The Effects of Postpartum Estrogen and Progesterone Withdrawal on ΔFosB Expression in the Nucleus Accumbens and Anxiety- and OCD-like Behaviors in Mice

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Abstract

Anxiety and OCD are debilitating psychiatric conditions characterized by somatic, cognitive, and or behavioral symptoms that occur in anticipation of potential threats. These symptoms result from the abnormal processing of fear- or stress-provoking stimuli and alterations in brain regions responsible for motivated behaviors. Anxiety and related disorders are seen with increased prevalence during the peripartum period, which refers to the period before, during, and after pregnancy (Forray et al., 2010). Most of the literature on the emergence and exacerbation of psychiatric conditions during the peripartum period focuses on depression. As a result, there is a lack of research on anxiety and OCD during the peripartum period and the neurobiological underpinnings of these disorders. The present study used a hormone stimulated pregnancy (HSP) model in female mice to assess the effects of estradiol-withdrawal on anxiety-like and OCD-like behaviors in mice and the induction of a transcription factor implicated in compulsive-like behaviors, ΔFosB, in the nucleus accumbens (NAc), the prime brain region in regulating motivated behaviors. In addition, the present study employed a novel HSP regimen that more accurately reflects the trajectory of progesterone during human pregnancy. We found that there were no differences in anxiety- or OCD-like behavior in mice in the hormone-sustained versus hormone-withdrawn groups, however the hormone-sustained group in the modified HSP cohort exhibited greater ΔFosB induction in the NAc core. These findings suggest that progesterone influences the estradiol-withdrawal-mediated induction of ΔFosB in this region.

KEY WORDS: anxiety, OCD, compulsive, hormone simulated pregnancy, ΔFosB
Hormonal Fluctuations during the Peripartum Period

The peripartum period refers to the time before, during, and after giving birth and it presents a period of increased susceptibility to psychiatric disorders such as depression, anxiety, and obsessive-compulsive disorder (OCD). Postpartum depression affects 10-16% of new mothers and a milder form of depression or “baby blues” affects 50-85% of postpartum women (Stoffel & Craft, 2004). Anxiety also occurs in rates as high as 35% during pregnancy, 17% immediately following childbirth, and 20% six weeks postpartum, rates that exceed those of depression (Nakić Radoš et al., 2018). Additionally, the comorbidity of anxiety and PPD is 75% (Radoš et al., 2018). OCD, an anxiety-subtype, has also been reported in the literature to occur in between 2-40% and 7-21% of mothers (Forray et al., 2010). The wide range reflects methodological inconsistencies across studies. Despite the prevalence and debilitating effects of these disorders, little is known about the neurobiological and neuroendocrine underpinnings of PPD, and even less is understood about postpartum anxiety and OCD. There have been no identified differences in baseline hormonal profiles during the peripartum period between women with and without these illnesses (Bloch et al., 2003), which suggests that these conditions do not arise simply through an excess or deficit of certain hormones. Longitudinal studies have revealed that untreated cases of women with PPD and OCD can result in impaired cognitive ability, increased marital difficulties, and an increased likelihood of abusing their children, committing infanticide, and raising children with impaired cognitive, motor, and social development (Galea et al., 2001).

Susceptibility to developing depression and anxiety in the peripartum period may be related to increased sensitivity to the fluctuations in hormones that support pregnancy, fetal
growth, labor, and postpartum behaviors like caregiving. Many hormones including estrogens and progesterone fluctuate dramatically throughout pregnancy and postpartum (Cárdenas et al., 2020; Green & Galea, 2008; Neumann, 2003). Estrogen levels steadily increase throughout pregnancy, peak in the third trimester where they rise 100-1000-fold, and drop to pre-follicular levels by the fifth postpartum day (Galea et al., 2001). Subsequently, estrogen levels remain low until ovulation resumes weeks to months later. During pregnancy, estrogens are important for development of the fetus, uterus growth, maintenance of uterine lining, milk duct development, among other things (Cárdenas et al., 2021). Researchers have referred to the dramatic decrease in estradiol with the expulsion of the placenta after birth as an “estrogen withdrawal state” which could precede PPD and other mood disorders (Galea et al., 2001; Hedges et al., 2021; Zhang et al., 2016).

Estradiol is known to have mood-enhancing effects but its mechanisms of action are less clear. For example, estrogen-replacement therapy has been shown to be effective in elevating mood for women experiencing menopause (Galea et al., 2001). It is hypothesized that estradiol influences mood by regulating the release of serotonin, dopamine, GABA, norepinephrine, and corticosterone through direct reduction of these neurotransmitters/ hormones or by decreasing the affinity or number of their receptors. Specifically, acute estradiol exposure may attenuate depressive symptoms by causing a significant increase in the binding density of 5-HT$_{2A}$ receptors, a decrease in 5-HT$_{1A}$ mRNA expression, an increase in the density of serotonin transporter binding sites, and normalization of serotonin receptor mRNA levels in a genetic rat model of depression (Galea et al., 2001). Also, withdrawals from estradiol have been reported to result in dopamine receptor supersensitivity and to increase dopamine transporter mRNA levels in the brain (Bloch et al., 2003). The effects of chronic estradiol exposure (akin to what is experienced during pregnancy) are not well documented.
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Similar to estrogens, progesterone levels also increase steadily throughout pregnancy (around 10-fold), but drop slightly before birth. Progesterone loosens ligaments and joints throughout the body and increases the size of internal structures like the uterus during pregnancy (Cárdenas et al., 2021). Progesterone likely also modulates anxiety and mood, but there are conflicting findings on what it does and how it may do this. Progesterone withdrawal has been shown to both have anesthetic and anticonvulsant effects through modulation at the GABA receptor, as well as have inhibitory effects at glutamatergic synapses and modulation of serotonergic and dopaminergic neurotransmission (Bloch et al., 2003).

Mood alterations occur during other time points, not just the peripartum period, and there is a subset of women who are particularly vulnerable to these hormonal fluctuations. Previous literature has shown that other periods involving hormonal fluctuations such as the premenstrual period and menopause are also associated with negative mood symptoms, suggesting that there is increased an vulnerability to the mood-destabilizing effects of reproductive hormones (Bloch et al., 2003). However, current research does not report consistent findings as to whether lower or higher levels of these hormones lead to these mood changes. Several studies have found that PPD symptoms can be alleviated by estradiol treatment in humans (Galea et al., 2001, Sichel et al., 1995), while others have failed to replicate this finding (Klier et al., 2007). Depression scores in women with PPD have been shown to improve with progesterone administration (Josefsson et al., 2002) while others have shown that administration of progesterone raises depression scores in a group without PPD (Cizza et al., 1997). In addition, experimental increases of estradiol and progesterone levels followed by an abrupt withdrawal causes significant depressive symptoms in women with previous PPD but not in controls (Bloch et al., 2000). This suggests that there is a subset of women, those with a history of mood disorders, who are more sensitive to changes in
hormone levels and that these changes (rather than absolute hormone levels) are what contribute to mood disorders. The biological basis of this differential sensitivity is unknown but is speculated to be a result of genetic polymorphisms in genes involved in regulating reproductive hormone signaling (Forray et al., 2010). Ultimately, the changes in hormones that drive neurobiological and cognitive changes across the peripartum period are critical for offspring development and caregiving but may also result in mood changes and anxiety in new mothers.

Research on mood changes caused by changes in gonadal steroids during the peripartum period in humans is sparse and has several limitations. For example, there are changes in other hormonal axes (like the HPA axis), and confounding factors such as pain, obstetrical complications, levels of support, and psychological stress surrounding childbirth and motherhood that could contribute to mood disorders. Since it is difficult to draw causal relationships between hormones and mood changes in humans, researchers have turned to animal models in order to directly test the role of peripartum estrogen and progesterone fluctuations in a controlled setting.

**Hormone Simulated Pregnancy (HSP) model**

One animal model used to test the impact of dramatic changes in ovarian hormones, namely estradiol, on the brain and behavior is a hormone-simulated pregnancy (HSP) model. This model is based on the estrogen withdrawal hypothesis and is consistent with clinical literature that estrogen withdrawal following HSP results in depressive- and anxiety-like behaviors in rodents. The original study carried out by Galea et al. (2001) administered estradiol and progesterone daily to ovariectomized rats to create a hormone-simulated pregnancy, then the hormones were abruptly withdrawn to mimic early postpartum. This group found that the “pregnant” rats demonstrated increased depressive-like behavior which was operationalized as increased immobility on the forced swim task (FST) and this depressive behavior is prevented by
continued estradiol treatment (Galea et al., 2001, Stoffel and Craft, 2004). Importantly, the increased immobility seen in “pregnant” rats was not due to an overall reduction in locomotor activity levels since they displayed more activity than the other groups in the Open Field Test (OFT), a measure of anxiety and locomotor activity. Additional rodent studies have supported these findings. These findings are also in line with human studies which have found that experimentally-induced estradiol withdrawal causes depressive symptoms in women with a history of PPD and that estradiol administration can relieve these symptoms.

The increase in depressive-like behavior in the postpartum period simulated by the HSP model is reported consistently, but there are conflicting findings as to whether this model also causes increased anxiety-like behaviors in rodents. In humans, anxiety is characterized by transient or long-lasting somatic, cognitive, and behavioral symptoms that occur in anticipation of possible future threats. It arises from the abnormal processing of fear- or stress-provoking stimuli in the limbic system of the brain and altered processings of regions that translate planning into motor behavior and ultimately mediate goal-directed behavior. Anxiety-like behaviors can be modeled in rodents and some of these studies have shown that estrogen withdrawal does not yield increased anxiety-like behaviors in rodents (Galea et al., 2001, Stoffel and Craft, 2004), while others have demonstrated that it does (Zhang et al., 2016; Hedges 2021). Hedges et al. (2021) found that estrogen-withdrawn female Syrian hamsters had increased anxiety-like behaviors in tests of anxiety like the Elevated Plus Maze (EPM) and Open Field Test (OFT), but did not differ from control females in measures of anhedonia (a core feature of depressed mood) in the sucrose preference test. Additionally, some research has shown that progesterone administration decreases anxiety-like behaviors in rodents. For example, ovariectomized females treated with progesterone show decreased anxiety-like behaviors in the Elevated Plus Maze.
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(Bitran et al., 1995). This is in line with the fact that female rats tend to show fewer anxiety-like behaviors during proestrus, when levels of progesterone and its metabolites are high (Stoffel & Craft, 2004). However, Stoffel & Craft (2004) failed to find any increases in anxiety-like behavior during the withdrawal phase in HSP-treated rats. The effects of hormones like estrogen and progesterone on the peripartum period are not consistent across studies and require additional research to clarify their effects and the mechanisms through which these hormones act.

The HSP doses were initially chosen for their ability to elicit maternal behavior but it does not accurately reflect the trajectory of progesterone in human pregnancy, so there should be some caution in translating its findings. Since rodents and humans exhibit different pregnancy profiles, humans may be affected by estrogen withdrawal differently. In rodents, progesterone levels are high and estradiol levels are low throughout the first and second trimester. During the third trimester, estradiol levels rise the final days before birth and progesterone levels drop. In humans, estradiol levels are high throughout pregnancy and steadily increase. Progesterone levels also remain high but are especially high during the third trimester. As a result, humans may be more affected by estrogen withdrawal since levels are consistently high throughout pregnancy whereas rodents may not be as affected since their levels are high only in the last couple days of pregnancy. In light of this, more recent studies have adapted the original HSP model to be more in line with human conditions. For example, Suda et al. (2008) withdrew 17β-estradiol and progesterone simultaneously at ‘delivery’ in attempts to better model the trajectory of human pregnancy. In addition, estradiol and progesterone are only some of the hormones that fluctuate during pregnancy - corticotropin-releasing hormone and oxytocin also play important roles and mediate mood changes. Nonetheless, the HSP model allows researchers
to isolate postpartum estrogen withdrawal as the single manipulated variable which allows them to make causal inferences about changes in the brain and behavior.

**Obsessive Compulsive Disorder (OCD)**

OCD is a severe psychiatric disorder that is characterized by obsessive thoughts and/or compulsive acts. This disorder is often very debilitating and potentially chronic. The obsessive-compulsive (OC) symptom manifestation exhibited in OCD is mostly driven by anxiety (de Brouwer et al., 2019). OCD is often accompanied by a range of symptoms but research reveals a consistent pattern with postpartum OCD in regards to the content of the obsessions and compulsions. Postpartum OCD obsessions often include ego-syntonic intrusive thoughts of harming the infant along with avoidance behaviors or checking rituals. In addition, postpartum OCD is characterized by the rapid onset of obsessional symptoms after birth and if untreated, this disorder can result in consequences for the mother, family, and child (Forray et al., 2010). The most updated treatments for OCD are high-dose selective serotonin reuptake inhibitors, a tricyclic antidepressant called clomipramine, and/or cognitive behavioral therapy. However, about two-thirds of OCD patients fail to reach a satisfactory response which highlights the need for a better understanding of the etiology disorder (Rapinesi et al., 2019). Deep brain stimulation (DBS) and transcranial magnetic stimulation are some treatments that have been shown to be efficacious for treatment-resistant forms of OCD. Although DBS is invasive, long term responder rates are greater than 60% and this treatment may not only reduce obsessions and compulsions but also anxiety and depression (Rapinesi et al., 2019). Additional research is required to elucidate the complex etiology of OCD in order to find additional efficacious treatments.
Despite OCD’s severity, most studies researching peripartum mood disorders focus on depression. Few studies delve into anxiety disorders and OCD. This is noteworthy because clinical research has consistently reported both the onset and worsening of preexisting OCD during this period (Forray et al., 2010; Stoffel & Craft, 2004). Research also supports the onset and worsening of OCD during the premenstruum, which highlights the role of hormones like estrogen and progesterone in this disorder. A study by Forray et al. (2010) found that women with pregnancy-related onset of OCD or perinatal worsening of pre-existing OCD are more likely to experience premenstrual exacerbation of OCD symptoms compared to women whose OCD onset was not related to pregnancy and whose symptoms were unaffected by pregnancy.

The influence of ovarian hormones on OCD has also been modeled in rodents. Flaisher-Grinberg et al. (2009) found that ‘compulsive’ lever-pressing, which is analogous to excessive and unreasonable behavior in OCD, in female rats fluctuates throughout the estrous cycle, being lowest at estrus and rising through diestrus. Specifically, the high amount of compulsive lever-presses during late diestrus and proestrus corresponds to points in the estrous cycle where estradiol levels are high and progesterone levels are also increasing. The low amount of compulsive lever-presses during estrus corresponds to a point where levels of progesterone and estradiol are low. The finding that high levels of estradiol and progesterone during late diestrous and proestrous increases compulsive-like behavior is interesting as the researchers also found that acute administration of estradiol to prepubertal female rats decreases compulsive lever-pressing and that withdrawal from repeated administration of estradiol results in increased compulsive lever-pressing in pre-pubertal female rats. The finding that there were cyclic changes in compulsive lever-pressing but not excessive lever-pressing in general suggests that compulsive lever-pressing is modulated by ovarian hormones. To further support the
relationship between estradiol and compulsive lever-pressing, inspection of plasma levels of ovarian hormones in this study revealed that there was a correlation between level of estradiol, but not progesterone, and compulsive behaviors. This finding suggests that estradiol is modulating compulsive behaviors and is consistent with studies using other models of OCD such as the 8-OH-DPAT model (Agrati et al., 2005). In addition, acute administration of estradiol decreases the amount of compulsive lever-pressing and withdrawal from repeated estradiol administration has the opposite effect - it increases compulsive lever-pressing. This increased compulsivity following withdrawal from repeated estradiol administration is consistent with the “estrogen withdrawal” hypothesis that suggests withdrawal from estradiol contributes to the worsening of OCD symptoms and other mood disorders.

Acute administration of estradiol likely exerts its anti-compulsive effects through genomic longer acting mechanisms, as opposed to non-traditional/ fast acting mechanisms. In support of this, Flaisher-Grinberg et al. (2009) found that estradiol only exerted anti-compulsive effects when administered 24 hours before the test, but not when administered one hour before the test. Estradiol can act through its genomic action to interact with systems involved in OCD such as the serotonergic and dopaminergic systems and the orbitofrontocortex (Flaisher-Grinberg et al., 2009).

**Brain Changes During the Peripartum and Their Involvement in Mood/ Anxiety Changes**

There are many structural and functional brain changes associated with the peripartum period. For example, there is a correlation between changes in the hippocampus and depression/ other mood changes. Specifically, reduced cell proliferation in the dentate gyrus of the hippocampus and a loss of hippocampal spine synapses is evident in some animal models of depression and in a hormone-simulated pregnancy model in rats (Baka et al., 2017; Green &
Galea, 2008). Since this plasticity is modulated by estradiol, fluctuations in this hormone during the peripartum period may contribute to PPD symptoms via its effects on hippocampal plasticity. This idea is supported by a study carried out by Green & Galea, (2008) which found that estrogen withdrawal in an HSP model decreased hippocampal cell proliferation in “pregnant” rats and this decrease was prevented by treatment with antidepressants or the ERbeta agonist diarylpropionitrile. This study also demonstrated that repeated, but not acute, exogenous doses of estradiol prevented depressive behavior in the FST but did not rescue the decrease in cell proliferation in the dentate gyrus. Also, the length of exposure time to estradiol changes its anxiolytic effects on measures of anxiety such as the Open Field Task and repeated exposure to estradiol increased cell proliferation in the hippocampus. Further, Galea et al. (2001) showed that chronic exposure to estradiol significantly decreased immobility time compared to controls but the suppressive effect of estradiol on immobility diminishes at high doses. These studies suggest that the effects of estradiol depend upon duration of administration, the animal’s hormonal environment, and the dose.

The findings of the Green & Galea (2008) study demonstrate that male and female rats show different responses in tests of depressive-like behavior such as learned helplessness and the FST, so it is likely that female rats have different neural responses to mood disorder pharmacological treatments and markers of depression. Males and females differ in their etiologies of mood disorders and past literature on mood disorders has primarily used male rodents, so future research should include increased representation of female rodents.

Abnormalities in several neurotransmitter systems such as dopaminergic, serotonergic, glutamate, GABA, as well as the neuropeptide oxytocin (OXT) and the corticotropin releasing factor (CRH) (in rodents) or cortisol (in humans and non-human primates) likely also contribute
to postpartum depression and OCD-related anxiety. These abnormalities are at least partly influenced by ovarian hormones like estradiol. Estrogen withdrawal during the postpartum period likely causes changes in these neurotransmitter systems to result in postpartum anxiety-like behaviors (Hedges et al., 2021, Neumann, 2003).

Many brain regions are implicated in the presentation of OCD, however those important for evaluation, affect regulation, and reward-based decision making, such as the nucleus accumbens (NAc), are of particular interest (Alonso et al., 2015). The nucleus accumbens, commonly regarded as the reward center of the brain, plays a role in the rewarding effects of certain stimuli and regulates responses to natural reinforcers like food, drink, sex, and social interactions including maternal care (Nestler et al., 2001). This region is plastic in response to hormone-mediated changes, especially those induced by estradiol (Eisinger et al., 2018). Previous studies have shown that altered structure and activity in this region is linked to depression and anxiety in the postpartum (Haim et al., 2014), so the nucleus accumbens may also play a role in other postpartum mood disorders like OCD.

In fact, ovarian hormones such as estradiol are known to have profound effects on the structure and function of the nucleus accumbens in order to support female motivational behaviors that lead to successful copulation as this brain region is responsible for motivated behaviors (Eisinger et al., 2018). Estradiol exerts striking effects through estrogen receptor-metabotropic glutamate receptor (ER/mGluR) complexes in the NAc where it can regulate gene expression and lead to long term changes in neuronal function (Staffend et al., 2011). The relationship between estrogen receptors and mGluRs across many brain regions appears to be unique to females (Staffend et al., 2011). One of the most prominent effects of ER/mGluR signaling is the change of functional circuitry in the nucleus accumbens which can be
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identified through structural changes in medium spiny neurons (MSNs), the primary neuronal subtype in this region. Estradiol injections in adult ovariectomized rats decreases MSN dendritic spine density in the NAc core and increases MSN spine density in the NAc shell (Staffend et al., 2011). These findings indicate that estradiol and other ovarian hormones result in structural and functional changes in the NAc.

Estradiol also encourages other motivated behaviors, such as those seen in drug addiction phenotypes, as drugs of abuse exert their effects by co-opting the brain’s reward system (the NAc). The interrelation between hormones and drugs of abuse is supported by sex differences between men and women in drug addiction, as women are more likely to reach an addicted state than men, begin psychostimulants at younger ages, have enhanced subjective responses, and become addicted at faster rates (Staffend et al., 2011). In addition, women report enhanced subjective experiences of stimulant drugs when estradiol levels are higher, whether this be through endogenous means or exogenous administration. These sex differences in addiction have been modeled in rodents where ovariectomies eliminate the sex differences in addiction and estradiol replacement restores these differences (Staffend et al., 2011). Drugs of abuse result in long-term changes in MSN excitability and structure in the NAc through ER/mGluR signaling that results in alterations in patterned motor programs to obtain rewards and reward processing which ultimately promotes drug addiction. These findings point to the fact that estradiol impacts motivated behaviors such as reproductive and addiction-promoting behaviors through neuronal changes in the NAc, which suggests that the NAc likely experiences changes during the peripartum period. Further, since drug addiction and OCD are caused by similar alterations in patterned motor activity and reward processing and estradiol influences both these phenotypes, changes that occur in the NAc as a result of prolonged drug exposure may be seen after
prolonged estradiol exposure and subsequent withdrawal. Understanding the influence of these hormone-mediated changes in the nucleus accumbens may be important in elucidating the etiology of postpartum anxiety and OCD.

**ΔFosB in the Nucleus Accumbens**

ΔFosB is a viable molecular marker for measuring neuroplasticity in the nucleus accumbens and it may be the means through which estrogen withdrawal impacts anxiety-like behavior in mice. The regulation of gene expression is known to be important in neuronal plasticity. As a transcription factor, ΔFosB can induce long term changes at the cellular level which have behavioral effects including the onset and worsening of psychiatric disorders like depression and anxiety (Sun et al., 2013). As a result, identifying the location and distribution of ΔFosB in the nucleus accumbens may yield insight into specific neural changes that occur following a hormone-simulated pregnancy.

Because ΔFosB is a member of the Fos family of transcription factors. It has a long half-life afforded to it by its unique stability which allows it to initiate and sustain changes in gene expression that persist well after the compulsive behaviors cease. ΔFosB can induce these behavioral changes by regulating gene transcription related to cell proliferation, differentiation, and transformation. Unlike other members of the Fos family, which are induced rapidly and transiently in response to an acute stimulus, ΔFosB accumulates in response to repeated stimulation and is stable across time. ΔFosB accumulation in the nucleus accumbens is important for reward and motivation and is implicated in drug addiction.

ΔFosB accumulates in some regions of the nucleus accumbens and dorsal striatum after repeated administration of many kinds of addictive drugs and compulsive running in mice which suggests that ΔFosB may accumulate in response to other compulsive behaviors (Nestler, Barrot,
& Self, 2001). Studies in transgenic mice that overexpress either ΔFosB or a dominant negative inhibitor of the protein suggest that ΔFosB causes increased sensitivity to the behavioral effects of drugs of abuse and likely also increased drug seeking behavior (Nestler, Barrot, & Self, 2001). McClung et al. (2004) found that FosB knockout mice show heightened sensitivity to the initial locomotor and rewarding effects of cocaine yet lack the behavioral sensitization that typically occurs with repeated drug administration. The same researchers also found that ΔFosB has been shown to be induced in the nucleus accumbens as a result of chronic stress. Interestingly, the level of accumulated ΔFosB is shown to be negatively correlated with the degree of learned helplessness in inescapable foot shock experiments (McClung et al., 2004). This suggests that ΔFosB induction serves as an adaptive coping mechanism that prevents the development of learned helplessness. Moreover, this supports ΔFosB’s role in pro-reward and pro-motivational behaviors. Together, these findings support the idea that ΔFosB operates as a molecular switch that can convert acute responses into long-term neural and behavioral changes that support motivated behaviors. Thus, it is likely that ΔFosB will be induced in this region alongside the altered motivated behavior and repetitive patterns seen in OCD.

**Measuring Anxiety-like and OCD-like Behaviors in Rodents**

The Elevated Plus Maze (EPM) and Open Field Test (OFT) are well-validated tests that have been used to measure anxiety-like behavior and spontaneous locomotor activity in rodents. In the EPM, rodents are placed at the junction of the four arms of the maze facing an open arm. Entries and duration in each arm are recorded over a period of time. In the OFT, rodents are placed in an arena and allowed to freely move for a period of time. The distance moved, velocity, and time spent in pre-defined zones is recorded. These tests require no training. Also, unlike other behavioral assays that measure anxiety such as electric shock or food/water deprivation,
these assays rely on the natural tendency of animals to explore a novel environment and exploit their innate aversion to open spaces. Lower amounts of time in the open arms of the EPM and in the center of the OF indicate increased anxiety and are correlated with significantly greater plasma corticosterone concentrations (Pellow et al., 1985). Previous studies have shown that late pregnancy and estrogen withdrawal increases the behavioral measures of anxiety by decreasing the number of entries and time spent in the open arms of the EPM (Hedges et al., 2021; Neumann et al., 1999). In addition, Galea et al. (2001) and others have used the OFT to discount that “pregnant” female rodents exhibit increased depressive or anxiety-like behavior because they are less active, since the “pregnant” mice exhibited more area crossings than the other groups.

Modeling OCD is difficult since obsessions are an internal experience and assessing them depends on verbal/ written communication. However, animal models can replicate compulsions, which are the repetitive overt or covert behavioral routines that are expressed in OCD to alleviate obsession-driven anxiety. Some researchers may argue that compulsions in OCD are not spontaneous and are instead provoked by obsessions, so they are phenomenologically distinct from other repetitive behaviors like tics or stereotypic behaviors seen in patients with head injuries. However, OCD can be conceptualized as a consequence of overactive habit-forming circuitry with a lack of sufficient top-down control over these habits, so animal models can still be useful (Alonso et al., 2015).

Marble burying by rodents is one type of behavioral assay to measure OCD-like anxiety. Marble-burying takes advantage of the natural proclivity of rodents to dig in both natural settings and in standard cage bedding and is a simple assay of repetitive, compulsive-like behavior in rodents. During the Marble Burying Test (MBT), a certain number of marbles are arrayed on the
surface of clean bedding and the number of marbles buried in a certain amount of time is scored. A higher number of buried marbles is interpreted as a higher level of compulsivity. Previous literature has demonstrated that this test is reflective of perseverative, repetitive behavior that is highly resistant to change based on familiarity (Ahmari, 2016). It is also cost-effective, easy to carry out, there is high accuracy in scoring the behavior and spontaneous exhibition of rodents’ behavior, and it can be used in the screening of genetically modified mice for abnormal behavioral phenotypes. As a result, the MBT is a viable option for shedding light on the hormonal influences of compulsive-like behavior and can ultimately facilitate the identification of novel-drug receptor interactions important for anxiety and OCD.

Present study

The present study used both the original HSP regimen and a novel modified HSP regimen in female mice to evaluate the impact of postpartum estrogen and progesterone withdrawal on several measures of anxiety and OCD-like anxiety as well as on plasticity in the nucleus accumbens. Since past HSP hormone regimens have not accurately reflected the trajectory of progesterone during the peripartum period in humans, we tested whether more closely modeling its trajectory during pregnancy would cause previously documented behavioral and brain changes. We hypothesized that estrogen withdrawal would lead to both increased anxiety-like behaviors and OCD-like behavior in mice, but have a greater impact on anxiety-like behavior. We also hypothesized that progesterone withdrawal would lead to both increased anxiety-like and OCD-like behavior in mice, but have a greater impact on OCD-like behavior. Finally, we hypothesized that we would see greater ΔFosB induction in the nucleus accumbens core of the hormone-withdrawn animals.

Methods and Materials
Subjects

30 adult female C57/Bl6 mice weighing 18-23 g purchased from Charles Rivers Laboratories were used as subjects in the present study. The mice were about 11 weeks old when delivered. They were housed in a temperature-controlled room with a 12:12 reverse light-dark cycle. As mice are social animals, they were housed with one other cage-mate in solid-bottom cages filled with aspen bedding. Food pellets and water were available *ad libitum*.

Ovariectomy

Ovariectomies were performed to remove endogenous sources of circulating hormones such as estradiol and progesterone before initiating the HSP. Mice were anesthetized using isoflurane anesthesia (2-5% vaporized in oxygen). Analgesic (meloxicam, 5 mg/kg) was administered subcutaneously before the start of surgery. Subjects’ bilateral flanks were shaved and then cleaned with three alternating swabs of 70% ethanol and betadine before being transferred to a sterile surgical field. Mice were under anesthesia via a nosecone for the duration of the surgery. Subjects’ ovaries were extracted by bilateral flank incisions and removed via cauterization of the uterine horns. Polydioxanone absorbable suture was used to close the smooth muscle and stainless steel wound clips were used to close the skin incisions. The statuses of the ovariectomized mice were checked 15 min, 30 min, and 1 hour post-surgery, and for 3 days after. Two subjects did not recover from the surgery (n=30).

Hormone Administration

Following ovariectomies, an HSP regimen was initiated. Each day, the ovariectomized female mice were subcutaneously injected with either hormone or oil at around 6:30pm EST for 28 days. Group 1 (n=8) and Group 2 (n=8) were injected with low estradiol (0.5 μg) high progesterone (0.8 mg) dissolved in 0.1 mL oil for 16 days. On days 17-28, Group 1 received high
estradiol (10 μg) dissolved in 0.1 mL oil. Group 1 represents a replication of the estrogen sustained group in the commonly-used HSP (Galea et al., 2001, Zhang et al., 2016). Group 2 received high estradiol (10 μg) on days 17-23 and 0.1 mL oil on days 24-28. Group 2 represents replication of the estrogen withdrawn group in the original HSP. Group 3 (n=7) and Group 4 (n=7) received low estradiol (0.5 μg) and low progesterone (0.4 mg) dissolved in 0.1 mL oil for 16 days. Group 3 received high estradiol (10 μg) and high progesterone (0.8 mg) dissolved in 0.1 mL oil on days 17-21 and just high estradiol (10 μg) dissolved in 0.1 mL oil on days 22-28. Group 3 represents the modified sustained model. Group 4 received high estradiol (10 μg) and high progesterone (0.8 mg) dissolved in 0.1 mL oil on days 17-21, just high estradiol (10 μg) dissolved in 0.1 mL oil on days 22-23, and 0.1 mL oil on days 24-28. Group 4 represents the modified withdrawn model and more accurately reflects the fluctuations of estradiol and progesterone in a real human pregnancy. Groups 3 and 4 (the modified cohort) more accurately reflect the trajectory of progesterone during a human pregnancy. The HSP doses were chosen based on previous studies to simulate pregnancy in nulliparous ovariectomized mice (Galea et al., 2001). No vehicle group was used because there is sufficient literature on how control mice respond in the tests for anxiety and OCD-like anxiety that are used in this study. A description of the four hormone groups is found in Table 1.

Behavioral Assays

Elevated Plus Maze

The Elevated Plus Maze was used to assess anxiety-like behavior since it has been shown to measure anxiety-like behavior following HSP in rodents (Hedges et al., 2021, Zhang et al., 2016). The apparatus consisted of a plus-shaped maze elevated 73 cm above the floor, with two open arms (51 x 11.5 cm) and two enclosed arms (51 x 11.5 x 39.5 cm). Animals were placed in
the center of the maze facing a closed arm and allowed to freely explore the maze for five
minutes. To counterbalance the groups, half of the mice performed the EPM on day 25 and the
other half performed the EPM on day 26. Tests were recorded and the amount of time spent in
each arm, velocity, as well as the total distance traveled were recorded via Noldus Ethovision.

**Open Field Test**

The Open Field Test was used to measure anxiety-like and locomotor behavior in mice
since it has been previously used following HSP in rodents (Hedges et al., 2021, Zhang et al.,
2016). The apparatus consisted of a 40.5 x 40.5 x 30 cm arena. Animals were placed in the center
of the apparatus and allowed to freely explore for five minutes. To eliminate the influence of test
order on the results, the mice that performed the EPM on day 26 did the OFT on day 25 and the
mice that performed the EPM on day 25 did the OFT on day 26. Tests were recorded and the
amount of time spent in the center of the field, periphery of the field, velocity, and total distance
traveled were recorded via Noldus Ethovision. Locomotor activity over the five minute period
was also recorded. The apparatus was cleaned in between testing for each animal.

**Marble Burying Test**

The Marble Burying Test was carried out to assess OCD-like anxiety. All mice were
handled daily for several minutes for 12 days before the baseline marble burying test was carried
out in order to habituate them to being handled. A second round of the Marble Burying Test was
carried out on day 27 of “pregnancy.” The test was conducted using 32 standard plexiglas mouse
cages with metal lids. Prior to the test, 15 marbles were dispersed evenly across Aspen bedding
(2.5 cm from the side of the cage, and 4 cm from each other) covering the bottom of the cage 3.5
cm deep. Each mouse was then placed into the cage for a 30 minute testing period. Marbles were
counted as ‘buried’ if they were more than two thirds covered and were logged on the marble burying behavior (MBB) counting form. All mice were tested at roughly the same time.

**Animal Sacrifice**

On postpartum day 5, all 30 subjects were sacrificed via anesthetized rapid decapitation. Prior to this procedure, the mice were anesthetized with 5% isoflurane anesthesia. The subjects were then decapitated with a razor blade. The brains were removed and immediately stored in a paraformaldehyde solution for approximately 48 hours before being transferred to a 30% sucrose cryoprotectant solution. The brains were stored in this solution at -20°C until sectioning.

**Tissue Histology**

**Sectioning**

Brains were washed with deionized water. Then, the olfactory bulbs and cerebellum were removed with a razor blade and brains were mounted onto a cryostat chuck using Optimal Cutting Temperature (OCT) compound. The brains were coronally sectioned at 35μm thickness using a manual cryostat set at -20°C. The sections were collected into wells containing cryoprotectant and stored at -20°C until staining.

**Immunohistochemistry**

Immunohistochemistry was performed in order to quantify and determine the distribution of FosB-containing cells in the nucleus accumbens. This technique is used to visualize proteins in cells and it is well-established, relatively low-cost, and has a fast turn-around time. Tissue sections containing the NAc were washed 5 X 5 minutes in PBS, incubated in 30% hydrogen peroxide, washed again in PBS, then incubated with rabbit anti-FosB primary antibody at the dilution of 1:2000 for 36 hours. The tissues were then washed, incubated with goat anti-rabbit biotinylated secondary antibody at the dilution of 1:600 for ~1 hour, and washed again. After, the
tissue was incubated with an avidin biotin complex (ABC) stock solution for 1 hour. Finally, a nickel-enhanced DAB reaction was used to visualize FosB-containing cells. The tissue was stored in 6 well plates in PBS at 4°C until mounting.

**Mounting and Microscopy**

The sections were mounted onto slides, dehydrated, cleared, and cover-slipped. The slices were visualized using a Nikon Eclipse E400 brightfield microscope with ‘SPOT basic’ software. Photomicrographs of the left and right hemispheres of the caudal NAc were taken at 10x magnification. Each image was post-processed using the ‘Dark 1’ filter in Google Slides, as this allowed for better visualization of the FosB-positive cells. Quantification of FosB-containing cells was carried out by observers blind to the condition of the animal by marking each FosB-positive cell on the image using the marking tool on a computer.

**Statistical Analyses**

All data were analyzed using Jamovi (Version 1.6.23.0) for Mac and significance was defined as p<0.05. One-way between subjects ANOVAs were performed to examine mean group differences in measures of OCD-like and anxiety-like behavior exhibited in the Marble Burying Test, Open Field Test, and Elevated Plus Maze. Specifically, the means for the total distance moved (cm), velocity (cm/s), difference score (time spent in periphery - time spent in the center), and relative score [time spent in periphery/ (time spent in center + time spent in periphery)] in the Open Field Test were compared. The means for the total distance moved (mm), mean velocity (mm/s), difference score (time spent in open arms - time spent in closed arms), and relative score [(time spent in open arms/ (time spent in open arms + time spent in closed arms)] in the Elevated Plus Maze were also compared. Independent samples t-tests were conducted to examine mean differences between anxiety-like behavior in the OFT and EPM within each
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cohort. Specifically, the independent samples t-test compared the means of the total distance moved (cm), velocity (cm/s), duration in periphery (s), duration in center (s), difference score, and relative score in the OFT and the total distance moved (mm), velocity (mm/s), duration in open arms (s), duration in closed arms (s), difference score, and relative score in the EPM between the sustained and withdrawn groups in each cohort (original and modified) separately. Independent samples t-tests were also used to examine the mean differences in FosB induction in the NAc core and shell between the sustained and withdrawn groups in the modified cohort.

Results

Marble Burying Test

Graphs for the Marble Burying Test results for each hormone group are shown in Figure 1. There was no significant effect of hormone group on the difference score in marbles buried postpartum compared to marbles buried during the baseline test, [F(3,26) = 0.288, p=0.834].

Open Field Test

Graphs for relevant measures in the Open Field Test are shown in Figure 2. There was a significant effect of hormone group on total distance moved [F(3,26) = 3.859, p=0.021] and velocity [F(3,26) = 3.869, p=0.021] at the p<0.05 level, but not for the difference score (p=0.673) or the relative score (p=0.707).

To examine between which hormone groups the differences in total distance moved and mean velocity lie, Tukey post-hoc tests were performed. This test revealed that the original EB withdrawn group moved a significantly greater distance in the Open Field Test (M=3499.532 cm, SD = 843.01 cm) than the modified EB+P4 withdrawn group (M=2223.724 cm, SD=989.96 cm), p=0.014. Tukey post-hoc tests also revealed that the original EB withdrawn group moved at a significantly higher mean velocity in the Open Field Test (M=11.68 cm/s, SD=2.809 cm/s) than
the modified EB+P4 withdrawn group ($M=7.42$ cm/s, $SD=3.302$ cm/s). There were no significant differences among any of the other hormone groups.

Graphs for the difference scores and duration of time spent in the center and periphery of the OFT for the original EB sustained and withdrawn and the modified EB+P4 sustained and withdrawn hormone groups are shown in Figure 3. The independent samples t-test found that the original EB withdrawn group moved significantly more ($M=3499.53$ cm, $SD=843.01$ cm) than the original EB sustained group ($M=2629.81$ cm, $SD=612.46$ cm), $t(14) = -2.36$, $p=0.033$. In addition, the EB withdrawn group moved at a significantly higher mean velocity ($M=11.68$ cm/s, $SD=2.809$ cm/s) than the EB sustained group ($M=8.767$, $SD=2.041$ cm/s), $t(14) = -2.37$, $p=0.033$. There were no significant differences between these groups in duration (s) in periphery ($p=0.807$), duration (s) in center ($p=0.934$), difference score ($p=0.874$), or relative score ($p=0.849$). Another independent samples t-test revealed that there were no significant differences between the modified EB+P4 sustained and withdrawn groups in total distance moved ($p=0.175$), mean velocity ($p=0.176$), duration in periphery ($p=0.426$), duration in center ($p=0.465$), difference score ($p=0.448$), or relative score ($p=0.454$).

**Elevated Plus Maze**

Graphs for the relevant measures in the Elevated Plus Maze are shown in Figure 4. There was no main effect of hormone group on total distance moved ($p=0.045$), average velocity ($p=0.045$), difference score ($p=0.414$), or the relative score ($p=0.372$). However, Tukey post-hoc tests revealed that the greater distance traveled in the EPM by the original EB withdrawn group ($M=65178.14$ mm, $SD=11806.14$ mm) was approaching statistical significance ($p=0.094$) when compared to the original EB sustained group ($M=50871.46$ mm, $SD=10206.395$ mm). In addition, the larger mean velocity of the original EB withdrawn group ($M=217.469$ mm/s,
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SD=39.42 mm/s) was approaching statistical significance (p=0.093) when compared to the original EB sustained group (M=169.644 mm/s, SD=34.01 mm/s).

Graphs for the difference scores and duration of time spent in the open versus closed arms of the EPM for the original EB sustained and withdrawn and the modified EB+P4 sustained and withdrawn hormone groups are shown in Figure 5. An independent samples t-test revealed that the original EB withdrawn group traveled significantly more distance (M=65178.1 mm, SD=11806.1 mm) than the original EB sustained group (M=51561.8 mm, SD=10206.4 mm), t(14) = -2.59, p=0.021. In addition, the original EB withdrawn group had a significantly greater mean velocity (mm/s) (M=217.5 mm/s, SD=39.42 mm/s) compared to the original EB sustained group (M=169.6 mm/s, SD=34.01 mm/s), t(14) = -2.59, p=0.021. No significant differences were found between these two groups in their difference score (p=0.799), relative score (p=0.931), total time spent in open arms (p=0.999), or total time spent in closed arms (p=0.649). Another independent samples t-test revealed that there were no significant differences between the modified EB+P4 sustained and withdrawn groups in total distance moved (p=0.125), average velocity (p=0.126), total time spent in open arms (p=0.156), total time in closed arms (p=0.226), difference score (p=0.178), or relative score (p=0.158).

Immunohistochemistry

An independent samples t-test was run to see if there were differences in the number of FosB-positive cells in the core and shell of the nucleus accumbens between the modified EB+P4 sustained and withdrawn hormone groups. The test revealed that the EB+P4 sustained group had significantly more FosB-positive cells in the core of the nucleus accumbens (M=783, SD=342) than the EB+P4 withdrawn group (M=439, SD=211), t(22.0) = -2.96, p=0.007 (Figure 6). The EB+P4 sustained group also had more FosB-positive cells in the shell of the nucleus accumbens
than the EB+P4 withdrawn group, however, this difference was not significant (p=0.192) (Figure 7).

Discussion

Brief Summary of Results

There were no differences between the sustained and withdrawn hormone groups in OCD-like or anxiety-like behaviors. However, the original EB withdrawn mice moved significantly more and at greater velocities than the original EB sustained mice in both the OFT and the EPM, indicating a greater degree of locomotor behavior. Also, all four groups of hormone-treated mice displayed an unusually low anxiety phenotype in the OFT, which is in contrast to the high anxiety phenotypes displayed in the EPM. Finally, the modified EB+P4 sustained mice had significantly greater FosB induction than the modified EB+P4 withdrawn mice in the NAc core.

OCD-like behavior

The four hormone groups (EB sustained, EB withdrawn, EB+P4 sustained, and EB+P4 withdrawn) did not differ in their marble burying behavior. These findings suggest that the different hormone conditions did not have an impact on OCD-like behavior in the MBT (Figure 1). Specifically, estradiol withdrawal did not yield an increase in compulsive-like behaviors, as was initially hypothesized. In addition, an increase in compulsive-like behaviors is not seen in the withdrawn mice that had a more accurate representation of the human trajectory of progesterone (the modified cohort). Together, these results suggest that withdrawal from both estradiol and progesterone did not increase compulsive-like behavior.

There are several explanations for this data. The lack of significant differences between the hormone groups on the MBT does not necessarily mean that the different hormone conditions
do not have an impact on compulsive behaviors. First, there was a low number of marble burying (1-2 marbles) in several mice, so a floor effect might have been in play which makes it difficult to determine a difference between the groups when the numbers are low. Also, these findings may be due to a lack of sensitivity of the marble burying test in capturing the changes in anxiety during the peripartum period. This explanation is plausible as previous studies have found that riluzole, a drug clinically used for the treatment of OCD, is ineffective in the Marble Burying Test (Angoa-Pérez et al., 2013). This suggests that any potential compulsive behaviors that the hormonal conditions invoke may take on a different behavioral phenotype in mice than what is seen in humans. In addition, other studies have also indicated the marble burying test may not be the best representation of compulsive-like behaviors in mice (de Brouwer et al., 2019; Thomas et al., 2009). So, future studies should determine how OCD-like behavior in mice can be best captured and additional behavioral analyses should be carried out to evaluate how hormonal conditions influence this behavior. Future research could use the video footage of the marble burying test used in this study to evaluate compulsive checking behavior, perseverative circling, and excessive digging or nest building as other measures of OCD-like behavior. Excessive nest building may be especially useful because it has reliable face, predictive, and construct validity for understanding compulsive behavior. In addition, since nest building is an example of maternal behavior, its presence of nest building would indicate that the HSP reflects behaviors induced by a real pregnancy (Mitra & Bult-Ito, 2021). Using multiple measures of OCD-like behavior in mice may yield a more holistic understanding of the impact of a hormone-simulated pregnancy on compulsivity.

Other studies have found that alterations in steroid hormones are associated with changes in OCD-like behavior in mice, however their relationship is not elucidated. A study using female
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mice found that there was an increase in compulsive-like nesting and marble burying behaviors as a result of ovariectomy in mice strains that engage in excessive and repetitive nest building and marble burying behavior and this increase was attenuated by acute subcutaneous administration of estradiol, but not progesterone (Mitra et al., 2016). However, there was no increase in compulsive-like behaviors in the other randomly bred mouse strains that were tested. This finding is in line with human studies that show that hormonal fluctuations can trigger mood disorders or exacerbate them in women with a history of mood disorders (Forray et al., 2010). These findings suggest that estradiol may be more effective than progesterone in reducing OCD symptoms in postpartum females that already exhibit compulsive-like behavior. However, other studies have found that administration of both estradiol and progesterone reduced compulsive perseverance in the T-maze (Fernández-Guasti et al., 2006) and that progesterone administration alone reduces compulsive-like marble burying in male rats (Umathe et al., 2009). Thus, it is likely that hormones influence compulsive-like behavior in rodents, however, there may be differential effects of hormones in different groups (i.e. males versus females, anxious females versus non-anxious females, etc).

Anxiety-like behavior

There was no significant difference between the sustained and withdrawn hormone groups in both the original (EB) as well as the modified (EB+P4) cohorts in anxiety levels as measured by the duration of time spent in the center and periphery of the OFT and the duration of time spent in the open and closed arms of the EPM, which is contrary to our initial hypothesis.

In the OFT, the sustained and the withdrawn groups in both the original and modified cohorts show similar differences in duration spent in the center versus the periphery in the open field (Figure 3). The lack of difference in anxiety-like behavior between these groups in the OFT
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is consistent with past data in the Been lab using Syrian hamsters (Benedetto, 2018; Carson, 2018). In the EPM, there was a larger difference between time spent in open versus closed arms in the sustained mice in the modified cohort compared to the old cohort (Figure 5). Yet there was a smaller difference between time spent in open versus closed arms in the withdrawn mice in the modified cohort compared to the old cohort. This suggests that progesterone has a role in anxiety-like behavior such that prolonged progesterone exposure may exacerbate anxiety when accompanied by continued estradiol exposure, however prolonged exposure may attenuate anxiety when accompanied by estradiol withdrawal. This finding contradicts findings that continued progesterone exposure attenuates anxiety (Bitran et al., 1995; Stoffel & Craft, 2004).

Together, this demonstrates the complex relationship of hormones on behavior and how reductions or increases in anxiety are not caused merely by high or low absolute levels of any one hormone, but rather how the hormones are operating together. The lack of a significant difference in anxiety-like behavior between the sustained and withdrawn groups in both the OFT and the EPM suggests that these tests may not accurately reflect anxiety-like changes in mice that occur following a hormone simulated pregnancy or that mouse pregnancy induces anxiety behaviors that are not captured by the OFT and EPM.

These findings are inconsistent with previous data that show that pregnancy (estrogen-withdrawal specifically) increases rodents’ anxiety in the Elevated Plus Maze. For example, one study by Neumann (2003) found that there was an increase in “emotionality” at the end of pregnancy, regardless of the initial level of anxiety in Wistar rats. In addition, the increase in anxiety was more pronounced in rats that displayed a high level of emotionality prior to pregnancy. Consistent with these findings, past research in the Been lab has shown that hormone-withdrawn Syrian hamsters spend significantly less time in the open arms of the EPM.
than the hormone-sustained animals, suggesting that the hormone withdrawn group exhibits more anxiety-like behavior than the other groups (Hedges et al., 2021). This is in line with human studies that cite heightened anxiety levels during the peripartum period (Bloch et al., 2003; Forray et al., 2010). Further, these hormones may impact different groups in different capacities. Mitra et al. (2016) showed that ovariectomy worsened anxiety-like behaviors only in the compulsive-like mice strains and not in the other strains of mice tested and that estradiol administration caused an increase of time spent in the center of the OFT in one compulsive-like strain but not the other, which indicates that the effects of estradiol treatment response were strain dependent. Progesterone treatment also decreased anxiety-like behavior in both of the compulsive-like mice strains in the OFT but only one of the compulsive-like strains in the EPM, which is suggestive of strain specific responses in anxiety-like behavior due to progesterone administration. This is similar to human studies which have found that exposure to hormones is known to have different behavioral effects in women with and without premenstrual syndrome (Schmidt et al., 1998). These findings support the notion that a subset of women, those with a history of mood disorders or anxiety, are especially vulnerable to experiencing increased levels of anxiety during the peripartum period.

Unexpectedly, both the sustained and withdrawn groups spent more time in the center of the Open Field versus the periphery. This finding was interesting as it suggests that these animals, at least in the OFT, regardless of their hormone condition and whether they were in the old or modified cohort, displayed an unusually low anxiety phenotype. This finding is partially consistent with previous studies that have found that hormone-withdrawn rats spend more time in the center than both the control and hormone-sustained groups in the OFT (Galea et al., 2001). This suggests that rats that experienced withdrawal from estradiol experienced less anxiety in the
OFT, which does not support the hypothesis that estrogen withdrawal induces postpartum anxiety. However, past research in the Been lab has found that both the sustained and withdrawn animals spend more time in the periphery than in the center of the OFT (Carson 2018). This finding is more typical of rodents and it suggests that both groups of hormone-treated animals experienced relatively high anxiety levels in this test.

The unusually low anxiety phenotype in the OFT in this group of mice could be explained by the physical nature of the test in that it is not as robust of a test of anxiety as other measures such as the EPM (Ramos 2008). Unlike the EPM which is elevated and has exposed arms, the OFT is just an open box. The mice could have been habituated to the space in the OFT since they lived in cages that were also box shaped. More likely, the low anxiety phenotype could be explained by the fact that all the animals had experienced behavioral tests, the baseline MBT and the postpartum MBT, prior to being tested in the OFT. This could have also gotten the animals habituated to testing and caused them to display low levels of anxiety in the OFT.

Overall, the behavioral results of this test indicate that the mice in the present study exhibit an uncharacteristically low-anxiety phenotype and that estrogen withdrawal did not lead to increased anxiety-like behavior in the OFT in either the old or modified cohorts, which is contrary to our initial hypothesis.

In contrast to the results from the OFT, a higher anxiety phenotype is suggested by the EPM results since mice in all hormone groups spent more time in the closed arms versus the open arms. This is in line with previous studies as more time spent in the closed arms reflects higher anxiety-like behavior. The differential results in anxiety levels suggested by the OFT and the EPM could be due to the fact that these tests measure different aspects of anxiety (Ramos 2008). Future research could use the video footage in the OFT and EPM to measure defecation
rates of these mice, which could be used to further evaluate their anxiety levels, considering they exhibited varying anxiety levels across the behavioral tests used.

In reconciling these differential findings, it is possible that the behavioral responses of pregnant animals may be more dependent on the behavioral test conditions and the type of behavioral test more than anything else, since there are conflicting findings on postpartum anxiety using the same behavioral tests for anxiety and across different behavioral tests for anxiety. However, the conflicting findings may also point to the complex relationship between hormones and anxiety. Pregnancy, or more accurately, the changing hormones that are associated with it, could lead to a reduction in anxiety in the postpartum which is an adaptive behavioral response that allows mothers to ensure the survival of their offspring (Agrati et al., 2008). At the same time, the changing hormones may lead to increased anxiety in the postpartum as a result of estradiol withdrawal in other groups of animals, primarily those who already exhibit high anxiety levels (Baka et al., 2017; Stoffel & Craft 2016; Zhang et al., 2016). This suggests that rodents, or humans, who have a history of mood disorders or anxiety may be more sensitive to fluctuations in hormones. So, periods of hormonal fluctuations like pregnancy may predispose this group to anxiety. Future studies may consider giving identical hormone regimens to anxious and non-anxious rodents and observing how each group’s anxiety levels change throughout the peripartum period. In addition, research could see if the novel hormone-simulated pregnancy regimen used in this study impacts other behaviors, such as depressive-like behaviors and maternal behaviors, since depressive-like behaviors are induced by the original HSP regimen (Galea et al., 2001).

**Non-specific locomotor behavior**
The withdrawn animals in the original cohort moved significantly more distance and at higher velocities than the sustained animals in the original cohort in the Open Field and in the Elevated Plus. However, there were no differences in the distance and velocities of animals in the hormone-sustained and -withdrawn groups in the modified cohort in either of these anxiety tests. These findings indicate that hormone withdrawal in the original cohort is modulating non-specific locomotor behavior. Consistent with the idea that estradiol modulates non-specific locomotor behavior, Luine et al. (1998) showed that estradiol increases locomotor activity in the OFT after short term estrogen treatment (5 days) but not long term treatment (35 days). This suggests the dose and duration of estradiol exposure has an impact on the response to estradiol. Hence, it is essential to test different lengths of exposure and different doses of estradiol to determine the exact effects of this hormone on behavior. The lack of difference between the sustained and withdrawn groups in the modified cohort of the present study suggests that progesterone may be eliminating the effect of estradiol-withdrawal on non-specific locomotor behavior during the postpartum. This finding is in opposition to past research in the Been lab that has found that the hormone-withdrawn animals moved significantly less and at lower velocities compared to the hormone-sustained animals (Benedetto, 2018). Taken together, these studies suggest that estradiol-withdrawal (as seen in the original cohort, when it is not accompanied by the typical trajectory of progesterone) modulates non-specific locomotor behavior, but it is unclear as to whether estradiol is increasing or decreasing non-specific locomotor behavior. Further, this modulation of non-specific locomotor behavior could explain the interesting findings on the measures of anxiety-like behavior, as time spent in the periphery versus the center of the Open Field could have been influenced by inactivity rather than effects of hormone group. This is especially the case since ambulatory distance is arguably the most important
measure in the OFT. Indeed, one mouse in the present study was essentially immobile during the OFT and had to be tested again.

**Immunohistochemistry**

Immunohistochemistry was carried out on the brain slices of mice in the modified EB+P4 sustained and withdrawn groups. The sustained group exhibited significantly greater FosB induction in the NAc core than the withdrawn group. However, the sustained group did not have significantly higher FosB induction in the NAc shell. This finding is in direct opposition to our initial hypothesis that the hormone-withdrawn group would exhibit higher ΔFosB induction in the NAc core. The present study’s results are interesting since higher ΔFosB induction is known to be induced in response to compulsive-like behaviors in rodents, which is not reflected in the behavioral tests (McClung et al., 2004; Nestler et al., 2001). Still, its greater induction in the sustained group suggests that there is chronic stimulation of the NAc core and this is likely due to, or is at least associated with, prolonged estradiol exposure.

Other studies have supported the finding that estradiol influences ΔFosB since males and females have different levels of ΔFosB in the NAc both endogenously and when induced by cocaine, which may be due to difference in dopamine receptor expression and binding (Kokane & Perrotti 2020). Estradiol exerts its effects in females by altering the activity of various intracellular signaling cascades in the NAc (Kokane & Perrotti 2020). Studies on drug-addiction in rodents have shown that activation of membrane-bound estrogen receptors on cells in the NAc increases sensitivity to drugs of abuse in females and changes dendritic spine morphology and density in this region. In addition, estradiol influences dopamine and mGluR signaling in the NAc core, which leads to structural alterations that ultimately lead to long-lasting activation of the reward pathway (Kokane & Perrotti 2020). Overall, estradiol and dopamine systems interact
to enhance sensitivity of dopaminergic neurons and modify signaling pathways and gene expression which ultimately produces altered reward and motivated behaviors. In light of this knowledge, it is unsurprising that there was no increase in FosB in the withdrawn animals if it is true that increases in ΔFosB are associated with increases in anxiety and compulsive behaviors, since the present study showed no increase in anxiety-like or compulsive-like behaviors in all of the behavioral measures tested.

However, the finding that FosB was increased in the sustained group is interesting and is in direct opposition to previous studies in the Been lab that show higher FosB induction in the nucleus accumbens core of mice in the hormone-withdrawn condition (Carson 2018). It is important to note that Carson (2018) used the original HSP regimen whereas the present study analyzed brains that belong to mice that underwent the modified HSP regimen. Since these two regimens differ in the amounts of and exposure length to progesterone, it is possible that progesterone may be influencing how ΔFosB gets induced in the NAc. This could explain the larger difference between time spent in the open versus closed arms of the EPM in the sustained group in the modified cohort compared to the withdrawn group. Even though the differences in anxiety-like levels in the EPM were not significant, this could have been due to a small number of subjects or the possibility that ΔFosB induction in the NAc is related to increased anxiety behaviors that are not measured by the EPM. Another possibility is that the sustained group could exhibit anxiety- or compulsive-like behavior if they were to be tested again at a later time point. This may suggest that ΔFosB induction is temporally decoupled from anxiety or compulsive behaviors. In other words, an increase in ΔFosB induction could precede any anxiety-like or compulsive-like behaviors instead of being seen simultaneously. Future studies should examine if there were differences in ΔFosB induction between the sustained and
withdrewn group in the original cohort. Since the original cohort differs from the modified cohort in the amount of and exposure length to progesterone, these results could shed light on the impact of progesterone specifically during pregnancy on ΔFosB induction in the NAc.

There was a significant difference in FosB induction in the NAc core but not the NAc shell between the sustained and withdrawn groups which suggests that hormonal fluctuations differentially affect different regions of the NAc. Studies have indicated the distinct nature of the NAc core and shell as lesions to the shell, but not core, are associated with disruptions of maternal behaviors including maternal memory and pup-retrieval (Haim et al., 2014). Additionally, the NAc core and NAc shell project to distinct regions of the brain and the former is associated with extrapyramidal motor movement while the latter is associated with the limbic system, which also indicates their differential modes of action (Sturm et al., 2007). Further, one study has shown that DBS treatment to the nucleus accumbens core, but not shell, of men and women improves OCD symptoms, suggesting that the core may be more influential in OCD symptoms possibly due to its modulation with extrapyramidal motor movement (Denys et al., 2010). The implication of these findings is that it is important to distinguish neural changes in these two regions, especially when interpreting behavioral results, and that OCD researchers may take more interest with the NAc core. Future research using hormone simulated pregnancies should investigate these regions separately as targeting one region or the other may be more efficacious for OCD-treatment purposes.

**Strengths and limitations**

The present study is novel because it incorporates a “modified” cohort of hormone-treated mice that received progesterone injections on a schedule that more accurately reflects the typical trajectory of progesterone in human pregnancy. Specifically, the EB+P4
withdrawn mice in the modified cohort of the present study underwent hormone injections that reflect this. The typical trajectory of hormones during mouse pregnancy is represented by the EB withdrawn group in the original cohort. Since human females experience high levels of estradiol throughout the entire pregnancy, it is likely that humans are more affected by estrogen withdrawal than mice, since mice only experience high levels of estradiol during the third trimester. As a result, it is reasonable to predict that the behavioral and neural changes that occur in humans are greater than those that occur following rodent pregnancy. Hence, it was important to capture the hormonal profile of humans in this study.

The present study focused on the role of estradiol and progesterone during the peripartum period. While focusing on these two hormones allowed for experimental control and to be able to discern cause and effect relationships, this is also a limitation of the present study as the HSP is just a model of pregnancy and estradiol and progesterone are only two hormones of many additional factors that fluctuate dramatically during pregnancy. Hormones such as corticotropin-releasing hormone (CRH) are also produced during pregnancy and the effects of estradiol on mood and anxiety could be mediated by interactions involving estradiol and CRH receptors, since CRH receptors have estrogen binding elements (Galea et al., 2001). In addition, neurotransmitters such as GABA, serotonin, dopamine, and norepinephrine and neuropeptides such as cholecystokinin, neuropeptide-Y, vasopressin, and oxytocin, Substance P, and corticotropin releasing hormone have also been implicated in anxiety (de Brouwer et al., 2019). Further, there are many structural and functional brain changes associated with the peripartum period. Structural changes include reductions in brain volume (both overall and in certain regions) and increases in ventricular size across the peripartum period in both humans and rodents (Galea et al., 2014; Zhang et al., 2016). In addition, reductions in cell proliferation over
the peripartum period in rodents is reported (Cárdenas et al., 2020). New human mothers have also been found to display more activity in several brain regions, not only those associated with reward and motivation. Also, increased connectivity between the left amygdala and the left nucleus accumbens, which has been shown to be positively associated with positive maternal behavior, and heightened PFC activity in response to threat and distress stimuli across gestation has been documented (Cárdenas et al., 2020). Some of these neural changes are transient and return to their pre-pregnancy state while others are more enduring. The host of structural and functional changes that occur during pregnancy, which play a role in mothers’ behaviors during the peripartum period and likely also contribute to the vulnerability to depression and anxiety, are not captured in the HSP.

An additional limitation is the validity of some of the behavioral assays used in the present study. The Marble Burying Test has poor predictive validity, i.e. it is not sensitive to all classes of anti-compulsive treatments and burying behavior is reduced by several drugs that do not have anti-compulsive activity. This suggests that this assay cannot distinguish between anti-compulsive and anxiolytic drugs or between anxiolytics. However, this test’s poor predictive validity may not be of concern as there is a lack of specificity of many medications in human patients. The Marble Burying Test also has poor constructive validity since little is known about the conserved neurocircuitry that underlies the burying behavior. Further, some research has indicated that marble burying is not correlated with other measures of anxiety such as the Open Field and light-dark tests, that mice do not spend more time in parts of the cage without the marbles, and that they bury objects like food pellets, which suggests that the marbles are not anxiety-provoking (Thomas et al., 2009). Finally, rodents may only bury marbles by accident in the process of digging. Despite these limitations, this assay is reflective of perseverative,
repetitive behavior that is highly resistant to change based on familiarity (Ahmari, 2016). It is also easy to carry out, there is high accuracy in scoring the behavior and spontaneous exhibition of rodents’ behavior, and it can be used in the screening of genetically modified mice for abnormal behavioral phenotypes. The MBT may be especially useful for shedding light on the hormonal influences on compulsive-like behavior. This behavioral model is cost-effective, does not require pharmacological intervention, and could ultimately allow for the identification of novel-drug receptor interactions important for anxiety and OCD.

Another behavioral assay used in this study, the Open Field Test, is limited by the fact that it seems to be a less robust test than other measures of anxiety. This may be due to the physical nature of the test as it is just an enclosed box and does not force the animal to choose between anxiety-inducing open arms and more anxiolytic closed arms as is the case in the Elevated Plus Maze. This may explain the present study’s findings of unexpectedly low anxiety behavioral phenotypes in the Open Field that is exhibited by mice in all four hormone conditions. Future studies using a HSP may consider looking at maternal care behaviors such as pup retrieval, as the evocation of maternal behaviors is an important measure of validity for a HSP model and may be more indicative of behavioral changes that occur as a result of the hormone injections. Incorporating these measures will be important to shed light on if a hormone simulated pregnancy models a typical pregnancy in mice.

Another limitation of the present study is that, during immunohistochemistry staining, we made the assumption that the FosB-positive cells were ΔFosB-positive cells. This is because immunohistochemistry staining with an antibody is not specific enough to pick up ΔFosB, so FosB was our protein of interest. However, ΔFosB is the primary splice variant in the NAc so we can assume that the staining in our slices is ΔFosB. This is an interpretational caveat of the
present study, since the staining is not specific for ΔFosB, but it is a feasible assumption to make. In addition, while performing the immunohistochemistry procedure, some tissue was lost and slices were dropped from their containers. This not only led to a reduction in available tissue for several mice, but it is also possible that some tissue was mixed up. Preserving the identities of the tissue was central to this experiment to determine neurological differences between hormone groups. Future researchers should pay extra attention to carefully carrying out procedures and maintaining the identities of the tissue.

A final limitation of this study is that there was no true control group. Due to the relatively small number of mice in each hormone group (n=7 or 8), we decided to have as many mice in each of the experimental conditions as possible instead of adding a true control group. The reason for this was because there is sufficient data from previous Been lab and outside studies that indicate the behavioral performances and neural changes of the control group in an HSP regimen that could be referenced. Despite this, having a control group in this study would have been useful to observe if this group of mice, when not given any hormone, would display the low levels of anxiety that are seen in the hormone-treated mice in this study in the OFT.

**Concluding Remarks**

The purpose of this research was to adapt the original HSP model to accurately reflect the pregnancy trajectory of both estradiol and progesterone in humans to observe the effects of these hormones on OCD and anxiety. Both the onset and worsening of OCD has been reported in women during the postpartum and there is a subset of women, those with a history of mood disorders, who are more vulnerable to having this occur. The behavioral analyses carried out were meant to elucidate whether there were differences in anxiety-like and compulsive-like behaviors among the different hormone groups. Contrary to our hypothesis, the results of the
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The present study suggests that regardless of whether the animals continued to be exposed to estradiol and progesterone or whether they were withdrawn from these hormones, their anxiety levels were not significantly different. However, non-specific locomotor activity was increased by estradiol-withdrawal in the original HSP hormone regimen. The goal of the neural analyses was to observe if there were differences in the ΔFosB number and distribution in the nucleus accumbens among the hormone groups, which would provide insight on neural plasticity associated with compulsive-like behavior. In line with past findings, as there was no increase in anxiety-like behavior in the hormone-withdrawn mice, there was no increase in FosB induction in the NAc. However, greater induction was seen in the NAc core of hormone-sustained mice which is contrary to our hypothesis that greater ΔFosB induction would be seen in the NAc core in the withdrawn mice.

This study begins to fill in gaps in the current literature of both the behavioral and neurological manifestations associated with anxiety and OCD in the peripartum period. However, given the discrepant findings in anxiety levels among hormone groups across HSP-studies, it is clear that further research is needed to identify the effects of pregnancy-related hormones on behavior and the brain. Identifying the neural and behavioral changes that occur in a hormone simulated pregnancy in mice, and observing whether these the behavioral changes and the neural changes are seen together, is important for understanding anxiety-like and compulsive-like behavior in mice and may be extrapolated to humans to better understand anxiety and OCD during the peripartum period. Subsequently, this knowledge can be used to identify vulnerable populations of women and develop efficacious treatments for anxiety and OCD.
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Tables and Figures

**Table 1.**

*Experimental Groups for the Mice*

<table>
<thead>
<tr>
<th>Group 1 (animals 111-118)</th>
<th>Early Pregnancy</th>
<th>Late Pregnancy</th>
<th>Postpartum</th>
</tr>
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<tbody>
<tr>
<td>Low estradiol/ high progesterone</td>
<td>High estradiol</td>
<td>High estradiol</td>
<td></td>
</tr>
</tbody>
</table>
n = 8

<table>
<thead>
<tr>
<th>Group 2 (animals 119-126) n = 8</th>
<th>Low estradiol/ high progesterone</th>
<th>High estradiol</th>
<th>oil</th>
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</thead>
<tbody>
<tr>
<td>Group 3 (animals 127-133) n = 7</td>
<td>Low estradiol/ low progesterone</td>
<td>High estradiol/ high progesterone</td>
<td>High estradiol</td>
</tr>
<tr>
<td>Group 4 (animals 134-140) n = 7</td>
<td>Low estradiol/ low progesterone</td>
<td>High estradiol/ high progesterone followed by high estradiol</td>
<td>oil</td>
</tr>
</tbody>
</table>

Figure 1. The graph labeled “Postpartum Marble Burried” is a comparison of the four hormone groups in the amount of marbles buried during the “postpartum” period. There are no significant differences. The graph labeled “P-B Marble Burried” is a comparison of the four hormone groups in the difference scores for the MBT. This value was found by subtracting the amount of marbles buried during the baseline test from the amount of marbles buried during the postpartum period.
Figure 2. The graph on the top left is a comparison of the four hormone groups’ difference scores for the OFT (time spent in the periphery minus time spent in the center). There are no significant differences. The graph on the top right is a comparison of the four hormone groups’ relative scores for the OFT [(time spent in periphery/ (time spent in center + time spent in periphery)]. There are no significant differences. The graph on the bottom left is a comparison of the four hormone groups in the mean velocity in the OFT (cm/s). The classic EB-withdrawn group has a significantly higher mean velocity in the OFT than the modified EB+P4 withdrawn group. The graph on the bottom right is a comparison of the four hormone groups in the total distance traveled in the OFT (cm). The classic EB-withdrawn group has a significantly higher total distance traveled in the OFT than the modified EB+P4 withdrawn group.
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Figure 3. The graph on the top is a comparison of the difference scores in the OFT for sustained and withdrawn hormone groups in both the classic (“EB”) cohort and the modified (“EB+P4”) cohort. Both the classic and modified cohorts show a relatively small difference in the difference scores of the sustained and withdrawn groups. The graph on the bottom left is the duration spent in open versus closed arms of the OFT in the sustained and withdrawn hormone groups in the classic (EB) cohort. Both the sustained and withdrawn groups spent more time in the center. There is no significant difference between the sustained and withdrawn groups. The graph on the bottom right is the duration spent in open versus closed arms of the OFT in the sustained and withdrawn hormone groups in the modified (EB+P4) cohort. Both the sustained and withdrawn groups spent more time in the center. There is also no significant difference between the sustained and withdrawn groups.
Figure 4. The graph on the top left is a comparison of the four hormone groups in the distance traveled (mm) in the EPM. There are no significant differences. The graph on the top right is a comparison of the four hormone groups in the mean velocity (mm/s) in the EPM. There are no significant differences. The graph on the bottom left is a comparison of the four hormone groups’ difference scores (the time spent in the open arms minus the time spent in closed arms). There are no significant differences. The graph on the bottom right is a comparison of the four hormone groups’ relative scores [(time spent in open arms/ (time spent in open arms + time spent in closed arms)]. There are no significant differences.
Figure 5. The graph on the top is a comparison of the difference scores in the EPM for sustained and withdrawn hormone groups in both the classic (“EB”) cohort and the modified (“EB+P4”) cohort. The modified cohort shows a greater gap in difference scores between the sustained and withdrawn groups than the original cohort, however this difference was not significant. The graph on the bottom left is the duration spent in open versus closed arms of the EPM in the sustained and withdrawn hormone groups in the classic (EB) cohort. Both the sustained and withdrawn groups spent more time in the closed arms. There is no significant difference between the sustained and withdrawn groups. The graph on the bottom right is the duration spent in open versus closed arms of the EPM in the sustained and withdrawn hormone groups in the modified (EB+P4) cohort. Both the sustained and withdrawn groups spent more time in the closed arms. There is a greater difference in time spent in open versus closed arms in the sustained group compared to the withdrawn group. There is no significant difference between the sustained and withdrawn groups.
Figure 6. FosB count in the NAc core. The EB+P4 sustained group has a significantly higher amount of FosB in the NAc core than the EB+P4 withdrawn group.
Figure 7. FosB count in the NAc shell. The EB+P4 sustained group has a higher amount of FosB in the NAc shell, but this difference is not significant.
Figure 8. FosB staining in a caudal image of the right NAc from a mouse in the modified EB+P4 sustained group. There were 889 FosB-positive cells in the core (blue rectangle) and 126 FosB-positive cells in the shell (red rectangle).