. A mixed effects analysis was used to compare weights of control vs HC-treated animals across days.

Estrous Cycle was assessed using vaginal cytology and visual assessments (Caligioni, 2009; Keiser et al., 2017). Unpaired t-tests were used to compare the number of estrous phase shifts between treatment groups.

Behavioral tasks

Apparatus. MedAssociate boxes (8.5”x 7.12” x 5”) within sound attenuating cubicles (MedAssociates, 25” x 17” x 17.5”) were used for the sucrose preference, conditioned taste avoidance, instrumental training, and progressive ratio tests. A square open field (1m x 1m) was used for the open field, novelty suppressed feeding, and light/dark emergence tests. Contextual fear conditioning was conducted in rectangular Perspex conditioning boxes (9 3/4′′ × 12 3/4′′ × 9 3/4′′; MedAssociates, VT) with evenly spaced grid floors.

Sucrose preference was used to assess anhedonic-like behavior. Mice were first habituated to 2% sucrose in their home cage and subsequently habituated to the MedAssociate boxes. Each box was equipped with a Lixit apparatus and 2 bottles of 2% sucrose. At test, mice were placed into the identical boxes with a 2-bottle choice between 2% sucrose and water for 30-min sessions. A second day of testing counterbalanced for side biases. All habituation and tests were conducted after overnight water fasts. The number of licks were automatically detected using MedPC software (MedAssociates, VT), and preference was calculated as a percentage of sucrose *vs* total licks [100*# sucrose licks/(#sucrose + #water licks)]. Sucrose preference was assessed using unpaired one-sample t-tests to compare the preference ratios of each treatment group to a theoretical mean of 50. Mice that had fewer than 10 total licks were excluded from the analysis. Sucrose preference data was replicated with a second cohort of animals.

Novelty Suppressed Feeding was used to assess the conflict between feeding and fear of novelty (Dulawa & Hen, 2005). After an overnight fast, animals were placed into the open field

apparatus with a pellet of standard chow in the center. Latency to explore the food was automatically detected using Ethovision XT. Differences in latency to the food pellet between treatment groups was assessed using an unpaired one-sample t-test.

Forced swim test is a commonly used measure of stress-coping behaviors (Commons et al., 2017). Mice were placed in a 2000 mL beaker filled 2/3 with room temperature water (22° C +/- 1°C) for 6 minutes. After the test, animals were dried off with paper towels and placed in their home cage with a heating pad for 10 minutes (Sunbeam, 43.3°C). Time spent immobile (no movement other than that required to stay afloat) was hand scored during minutes 2-6 of the task by three researchers masked to condition. An unpaired t-test was conducted to compare time spent immobile between HC and control treatment groups.

Light/Dark Emergence was used as a sensitive measure of anxiety and risk assessment (Arrant et al., 2013). Animals were placed in a black box (2.5 x 5 x 3 in) within the open field. The door was opened, and latency for nose, arm, and full body to emerge from the box was hand scored by two researchers who were blind to experimental conditions. EthoVision XT (v.16) was used to record videos. Unpaired t-tests were used to compare first nose emergence, first arm emergence, and whole-body emergence between treatment groups.

Open field test was used to assess exploratory and anxiety-like behavior (Seibenhener & Wooten, 2015). Animals were placed in an open field for 10 minutes. EthoVision was used to record and analyze the animals' movement from the center of the body. Total distance travelled and cumulative duration at the field edges and center were measured. Less time in the center of the arena is an index of increased anxiety-like behavior. Unpaired t-tests were used to compare distance travelled and time-in-center between treatment groups.

Context Fear Conditioning was used to assess fear learning (Kim & Cho, 2020). The task was conducted as previously described (Keiser et al., 2017). Mice were placed into the chamber for 3 minutes, followed by a 2s, 0.8mA footshock. Mice were returned to their home cage in the colony room immediately following training. 24-hours later, animals were placed into the

identical chamber for 3-minutes. Locomotor activity and freezing were automatically assessed throughout training and testing via VideoFreeze (MedAssociates, VT). The chambers were cleaned with 70% ethanol between each mouse. Unpaired t-tests were used to compare locomotor activity during training, shock reactivity during training, and freezing during test between control vs HC-treated groups.

Conditioned Taste Avoidance Task was used to assess whether EE + LVNG administration caused a taste aversion to sucrose. Hormone and sucrose or sucrose alone were paired with different flavors, orange and cucumber (SodaStream). Flavor A was added to 10% sucrose at a 1:1000 dilution and placed in the home cage of all animals at the beginning of the light cycle, while Flavor B was added to either 10% sucrose + hormone or 10% sucrose alone, depending on which treatment group the mice were in, at the end of the light cycle. Flavors were counterbalanced to avoid a bias for one flavor or the other. A two-bottle choice was used (cucumber vs orange, each 1:1000 in tap water) and the number of licks on each bottle was measured using MedPC software. Preference ratio was calculated as described above. Unpaired one-sample t-tests were conducted for each treatment group to compare the group mean preference ratio to a theoretical mean of 50.

Instrumental Training and Progressive Ratio were used to assess appetitive learning and motivation (Sharma et al., 2012). Mice were food restricted up to 5 days a week with *ad lib* food at least 2 days a week to maintain at 85% of their free-feeding weight. Mice were habituated to grain food pellets (F0163, Bio-Serv) in a dish in their home cage and subsequently trained to nosepoke for the food pellet reward. Animals were trained on an FR1 schedule to a criterion of at least 20 total food pellets and 75% accuracy for three consecutive days, followed by an FR4 schedule to the same criterion, and then a progressive ratio task using a grain pellet reward, in which the number of nosepokes required to earn one food pellet increased exponentially ($[5e^{(reward\ number\ \times 0.2)}] - 5$) (Richardson & Roberts, 1996). Mice were retrained and tested according to the previous schedules with chocolate-flavored sucrose pellets (F05301, Bio-Serv). An unpaired t-test was conducted to compare the number of days it took for the animals in each treatment group to reach criteria. A 2-way ANOVA was used to compare

the number of control vs HC-treated animals that reached criteria across days. Unpaired t-tests were conducted to compare break points across treatment groups in the PR task with the grain pellets and the PR task with the chocolate pellets.

Acute Restraint stress. Animals were anesthetized using 0.1 mL isoflurane in a drop box and placed prone on a flat surface. Tape was applied to the elbow joints of the forelimbs and the knee joints of the hind limbs so that animals were restrained for 1 hour (Tronson et al., 2010), after which the tape was carefully removed to minimize unnecessary discomfort and animals were returned to their home cage.

Blood collection. Thirty minutes after the end of the acute stressor, mice were deeply anesthetized with an intraperitoneal injection of 250 mg/kg of Avertin (2,2,2-tribromoethanol), and blood was collected from the heart using cardiac puncture. Blood was centrifuged at 12000 rpm for 15 minutes. Serum supernatant was extracted and stored at -20°C.

Corticosterone Enzyme Immunoassay Kit (Arbor Assays, Ann Arbor, MI) was used according to the protocol (Epoch plate reader, Biotek). A 2-way ANOVA was conducted with treatment (EE+LVNG, EE+DRSP, and control) and stress exposure (stress vs non-stress) as factors. Post hoc tests with Bonferroni correction were conducted to determine the effects of stress within treatment groups.

RESULTS

Daily HC exposure disrupted the estrous cycle in mice

Vaginal cytology was conducted, and cell types were imaged to determine phase of the estrous cycle (as previously described in Tronson et al., 2010). Figure 1a displays representative images of cell types from each phase. Phases were graphed and control animals were compared to EE+LVNG-treated animals (Fig. 1b). In Cohort 1, EE+LVNG- treated animals experienced fewer estrous phase shifts ($t(14) = 6.76$, $p < 0.01$, Fig. 1c). In Cohort 2, there was no difference in phase shifts between treatment groups ($t(21) = 0.22$, $p = 0.83$, Fig. 1c). HC exposure disrupted the estrous cycle.

HC exposure did not cause weight gain

The weights of EE+LVNG-treated animals did not differ from control animals throughout the experiment. Both groups gained weight during free-feeding and maintained weight during food restriction. There is a main effect of time ($F(11,235) = 107.2$, $p < 0.001$), but no effect of treatment and no time x treatment interaction (time: $F(1,22) = 0.50$, $p = 0.49$; interaction: $F(11,235) = 0.43$, $p = 0.94$).

Daily EE+LVNG, but not EE+DRSP, suppressed the acute stress response

HC-exposure, specifically EE+LVNG, resulted in a significant blunting of the corticosterone response to an acute stressor (Main effect Stress: $F(1,56) = 13.05$, $p < 0.001$; Hormone: $F(2,62) < 1$; Stress x Hormone: $F(2,62) = 5.54$, $p = 0.02$; Fig 2). Whereas control mice and EE+DRSP-treated mice showed a robust increase in circulating CORT 30-min after stress (Control: $p < 0.001$; EE+DRSP: $p = 0.01$), EE+LVNG-exposed mice failed to do so ($p = 0.93$). Moreover, after acute stress, EE+LVNG- but not EE+DRSP-treated mice showed lower CORT levels than control mice (control *vs* EE+LVNG: $p = 0.044$; *vs* EE+DRSP: $p = 1.00$).

There were no significant differences between the HC groups and control mice in CORT levels of non-stressed mice (Control *vs* EE+LVNG: $p = 0.74$; *vs* EE+DRSP: $p = 1.00$), suggesting that the blunted CORT response in EE+LVNG-treated mice is not due to elevated circulating CORT in non-stressed mice.

EE+LVNG exposure decreased sucrose preference without other depression-like behaviors

We assessed preference for sucrose in both treatment groups. In two independent cohorts, Control animals, as expected, showed a robust preference for 2% sucrose (cohort 1: $t(8) = 3.91$, $p < 0.05$; cohort 2: $t(9) = 7.87$, $p < 0.001$; Figure 2); whereas EE+LVNG-treatment showed no preference (cohort 1: $t(8) = 2.10$, $p = 0.07$; cohort 2: $t(9) = 1.77$, $p = 0.11$). Overall consumption during the sucrose preference did not significantly differ between EE+LVNG-treated and control animals (cohort 1: $t(22) = 1.68$, $p = 0.011$, cohort 2: $t(18) = 0.56$, $p = 0.58$; Figure 3). When comparing sucrose preference data between the two cohorts, there was not a main effect of cohort ($F(1,34) = 1.97$, $p = 0.17$) or cohort x hormone interaction ($F(1,34) = 0.10$,

$p = 0.76$); however, there was a main effect of hormone ($F(1,34) = 5.26$, $p < 0.05$).

In contrast to this anhedonia-like effect in EE+LVNG-treated mice, we did not see evidence of EE+LVNG-driven differences in the novelty suppressed feeding task, where latency to approach food did not differ between groups ($t(22) = 0.40$, $p = 0.69$, Figure 3), nor the forced swim test, where time spent immobile was similar between control and EE+LVNG-treated mice ($t(21) = 0.70$, $p = 0.49$, Figure 3). Together, these data demonstrate that EE+LVNG causes specific anhedonia-like changes, and not a global change in affective processes.

EE+LVNG exposure caused mild changes in risk assessment behavior

EE+LVNG exposure increased risk-assessment in light-dark emergence but not open field test. In the light-dark emergence task, EE+LVNG-exposed animals had a longer latency for first paw emergence ($t(22) = 2.09$, $p < 0.05$), but showed no difference in first nose emergence, $t(22) = 1.17$, $p = 0.25$, or whole body emergence, $t(22) = 0.96$, $p = 0.33$. In the open field task, neither distance traveled ($t(21) = 0.96$, $p = 0.35$, Figure 2) nor time spent in the center of the open field ($t(21) = 0.04$, $p = 0.97$, Figure 2) differed between treatment groups.

EE+LVNG exposure did not alter context fear conditioning

To assess hippocampal-dependent memory for stress-related information, we used context fear conditioning. There was no effect of EE+LVNG exposure on locomotor activity during training ($t(20) = 1.44$, $p = 0.17$), animals exhibited no substantial freezing to the context during training, and there was not a difference in shock reactivity, ($t(19) = 0.47$, $p = 0.64$) (Figure 4). There was no difference in freezing ($t(21) = 1.00$, $p = 0.33$) or locomotion ($t(21) = 1.13$, $p = 0.27$) during the context test.

No evidence of a taste aversion to EE+LVNG

To determine whether the decreased sucrose preference might be due to aversion caused by hormone-delivery in sucrose, we conducted a conditioned taste aversion task. There was no evidence of a taste aversion for control ($t(9) = 1.24$, $p = 0.25$) or EE+LVNG-treated animals ($t(9) = 0.11$, $p = 0.92$; Fig 4).

EE+LVNG do not decrease global motivation

We used an instrumental task and progressive ratio to determine whether EE+LVNG decreased instrumental learning or motivation. All mice acquired the instrumental task, and EE+LVNG-treated animals did not differ from controls in the number of days to reach criteria ($t(18) = 0.16$, $p = 0.87$; Fig. 4), average total consumption ($t(18) = 1.46$, $p = 0.16$), nor break point of the progressive ratio task for a grain pellet reward ($t(17) = 0.80$, $p = 0.43$; Fig. 4). Contrary to our hypothesis, EE+LVNG-treated animals had a higher break point than control animals with a sucrose pellet reward ($t(17) = 2.16$, $p < 0.05$) with no difference in average total consumption between treatment groups ($t(18) = 1.57$, $p = 0.13$, Fig. 4).

DISCUSSION

Here we validate a new mouse model of HC exposure, designed to mimic daily oral contraceptive exposure in humans. Chronic daily EE+LVNG was well tolerated in mice, readily and rapidly ingested when given in 10% sucrose, and did not cause excessive weight gain. We observed suppressed estrous cycles in mice in this model, as assessed by vaginal cytology; however, complete blockade of cycles is not essential for HC effectiveness or for the presence of side effects (Pritschet et al., 2020). Consistent with reports in human HC-users, we also observed a blunting of the acute corticosterone response to stress response in EE+LVNG-treated animals without changes in corticosterone levels in non-HC-treated mice. In contrast, EE+DRSP-treated mice showed a robust corticosterone response, suggesting that the blunted stress response might be specific to androgenic- or second generation progestins. Behaviorally, we observed that chronic daily EE+LVNG triggers only very mild modulation of depression-like and anxiety-related behavioral phenotypes, with a reliable, replicable, and specific anhedonia-like effect in EE+LVNG-treated animals, without changes in other stress-coping or depression-like behaviors. We also observed a mild increase in anxiety-like behaviors, with sensitivity in the light-dark emergence test showing a small increase in risk assessment behaviors in HC-treated animals.

These findings are consistent with those from human HC users – most individuals do not experience major changes in mood, depression, or anxiety. Whereas there is a persistent misconception that the pill contributes to weight gain, neither individual studies nor meta-

analyses support weight gain on hormonal contraceptives (Brynhildsen, 2014; Gallo et al., 2014). Nevertheless, human HC users do show substantial regulation of stress-responsiveness, with HCs consistently blunting acute stress response for many individuals, with limited evidence for changes in baseline cortisol levels (Hertel et al., 2017; Kirschbaum et al., 1995; Merz & Wolf, 2017). In light of these observations in human subjects, our data provide strong support for the validity and utility of this model, and more broadly demonstrates that using translational-relevant daily oral dosing of EE+LVNG to mimic how humans take combined oral contraceptives effectively models HC use in humans.

This model builds upon a growing recent body of literature identifying important effects of hormonal contraceptives on the brain, with both mood-related benefits for many (Keyes et al., 2013) and increased risks for depression in a smaller proportion of individuals (Poromaa & Segebladh, 2012; Schaffir et al., 2016; Skovlund et al., 2016; Worly et al., 2018). It also builds on extant rat models of hormonal contraceptive exposure (Follesa et al., 2002; Porcu et al., 2012; Simone et al., 2015), which together demonstrate the importance of exogenous hormone treatment on cognition (e.g., Lacasse et al., 2022), affective behaviors (e.g., (Heinzmann et al., 2014; Planchez et al., 2019), and stress (e.g., (Porcu et al., 2019). Here, we independently replicate some of these effects (e.g., blunted stress response) and demonstrate a lower-dose oral regimen is effective for studying more subtle effects, like those observed at a population-level in women, transmen, and all individuals using hormonal contraceptives.

Our finding that HC-treated animals showed decreased sucrose preference – an effect not attributable to taste aversion to sucrose or a global decrease in motivation – indicates an anhedonia-like effect, or not finding pleasure in things that are typically pleasurable, a key symptom of depression (Gulfo et al., 2019). In contrast, we did not find any evidence of changes in other stress-coping, anxiety-, or depressive-like behaviors. One possible mechanism for this specific anhedonia-like effect is *via* direct and indirect effects of the progestin on endocrine receptors. Progestins, and their metabolites including allopregnanolone, have been linked with depression (Schüle et al., 2014) and with mood-related side effects of HCs (Porcu et al., 2012), whereas estrogenic components have been more closely linked with cognitive changes in HC users (Hampson, 2018). Anhedonic-like vulnerability is also likely related to the blunted stress response finding, as a dysregulated stress system is a dissociable aspect of depression, which is

in turn regulated by estrogens and progestogens (Chen et al., 2018; Worly et al., 2018).

The lack of major changes in anxiety and depression-like behaviors, together with the predicted changes in corticosterone response to acute stress in this translational-dose of contraceptive hormones is a strength of this model. Most HC-users do not experience adverse mood effects on HCs; thus, this baseline allows us to begin to systematically manipulate individual differences that contribute to increased vulnerability to depression and anxiety in some people; and to examine specific mechanisms by which HCs modulate the HPA axis. For example, given the interactions with stress responsiveness, this model can be used to examine how HCs alter response to chronic stress exposure, and vulnerability to depression-like consequences of stress. Importantly, together with existing models (Porcu et al., 2019), rodent models are an essential tool with which to begin to examine questions arising from human studies on HC use that are not feasible or ethical to consider in human participants (Tronson & Schuh, 2022). These questions include the identification of individual differences that predict beneficial or detrimental mood effects of specific HCs and how contraceptive hormones modulate the hypothalamic-pituitary-adrenal axis and blunt the stress response. Thus, this model—together with complementary animal and human studies—will be critical for identifying mechanisms and specific risk factors for adverse effects of HCs on mood. In the future, this work will be essential for developing a optimized and personalized approach to HC use.

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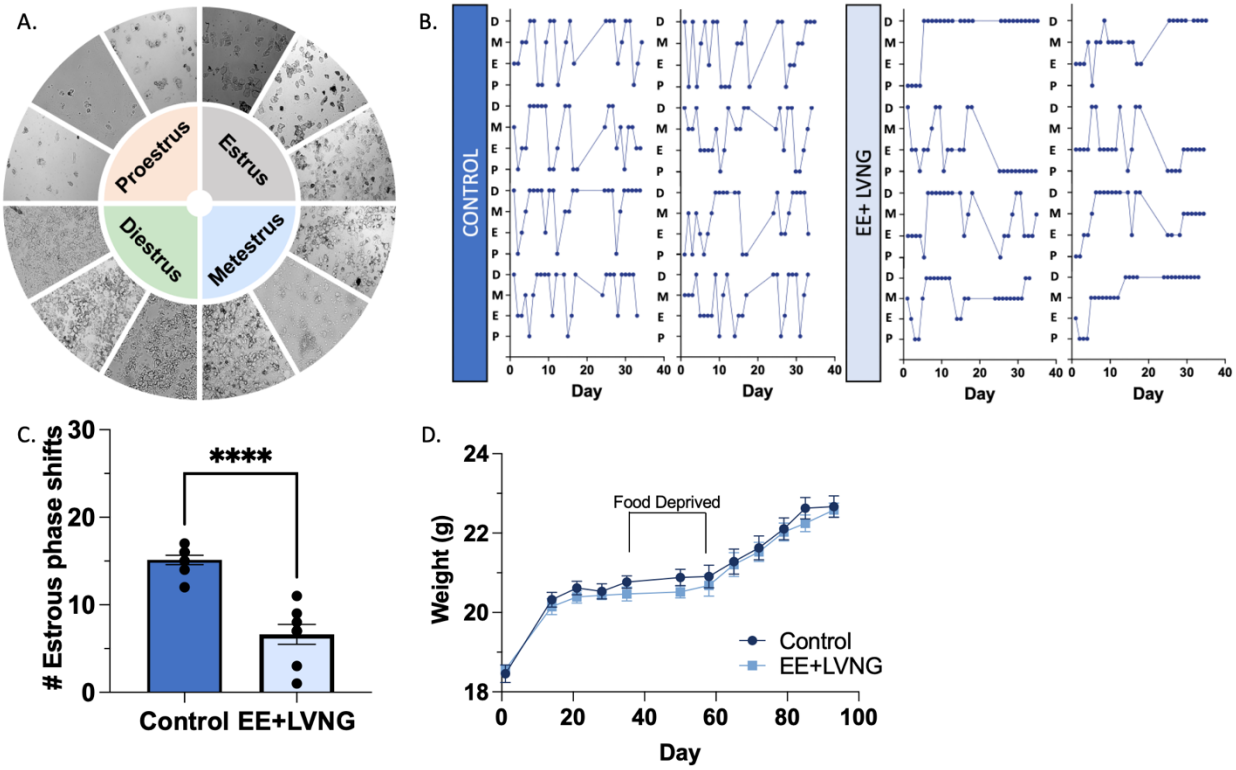

Figure 1. Estrous cycle and weight tracking. (A) Representative images of vaginal smear cell types in each phase of the estrous cycle. P: proestrus, mostly nucleated epithelial cells. E: estrus, mostly cornified cells. D: diestrus, mostly leukocytic cells. M: metestrus, a mix of all three cell types. (B) Visualization of how control and EE+LVNG mice cycled through the four phases of the estrous cycle. (C) Control mice seemed to cycle through all four phases, while EE+LVNG-treated mice did not, **** $p < 0.01$. (D) Average animal weights throughout the experiment. The bar represents when the animals were food deprived due to behavioral training. Both treatment groups gained weight during free-feeding and maintained weight during food restriction.

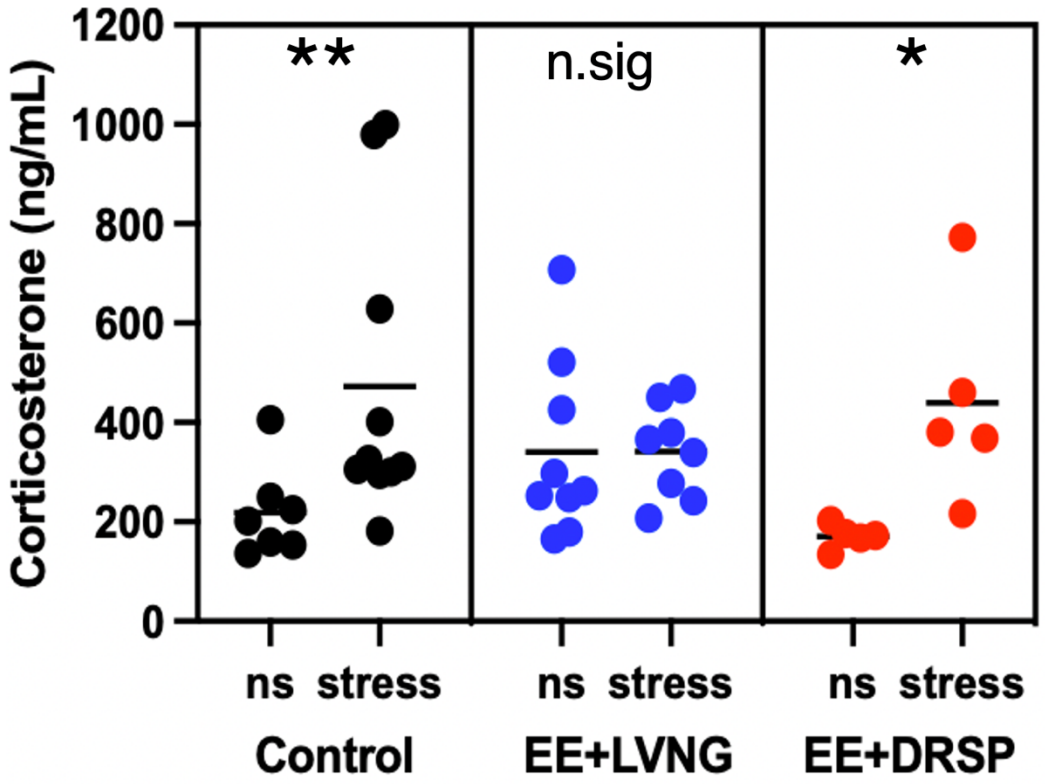

Figure 2. EE+LVNG blunts the acute stress response. Corticosterone levels (ng/ml) after no stress (ns) or acute restraint stress (stress). EE+ LVNG treatment resulted in a blunting of the corticosterone response. However, Control and EE+DRSP mice showed a robust corticosterone response to acute stress. ** $p < 0.001$, * $p = 0.01$.

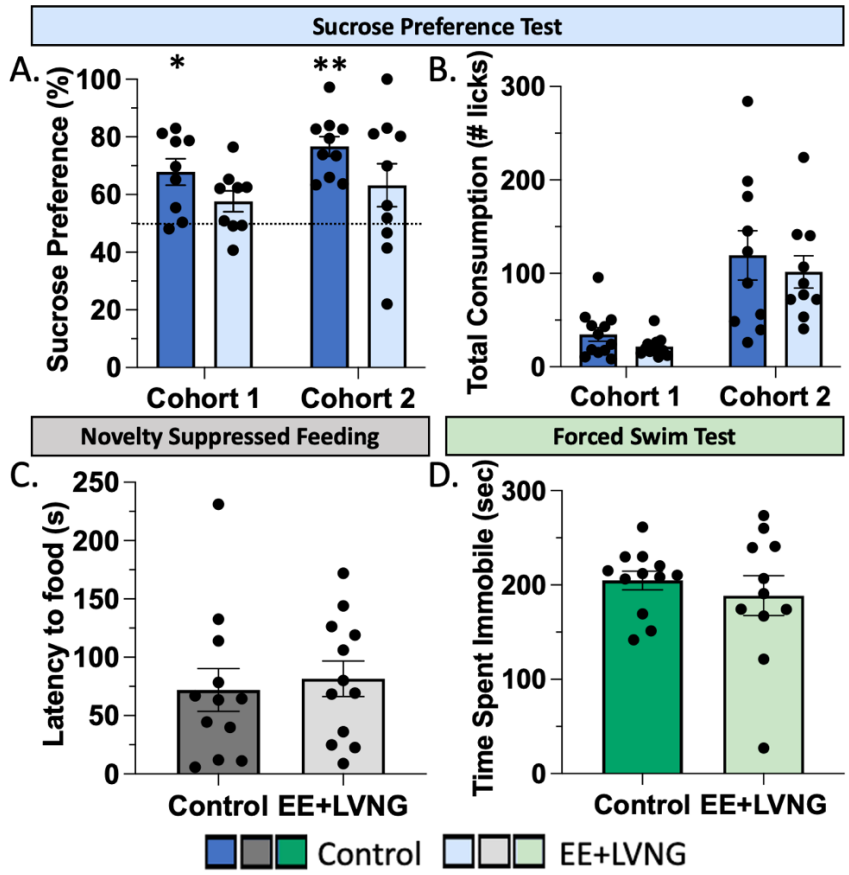

Figure 3. Behavioral tests of depression-like endophenotypes and stress-coping behaviors. (A) EE+LVNG mice in two independent cohorts of animals showed a disruption of preference for 2% sucrose compared to tap water. * $p < 0.05$ *cf* 50% ** $p < 0.001$. (B) There was no difference between groups in overall consumption in the sucrose preference test in either cohort. (C) There was no difference between Control and EE+LVNG in latency to approach a food pellet in the novelty suppressed feeding task, nor (D) time spent immobile in the forced swim test. Dark bars: Control-treated mice, Light bars: EE+LVNG-treated mice.

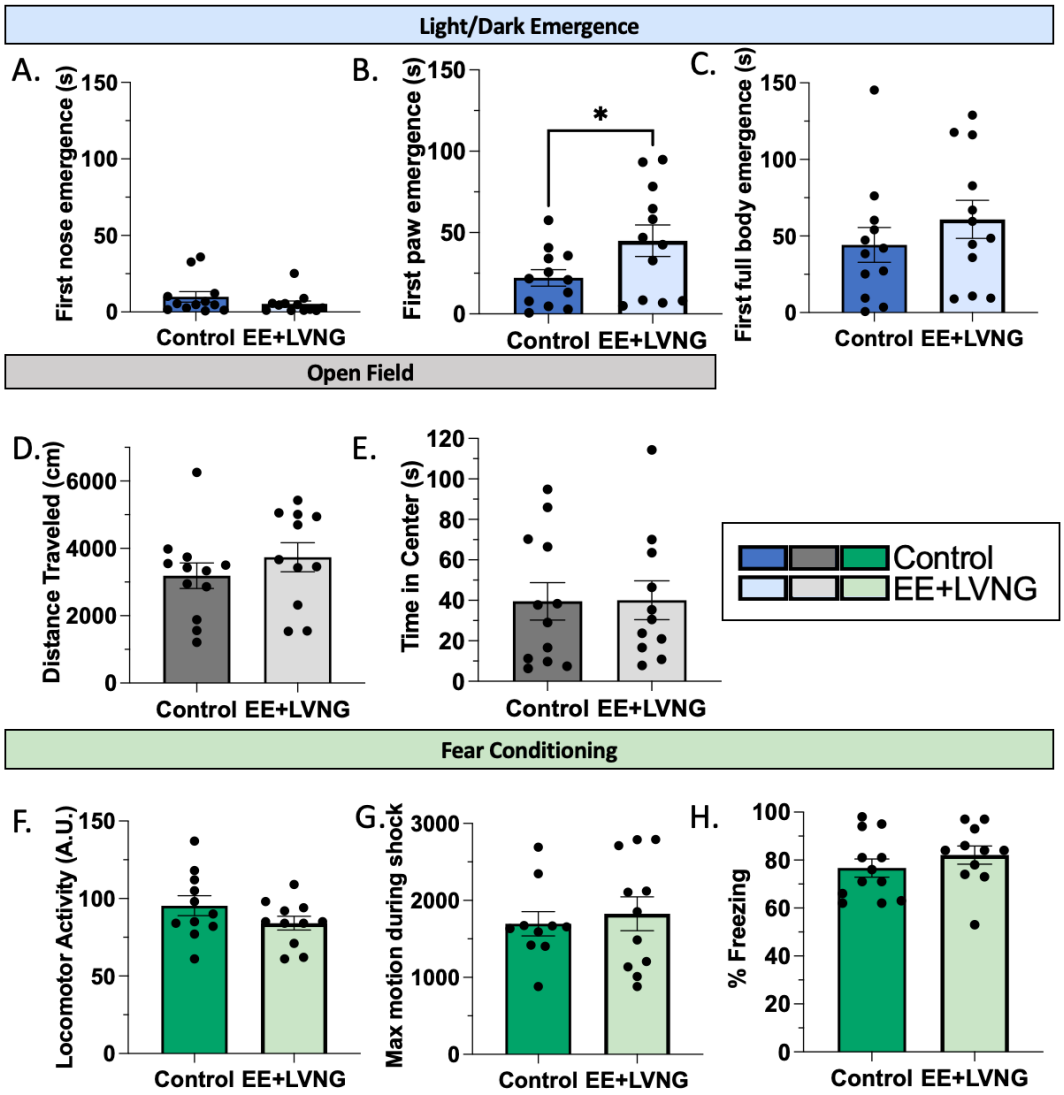

Figure 4. EE+LVNG exposure caused mild changes in risk assessment behavior, without overall anxiety-like effects. (A) EE+LVNG treated and control mice had no difference in latency for first nose emergence during the light/dark emergence task; however, (B) EE+LVNG treated mice had a longer latency for first paw emergence * $p < 0.05$. C) There was no difference in latency of first full body emergence. (D, E) Neither distance traveled, nor time spent in the center of the open field differed between treatment groups in the open field task. EE+ LVNG exposure had no effect on (F) locomotor activity during training, (G) shock reactivity, (H) percent freezing during the context test, nor (I) locomotor activity during the context test. Dark bars: Control-treated mice, Light bars: EE+LVNG-treated mice.

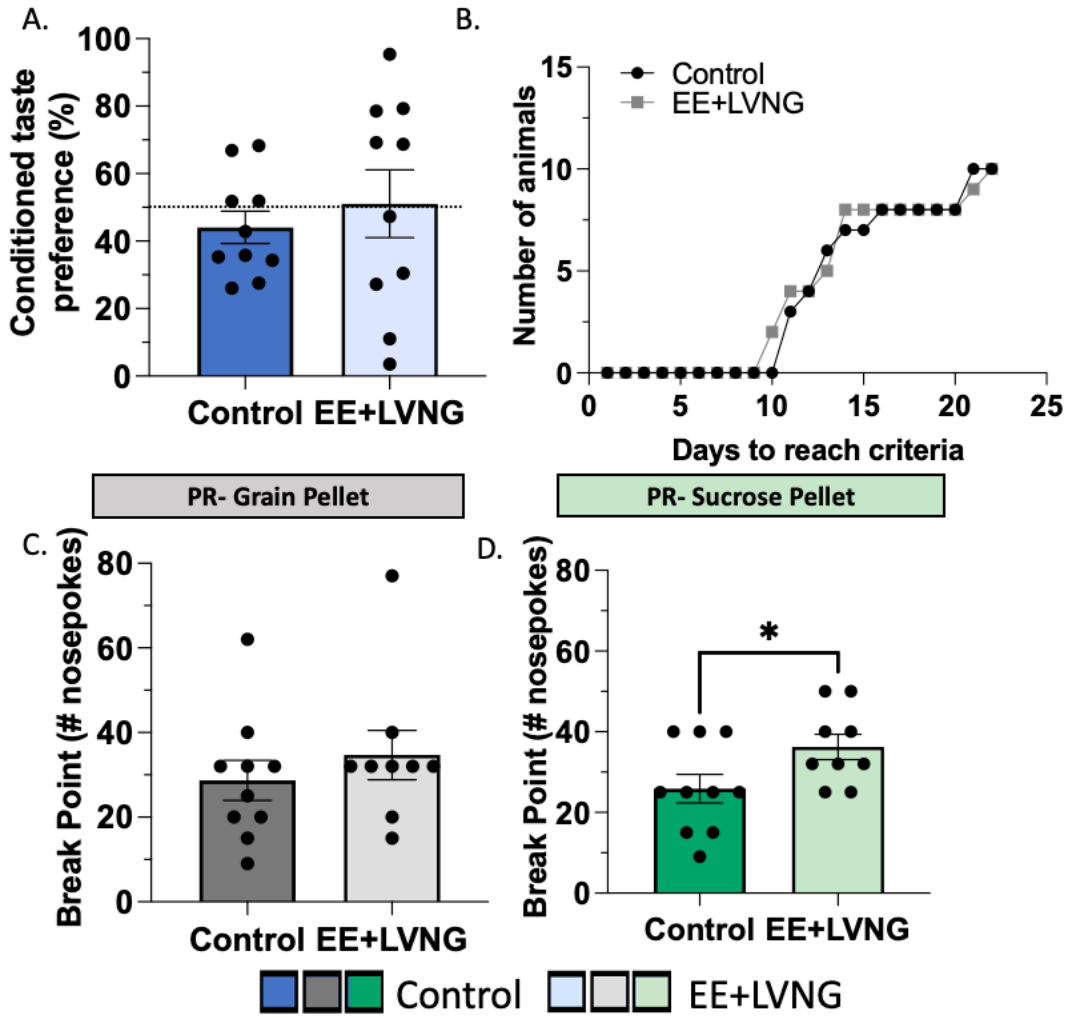

Figure 5. The sucrose preference findings could not be explained by a taste aversion nor a global decrease in motivation. (A) There was no evidence of a taste aversion to the daily oral EE+LVNG treatment. (B) There was no difference in the number of days to reach criteria in instrumental training between treatment groups. (C) The EE+LVNG-treated animals did not differ from control animals in the progressive ratio (PR) task with grain pellet rewards; however, (D) EE+LVNG-treated animals had a higher break point compared to control animals in the PR task with sucrose pellet rewards, *$p < 0.05$. Dark bars: Control-treated mice, Light bars: EE+LVNG-treated mice.