

**AN INVESTIGATION INTO THE IMPACT OF PREGNANCY AND STRESS  
ON NEUROIMMUNE FUNCTION: ARE THERE POTENTIAL LINKS TO  
POSTPARTUM DEPRESSION?**

by

Caitlin K. Posillico

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Behavioral Neuroscience

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## **ABSTRACT**

Postpartum depression is a specific type of major depression outlined by the National Institute of Mental Health that affects approximately 10-15% of mothers. While many have attributed postpartum depression to a dramatic change in hormone levels throughout pregnancy and the immediate postpartum period, the exact causes of postpartum depression are not well-understood. It is well-known; however, that pregnancy is associated with a number of dramatic changes in the pregnant mother's body. As mentioned above, pregnancy induces a striking increase in the levels of circulating hormones including progesterone, estrogen, prolactin and oxytocin. In addition, pregnancy induces significant changes in the peripheral immune system in order to foster the development of the growing fetus and to prevent it from being "attacked" by the mother's immune system. It is also well-known that changes in immune function, specifically within the brain, have been linked to several neuropsychiatric disorders including depression. Despite the fact that pregnancy is associated with dramatic changes in peripheral immune function, no one has ever examined whether pregnancy produces similar changes in immune function within the brain. Therefore, we hypothesize that functional changes in the immune system associated with pregnancy and parturition may increase the risk for developing postpartum depression. Microglia are the immune cells of the brain. They produce both pro- and anti-inflammatory cytokines in response to various insults such as infection, stress, and injury. Using real-time PCR analysis of gene expression within the brain, we identified significant changes in the expression of microglial activation

markers and pro-inflammatory molecules within the medial prefrontal cortex (mPFC) and the hippocampus of pregnant and postpartum rats. Separate cohorts of pregnant and non-pregnant rats were exposed to either forced swimming stress during the last week of gestation or an injection of an immunogenic compound, lipopolysaccharide (LPS), one day prior to parturition. These challenges induced differential changes of two inflammatory cytokines,  $IL-1\beta$  and  $IL-6$ , in the mPFC after birth. Both stress and pregnancy caused depressive-like anhedonia and increased anxiety as measured using sucrose preference testing and performance on an Elevated Plus Maze, respectively. Thus, our data show novel findings of neuroimmune changes and depressive-like behaviors induced by pregnancy alone. Additionally, stress and immune activation interact with pregnancy resulting in unique neuroimmune changes and depressive-like behavioral outcomes.

## **Chapter 1**

### **INTRODUCTION**

Postpartum depression is a specific type of major depression outlined by the National Institute of Mental Health that affects approximately 10-15% of mothers after birth (Wisner et al., 2013; National Institute of Mental Health, 2011). At least 30% of women will experience minor symptoms of anxiety and sadness, called “baby blues”, immediately following birth (Faisal-Cury et al., 2008; Harris et al., 1994; Kennerly and Gath, 1989). However, these less serious symptoms typically only last one to two weeks while the much more severe symptoms of postpartum depression may take anywhere from four weeks to six months to be expressed (Miller, 2002; Cox et al., 1982). Postpartum depressive symptoms include erratic mood swings, anhedonia, increased anxiety, insomnia, and social withdrawal. These mothers often feel disconnected to their baby, their family, and the world around them; this can significantly impact both the mother and the child’s health and well-being.

Estrogen and progesterone are two hormones that increase exponentially during pregnancy and peak during the third trimester. After the mother gives birth, these hormones drop dramatically down to pre-pregnancy levels within the first few days (Harris et al., 1994). This dramatic change in hormones is believed to be the major contributing factor in developing postpartum mood disorders like baby blues and postpartum depression. In rats, experiments that mimic these fluctuations in hormones between gestation and the postpartum period resulted in a depressive-like phenotype (Suda et al., 2008; Galea et al., 2001). However, little research has been

done to determine whether other mechanisms contribute to the development of postpartum depression. Using a rodent model, our experiments sought to examine whether pregnancy produces changes in the function of microglia, the immune cells of the brain, and whether exposure to either a chronic stressor or an acute immune challenge during late gestation pose as additional risk factors that alter microglial function and, in turn, increase the likelihood of developing postpartum depression. We examined the gene expression of specific cytokines and immune molecules to study the potential effects of pregnancy and stress on the central immune system, and we performed behavioral analysis of anhedonia and anxiety to examine changes in these behaviors during the postpartum period.

Microglia are the resident immune cells of the brain. They comprise approximately 10-20% of the neural cell population, and they express both pro- and anti-inflammatory cytokines in response to various types of insults including infection, stress, and injury. For example, both physiological stress and infection can activate microglia to induce the expression of numerous cytokines and chemokines in the brain (Raison and Miller, 2011; Dantzer et al., 2008). Microglia induce the expression of cytokines and chemokines in order to respond to the disturbance, protect the brain, and return the brain to homeostasis (Walkera et al., 2013; Kraft and Harry, 2011; Silverman et al., 2005). The increase in microglial activation and cytokine production results in specific sickness and depressive-like behaviors including increased anxiety, social withdrawal, and anhedonia (Schiepers et al., 2005). This is an adaptive response of the immune system, and these changes in behavior help to facilitate the rest and recuperation of the body during stress or infection. In fact, previous studies have shown that the expression of these sickness or depressive-like behaviors are the

direct result of altered immune function and cytokine expression in the brain as opposed to the stressor or infection itself (Raison and Miller, 2011; Dantzer et al., 2008). Thus, changes in neuroimmune function and the expression of cytokines in the brain as a result of pregnancy and parturition may also be factors underlying the etiology of postpartum depression.

Pregnancy results in many significant changes to the peripheral immune system in order to protect the growing fetus and to prevent it from being attacked or rejected as a foreign identity by the mother's immune system. One explanation suggests that pregnancy induces a temporary altered immune state in order to accommodate for such drastic changes (Klein et al., 2010; Veenstra van Nieuwenhoven et al., 2001). As a result, the expression of classical inflammatory immune molecules, including IL-1 $\beta$ , are decreased over the course of gestation while a unique composition of "alternate" immune molecules increases. These changes have all been observed and measured exclusively in the periphery of pregnant females (Marzi et al., 1996). To date, no one has examined whether similar or significant changes in immune function / microglial function occur in the brain during pregnancy. Our experiments provide an opportunity to fill this gap in knowledge by studying neuroimmune function during pregnancy and post-parturition in the hippocampus and medial prefrontal cortex, two areas highly implicated in the etiology of depression.

Additionally, previous research has shown that maternal stress during gestation may have negative impacts on the offspring later in life (Mulder et al., 2002; Glavin, 1984). Stressed dams show significant maltreatment of their offspring postpartum, as opposed to normal nurturing maternal care (Roth et al., 2009; Ivy et al., 2008; Champagne and Meaney, 2006). Poor maternal care prevents proper development of

the pups that may lead to cognitive deficits and neuropsychiatric disorders in the offspring later in life (Roth et al., 2009). Thus, understanding how postpartum depression presents itself and outlining potential risk factors for developing it is not only crucial for proper treatment of the mother, but it is also important to prevent cognitive deficiencies in offspring as well.

The findings from this experiment seek to provide novel lines of research necessary to properly understand the development and etiology of postpartum depression. Currently, treatment of postpartum depression does not significantly differ from that of major depressive disorder. Tricyclic antidepressants are prescribed during pregnancy and postpartum in order to prevent major depressive episodes; however, it is unclear as to how the hormonal and immune changes occurring during pregnancy are interacting with the effectiveness of the drugs (Deligiannidis et al., 2014). Interestingly, current animal models using typical antidepressants prescribed for humans have not seen effective treatment of depressive-like behaviors in pregnant or postpartum animals (Bourke et al., 2013; Craft et al., 2010). These data suggest the possibility that postpartum depression may not be adequately treated with typical administration of antidepressants.

First, our experiments intended to characterize neuroimmune changes during late gestation and the early postpartum period. We hypothesized that pregnancy would cause a decrease in the expression of typical inflammatory molecules (such as IL-1 $\beta$ ) while subsequently increasing expression of alternate cytokines and chemokines (such as IL-4 and Arginase I). Previous studies have shown that stress-induced models of depression can alter the expression of various cytokines (Walkera et al., 2013; Miller et al., 2009; Anisman and Merali, 2002), impact the morphology of

surrounding neurons (Pawluski et al., 2012), and generate the expression of depressive-like behaviors (Baker et al, 2008). Therefore, our study adopted a similar model of stress-induced depression using a chronic forced swim stressor during late gestation in order to determine the effect of this stressor on a depressive-like phenotype postpartum. We hypothesized that changes induced by pregnancy or parturition might interact with stress-induced neuroimmune changes to produce a unique expression of immune factors and depressive behaviors after birth. Finally, our third experiment used a model of acute immune activation (similar to sickness) in the form of an injection of lipopolysaccharide (LPS) one to two days prior to giving birth in order to analyze potential neuroimmune and behavioral changes postpartum. We hypothesized that LPS (Dantzer et al., 2008) would interact with the altered immune state of pregnancy to produce a unique set of neuroinflammatory genes and depressive-like behaviors postpartum. Overall, we predicted that physiological stress as well as the stress of an infection during the gestation period would increase the risk for developing postpartum depression by triggering a unique neuroinflammatory state to induce the expression of a depressed phenotype after parturition.

## Chapter 2

### MATERIALS AND METHODS

#### 2.1 Animals

All experiments used female Sprague-Dawley rats ordered from Harlan Laboratories (Indianapolis, IN). Rats were housed in clear polypropylene cages with *ad libitum* access to food and water in rooms under a 12:12-hour light:dark cycle maintained under controlled temperature and humidity. All experiments were performed in accordance with the Institutional Animal Care and Use Committee of the University of Delaware and under the *Guide for the Care and Use of Laboratory Animals* of the National Institute of Health.

In Experiment 1, we examined the expression of cytokines and microglial activation markers in the brain during late pregnancy and immediately postpartum. 10 female rats were bred and assigned to one of two experimental groups: Embryonic (E) Day 21 (n=5), or Postnatal (P) Day 2 (n=5), with E1 assigned to the day of the sperm plug and P0 being assigned to the day of birth. An additional set of females were not bred and assigned to the Virgin (non-pregnant) group (n=5). All rats remained undisturbed during their pregnancy or time-matched period. Dams were euthanized on either E21 during gestation or P2 after parturition, and non-pregnant females were euthanized as controls to analyze and compare gene expression of neuroimmune activation markers at these time points of pregnancy and the postpartum period.

In Experiment 2.1, we examined the expression of cytokines and microglial activation markers in the brain of pregnant and non-pregnant rats exposed to stress or

no stress. 16 females were bred and assigned to one of two experimental groups: Pregnant, No Stress (n=8) or Pregnant, Stress (n=8). An additional 16 non-pregnant females were assigned to one of two groups: Non-pregnant, No Stress (n=8) or Non-pregnant, Stress (n=8). Stressed rats underwent a forced swimming stressor once each day for the last week of gestation in pregnant rats or a time-matched period in non-pregnant rats. The No Stress rats remained undisturbed during the equivalent time frame. These rats were sacrificed on either the day of birth (P0) or the time-matched day for non-pregnant animals to assess the gene expression of cytokines and microglial activation markers in the brain in response to pregnancy and the stress of forced swimming during gestation.

In Experiment 2.2 we examined the expression of depressive-like behavior and anxiety behavior following the same stressor used in Experiment 2.1. A separate cohort of 16 female animals was bred for two experimental pregnant groups: Pregnant, No Stress (n=8) or Pregnant, Stress (n=8). An additional 16 animals were assigned to non-pregnant groups: Non-pregnant, No Stress (n=8), or Non-pregnant, Stress (n=8). These animals were sacrificed on P3 after undergoing post-parturition or time-matched behavioral testing of anxiety- and depressive-like behaviors in response to pregnancy and stress of forced swimming during gestation.

Experiment 2.3 used an additional cohort of animals to expand upon the results from Experiment 2.2 into additional time points. Sixteen female rats were bred for two experimental pregnant groups as before: Pregnant, No Stress (n=8) or Pregnant, Stress (n=8). Sixteen females remained virgins for the two opposing non-pregnant groups (n=8/group). Female rats used for Experiment 2.3 were euthanized on P10

after undergoing two time points of postpartum (or time matched) behavior analysis in response to the stress of pregnancy and forced swimming.

In Experiment 3.1, we examined the expression of cytokines and microglial activation markers in the brain of pregnant and non-pregnant females exposed to LPS or vehicle during late gestation. This experiment used a total of 32 female rats. Sixteen females were bred and assigned to one of two experimental groups: Pregnant, Saline (n=8) or Pregnant, LPS (n=8). Another cohort of non-pregnant animals were assigned to either Non-pregnant, Saline (n=8) or Non-pregnant, LPS (n=8). Females were euthanized on P0 or a time-matched day to analyze gene expression in response to an intraperitoneal injection of LPS during gestation.

In Experiment 3.2, we examined the expression of depressive-like behavior and anxiety behavior following the same LPS exposure used in Experiment 3.1. This experiment used a separate cohort of 32 females. Sixteen females were bred and assigned to either Pregnant, Saline (n=8) or Pregnant, LPS (n=8), and 16 non-pregnant females were assigned to either Non-pregnant, Saline (n=8) or Non-pregnant, LPS (n=8). These rats were euthanized on P10 after undergoing two time points of behavioral testing to analyze anxiety- and depressive-like behaviors postpartum (or the time-matched period) in response to LPS injection during gestation.

## **2.2 Forced Swim Test**

Female animals in Experiments 2.1 and 2.2 that were assigned to a Stress group underwent the Forced Swim Test (FST) every day for 7 days. Rats were forced to swim in a clear cylinder of  $20\pm 1^{\circ}\text{C}$  tap water with no option of rest or escape for five minutes each day for the last week of their gestation (E16-E22) or a time-matched

period for non-pregnant rats. Rats were monitored by researchers to prevent accidental drowning. All rats were housed separately prior to the first day of testing.

### **2.3 Lipopolysaccharide**

Lipopolysaccharide (LPS) derived from *Escherichia coli* 0111:B4 was obtained from Sigma-Aldrich® (Cat. No. L2630). LPS was diluted with deionized phosphate buffered saline (DPBS) to a concentration of 100µg/mL for injections.

### **2.4 Injections**

Experiments 3.1 and 3.2 used injections of either saline (DPBS) or lipopolysaccharide (LPS). Between E21 and E22 of gestation (or the time-matched equivalent), female rats were given a one-time intraperitoneal injection of LPS at 100µg/kg of body weight or saline at 1mL/kg as a control. All rats were housed separately post-injection to eliminate any risk of infecting other animals or causing stress to cage mates.

### **2.5 Behavioral Testing**

Experiments 2.2, 2.3, and 3.2 used identical measures of analysis to assess anxiety- and depressive-like behaviors. A sucrose preference test was used on day of birth (P0) and P1 (or a time-matched equivalent) to measure anhedonia for Experiments 2.2, 2.3, and 3.2. Females were individually housed in clean testing cages restricted from food from 3pm-6pm on days of testing. The lights in the room remained off for the duration of the test to foster activity in an attempt to prevent sleeping during the three-hour period. On each day, the rats were given two bottles: one with regular tap water, and the other with a 1% sucrose solution. Both bottles were weighed prior to the test and after the completion of the test to measure the

amount of water or sucrose, in grams, the rats had consumed. The orientation of the bottles was randomized on the first day of testing and switched on the second day of testing to prevent any place preference. The test was performed over two days in order to obtain an average preference for sucrose. The resulting average sucrose preference score was calculated using the following formula:

$$\text{Sucrose Preference Score} = \left( \frac{\text{Average Sucrose Consumed (g)}}{\text{Average Total Liquid Consumed (g)}} \right) * 100 \quad 1$$

For Experiments 2.3 and 3.2, this test was repeated one week later (P7 and P8, or the time-matched equivalent) to assess the potential time course of the expression of this behavior.

Elevated Plus Maze (EPM) was used as an additional behavioral measurement for Experiments 2.2, 2.3, and 3.2 to measure levels of stress and anxiety. This test was performed on P2 or the time matched equivalent, one day after the completion of sucrose preference testing. The apparatus consisted of a plus-shaped platform raised 40 cm above the ground with two opposing “closed” or walled arms and two opposing “open” or no-walled arms of equal length. ANY-Maze Behavior Tracking Software was used to video monitor the animals, qualify, and quantify the behaviors of the rats during the 5-minute test. Time spent in the open arms throughout the test was specifically used to analyze the stress and anxiety-like behaviors of the rats. This test was repeated one week later (on P9) for animals in Experiments 2.3 and 3.2 to gather information about the time course of the depressive-like and anxiety behaviors.

## **2.6 Euthanasia, Perfusion, and Tissue Collection**

All rats were administered an overdose of the barbiturate Euthasol® (ANADA 200-071) via intraperitoneal injection. Sufficient anesthesia was assessed after the rat

did not respond to a toe pinch. Once anesthetized, rats were perfused with 0.9% saline solution to remove blood. After perfusion, the medial prefrontal cortex (mPFC) and whole hippocampus (HP) were collected from brain tissue using the guide of a rat brain atlas. Once extracted, tissue was immediately frozen on dry ice and stored at -80°C until ready for processing.

## **2.7 Real-Time PCR**

Messenger RNA (mRNA) was extracted from frozen brain tissue using Isol-RNA Lysis Reagent (Cat. No. 2302700, 5 PRIME). Extracted RNA was then subjected to DNase treatment to remove any genomic DNA prior to cDNA synthesis using the QuantiTect® Reverse Transcription Kit (Cat. No. 205314, Qiagen). Relative gene expression was measured using the RealMasterMix™ Fast SYBR Kit (Cat. No. 2200830, 5 PRIME) in 10 µL reactions on a CFX96Touch™ real time PCR machine. The primer for Il-6 was a QuantiTect® Primer Assay Rn\_Il6\_1\_SG (Cat. No. QT00182896, Qiagen) and diluted as per the Qiagen protocol for the real-time PCR reaction. All other primers were ordered through Integrated DNA Technologies and diluted to a final concentration of 0.65 µM for the real-time PCR reaction. The sequences of primers were as follows: GAPDH forward:

GTTTGTGATGGGTGTGAACC, reverse: TCTTCTGAGTGGCAGTGATG; CD11b

forward: CTGGGAGATGTGAATGGAG, reverse: ACTGATGCTGGCTACTGATG;

IL-1β forward: GAAGTCAAGACCAAAGTGG, reverse:

TGAAGTCAACTATGTCCCG; BDNF forward:

ATCCCATGGGTTACACGAAGGAAG, reverse:

AGTAAGGGCCCGAACATACGATTG; TNFα forward:

CTTCAAGGGACAAGGCTG, reverse: GAGGCTGACTTTCTCCTG; and Arginase

I forward: GTGCCGTTGACCTTGTCTTG, reverse:

GCCTGGTTCTGTTCGGTTTG. GAPDH was used as the reference/housekeeping gene for all samples. For each reaction, the quantitative threshold amplification cycle number ( $C_q$ ) was determined, and the  $2^{-\Delta\Delta C_q}$  method was used to calculate the relative gene expression of each gene in question.

## **2.8 Statistical Analysis**

A one-way ANOVA was used to analyze the expression of immune molecules from the three groups (virgin, E21 pregnant, and P2 postpartum females) examined in Experiment 1. Significant overall effects ( $p < 0.05$ ) were followed up with Tukey's post hoc test to examine individual differences across groups ( $p < 0.05$ ). Two-way ANOVA tests were used to assess the statistical significance of all other data in this study using either stress and pregnancy (Experiment 2) or LPS and pregnancy (Experiment 3) as variables. Significant main effects and significant interactions of variables are reported using  $p < 0.05$ . Significant interactions were followed up with Tukey's post hoc test ( $p < 0.05$ ) to analyze individual comparisons.

### Chapter 3

#### **EXPERIMENT ONE: EFFECT OF PREGNANCY ON NEUROIMMUNE FUNCTION IN THE BRAIN**

Research has yet to examine whether pregnancy might induce changes in the expression of immune molecules of the brain as it does in the periphery. Experiment 1 sought to determine whether pregnancy induces changes in the expression of inflammatory cytokines and microglial activation in the brain. In addition, we also examined a group of rats collected two days postpartum to determine whether these changes in neuroimmune function are also seen in the postpartum period. Pregnant females were either euthanized on E21, two days prior to giving birth, or on P2, two days after giving birth. Virgin females were also euthanized to examine how the expression of immune molecules may change relative to non-pregnant rats. See **Figure 1** for a timeline of this experiment. We predicted that pregnancy would decrease the expression of classical pro-inflammatory immune molecules such as IL-1 $\beta$  and increase the expression of alternate immune molecules like Arginase I in comparison to non-pregnant females.

### Experiment 1 Timeline

#### **Pregnant and Postpartum Rats:**

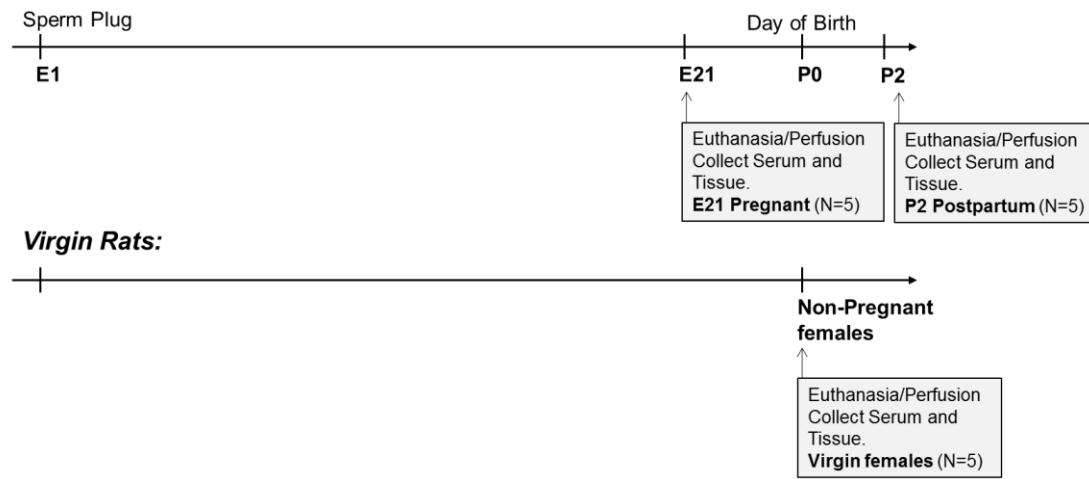


Figure 1. Timeline of Experiment 1. This figure depicts the timeline of events for pregnant, postpartum, and virgin rats used for Experiment 1. Rats in the pregnant and postpartum groups were determined to be pregnant by the presence of a sperm plug at Embryonic (E) day 1. On E21, pregnant rats were euthanized for tissue and serum collection. On Postnatal (P) day 2, postpartum rats were euthanized. Virgin rats followed a matched timeline.

### 3.1 Hippocampus

Hippocampal (HP) tissue was processed for gene expression analysis of microglial activation markers, immune molecules, and neurotrophic factors. IL-1 $\beta$  is a typical pro-inflammatory cytokine. Statistical analysis showed a significant effect of pregnancy or parturition ( $F_{2,11} = 9.20$ ,  $p = 0.004$ ) on the expression of IL-1 $\beta$ . Tukey's post hoc test revealed that both the pregnant females sacrificed at E21 and the new mothers sacrificed at P2 postpartum showed a significant decrease in expression compared to the non-pregnant controls ( $p = 0.006$  and  $p = 0.019$ , respectively; **Figure 2**). The inflammatory cytokine IL-6 also showed a significant effect of pregnancy state ( $F_{2,11} = 4.227$ ,  $p = 0.043$ ). However, in contrast to IL-1 $\beta$ , IL-6 showed

significantly increased expression in P2 postpartum mothers compared to the non-pregnant/virgin animals ( $p = 0.040$ ; **Figure 2**). We did not see any significant changes across groups in the expression of  $TNF\alpha$ , another classical pro-inflammatory immune molecule.

Arginase I is an enzyme that is expressed at elevated levels in the peripheral immune system during the altered immune state of pregnancy. Therefore, we expected that pregnant females would also show an increase in this gene compared to non-pregnant groups in the brain. Our findings supported this theory and showed a significant increase in Arginase I in only the E21 females euthanized during late gestation ( $p = 0.042$ ; **Figure 2**).

We did not find any significant differences in the expression of CD11b, a marker for microglial activation, or in the expression of BDNF, a neurotrophic factor implicated in depression and important for neurogenesis, plasticity, and learning and memory.

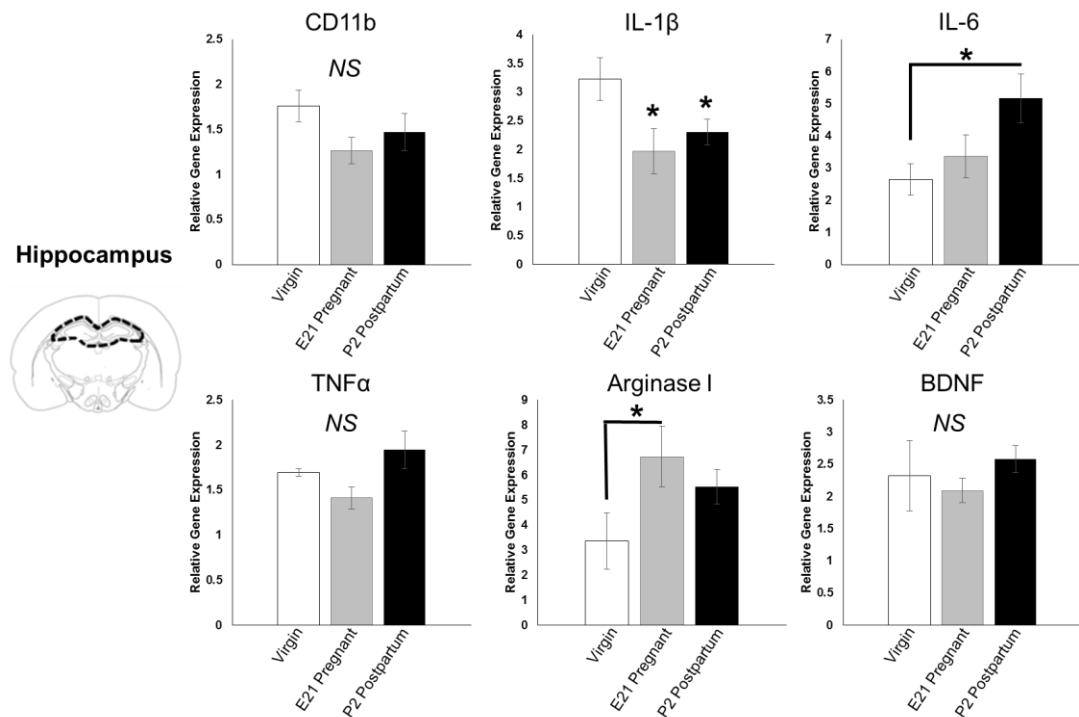


Figure 2. Analysis of Relative Gene Expression in the Hippocampus during Late Pregnancy and Immediately Postpartum. The hippocampal tissue dissected from female rat brains on Embryonic (E) day 21, Postnatal (P) day 2, or from time-matched virgin females is shown. Analysis of IL-1 $\beta$  and IL-6 show a main effect of pregnancy or parturition. Analysis of Arginase I shows increased relative gene expression of E21 females compared to virgin females. Analysis of CD11b, TNF $\alpha$ , and BDNF did not show significance. \*:  $p < 0.05$

### 3.2 Medial Prefrontal Cortex

We also analyzed the expression of these same neuroimmune molecules in the medial prefrontal cortex (mPFC) of the same rats. Again, we predicted that pregnancy would evoke a significant decrease in the expression of classical pro-inflammatory immune molecules while increasing expression of molecules associated with an altered immune state. Contrary to this, we did not find any statistical significance across groups with any of the genes examined in this brain region (**Figure 3**).

However, there was a significant trend in the analysis of IL-1 $\beta$  in the mPFC and in the analysis of IL-6 in the mPFC. Specifically, IL-1 $\beta$  expression decreases while IL-6 increases in the mPFC during late pregnancy and at P2 postpartum in the mPFC. These trends are similar to the significant effects that we found in the HP. In addition, we see trend in the expression of BDNF in the mPFC, such that it increases during late pregnancy and at P2 postpartum.

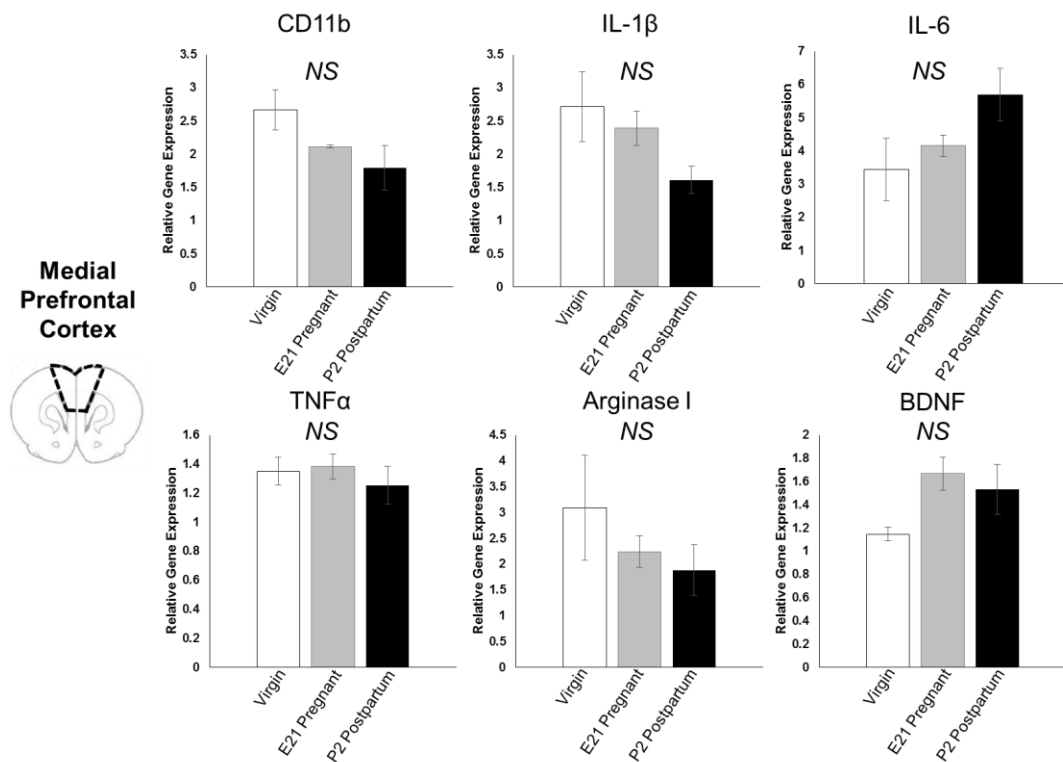


Figure 3. Analysis of Relative Gene Expression in the Medial Prefrontal Cortex during Late Pregnancy and Immediately Postpartum. The dissected region of medial prefrontal cortex tissue from females euthanized on Embryonic (E) day 21, Postnatal (P) day 2, and time-matched virgin females is shown. Analysis did not reveal significance in genes for CD11b, IL-1 $\beta$ , IL-6, TNF $\alpha$ , Arginase I, or BDNF.

### **3.3 Conclusion**

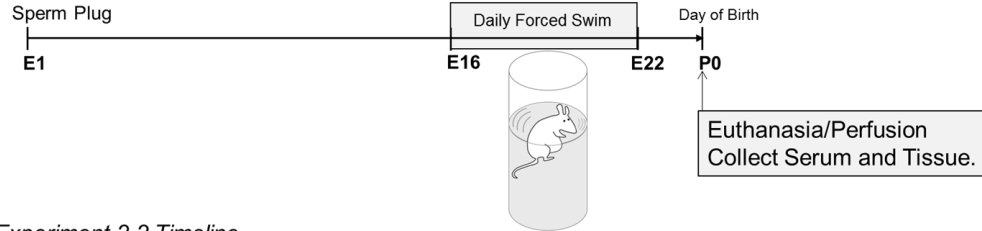
While studies had shown clear changes in the function of the peripheral immune system during pregnancy, the brain had yet to be examined for similar effects. This experiment showed novel changes in the expression of neuroimmune molecules within two brain areas implicated in depression, the hippocampus and medial prefrontal cortex. IL-1 $\beta$  showed a significant decrease in expression in HP tissue of E21 females that continued into the postpartum period at P2. In contrast, IL-6 showed a significant increase in expression only after parturition at P2 in the same brain region. Finally, a molecule thought to be expressed in an altered immune state, Arginase I, showed a significant increase in expression only during the pregnancy period at E21 in the HP. These novel findings provide interesting information about how pregnancy and parturition affect the brains of new mothers.

## Chapter 4

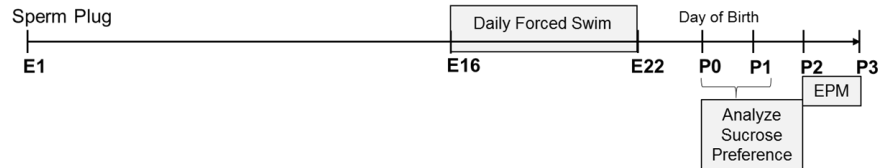
### EXPERIMENT TWO

In this experiment, we sought to examine the effects of a forced swimming stressor during the last week of gestation and its impact on neuroimmune function (Experiment 2.1) and depressive-like behaviors (Experiment 2.2 and 2.3) postpartum. See **Figure 4** for timelines of the pregnant groups for all three experiments. Virgin rats followed time-matched schedules. We hypothesized that the effects of stress would be additive with pregnancy and evoke more drastic changes in gene expression as outlined in Experiment 1. We also predicted that either pregnancy or stress alone may induce anxiety- and depressive-like behaviors, but that the effects of both pregnancy and stress would be additive and specifically induce significantly greater changes in neuroimmune function or the expression of depressive-like behaviors.

*Experiment 2.1 Timeline*



*Experiment 2.2 Timeline*



*Experiment 2.3 Timeline*

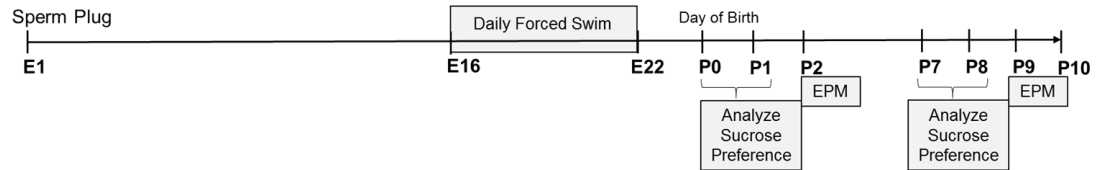


Figure 4. Timeline of Experiments 2.1, 2.2, and 2.3. This figure depicts the timeline of events for pregnant rats used for Experiments 2.1, 2.2, and 2.3. In all experiments, pregnancy was determined by the presence of a sperm plug on Embryonic (E) day 1. Daily forced swim occurred for 5 minutes from E16 through E22. In Experiment 2.1, rats were euthanized one day after completion of forced swim on day of birth (P0) to collect tissue and serum for further analysis. In Experiments 2.2 and 2.3, rats underwent sucrose preference testing on P0 and P1 and Elevated Plus Maze testing on P2. In Experiment 2.3, rats repeated sucrose preference testing on P7 and P8 and Elevated Plus Maze testing on P9. Virgin rats followed a time-matched schedule.

#### **4.1 Experiment 2.1: Impact of pregnancy and stress on postpartum neuroimmune function**

Stress groups received one week of a forced swimming stressor as earlier described while non-stressed groups remained undisturbed during the same period. Pregnant females received the stressor during the last week of gestation or the time-matched equivalent for non-pregnant females. Rats were euthanized on the day of birth (P0; **Figure 4**), one day after the completion of the forced swim stress, in order to analyze gene expression in response to this stressor and pregnancy/parturition.

In this and the following experiments, we narrowed our focus of analysis to the examination of genes that were significantly different (see Experiment 1) during pregnancy and immediately postpartum including the expression of CD11b, a microglial activation marker, IL-1 $\beta$ , IL-6, two pro-inflammatory immune molecules, and BDNF, important for neurogenesis, synaptic plasticity, and implicated in depression. We did not find significant changes in the expression of these genes across groups within the HP of these animals (**Figure 5**), though there appears to be a trend in the expression of IL-1 $\beta$  following pregnancy (similar to the findings in Experiment 1).

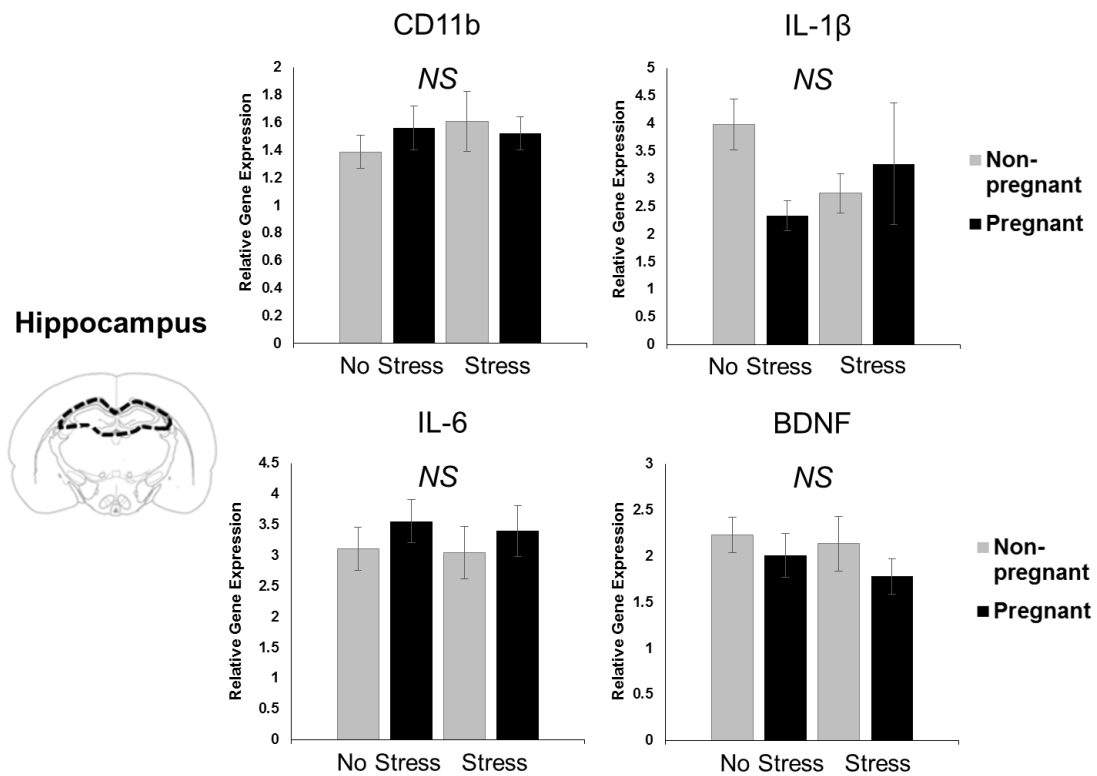


Figure 5. Effects of Forced Swim Test on Relative Inflammatory Gene Expression in the Hippocampus Postpartum. Stressed rats received daily forced swim test from Embryonic (E) day 16 through E21 while rats in the No Stress groups remained undisturbed. Rats were sacrificed on day of birth or 24 hours after the last day of forced swim test for non-pregnant animals. The hippocampal tissue dissected from their brains is shown. Analysis did not reveal significant changes in relative gene expression of CD11b, IL-1 $\beta$ , IL-6, or BDNF.

Interestingly, in the mPFC, we found a significant main effect of pregnancy across all genes examined. Similar to our findings in Experiment 1, we found a significant decrease in the expression of IL-1 $\beta$  in post-parturition animals compared to their non-pregnant counterparts ( $F_{3,27} = 10.664$ ,  $p = 0.003$ ; **Figure 6**). Additionally, we found a main effect of stress on the expression of IL-1 $\beta$  such that animals that

received forced swimming stress had decreased expression of IL-1 $\beta$  compared to non-stressed animals ( $F_{3,27} = 16.312$ ,  $p < 0.001$ ), and the effect of the stress was additive with the effect of pregnancy (**Figure 6**).

In contrast to this, we found that pregnancy or parturition caused a significant increase in the expression of IL-6, a typical inflammatory immune molecule, compared to non-pregnant females ( $F_{3,29} = 6.236$ ,  $p = 0.018$ ; **Figure 6**). This finding replicates the same increase we observed in Experiment 1 postpartum. Stress had no effect on the expression of IL-6.

CD11b, a marker for activated microglia, was significantly decreased in females postpartum compared to non-pregnant animals ( $F_{3,29} = 6.369$ ,  $p = 0.017$ ; **Figure 6**). Forced swim stress did not affect the expression of this microglial marker.

Lastly, BDNF was significantly increased in females post-parturition in comparison to non-pregnant animals ( $F_{3,29} = 130.64$ ,  $p < 0.0001$ ; **Figure 6**). Stress did not have any significant impact on the expression of BDNF in mPFC tissue.

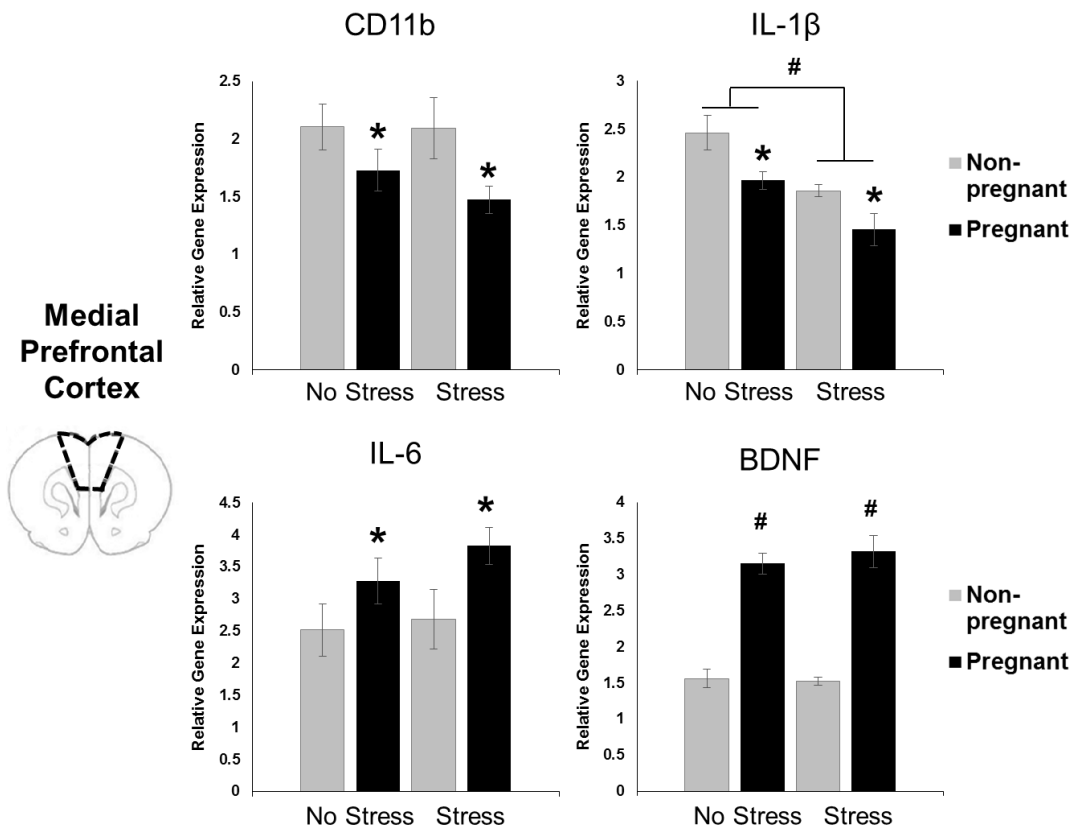


Figure 6. Effects of Pregnancy or Parturition and Forced Swim Test on Relative Inflammatory Gene Expression in the Medial Prefrontal Cortex Postpartum. Stressed rats received daily forced swim test from Embryonic (E) day 16 through E21 while rats in the No Stress groups remained undisturbed. Rats were sacrificed on day of birth or 24 hours after the last day of forced swim test for non-pregnant animals. The medial prefrontal cortex tissue dissected from their brains is shown. Analysis of CD11b, IL-6, and BDNF showed main effects of pregnancy/parturition. IL-1 $\beta$  analysis showed significant main effects of both pregnancy/parturition and forced swim stress. \*:  $p < 0.05$ ; #:  $p < 0.001$

#### **4.2 Experiment 2.2: Impact of pregnancy and stress on postpartum depressive-like behaviors**

For this experiment, the same four groups were used as previously described: Non-pregnant, No stress (n=8); Pregnant, No Stress (n=9); Non-pregnant, Stress (n=8); Pregnant, Stress (n=8). Identical to Experiment 2.1, stress groups received the forced swimming stressor as previously described during the last week of gestation or the time-matched equivalent period (**Figure 4**). Sucrose preference testing was used to measure anhedonia immediately postpartum (P0-1), and the Elevated Plus Maze (EPM) was used to identify anxiety-like behaviors two days postpartum (P2).

Baseline measurements for sucrose preference were taken prior to the start of the experiments. No significant differences existed between the groups in the expression of sucrose preference or anxiety before pregnancy or stress (**Figure 7**). Post-stress measurements identified a significant interaction of pregnancy and stress ( $F_{3,28} = 5.006$ ,  $p = 0.033$ ) such that there was a decrease in sucrose preference in the Pregnant, No Stress animals ( $p = 0.006$ ) and the Non-Pregnant, Stress animals ( $p = 0.032$ ; **Figure 7**) compared to the Non-pregnant, No Stress controls. This suggests that both the stress of forced swimming and the stress of pregnancy are inducing anhedonia behaviors postpartum and post-stress. However, the effects of stress and pregnancy were not additive in that Pregnant, Stress rats did not significantly differ from controls or any other groups in the expression of depressive-like behaviors. These data indicate that the experience of pregnancy may modulate how the animals respond to the stressor and subsequently influence the expression of anhedonia or depressive-like behavior immediately postpartum or following stress.

## Sucrose Preference Test

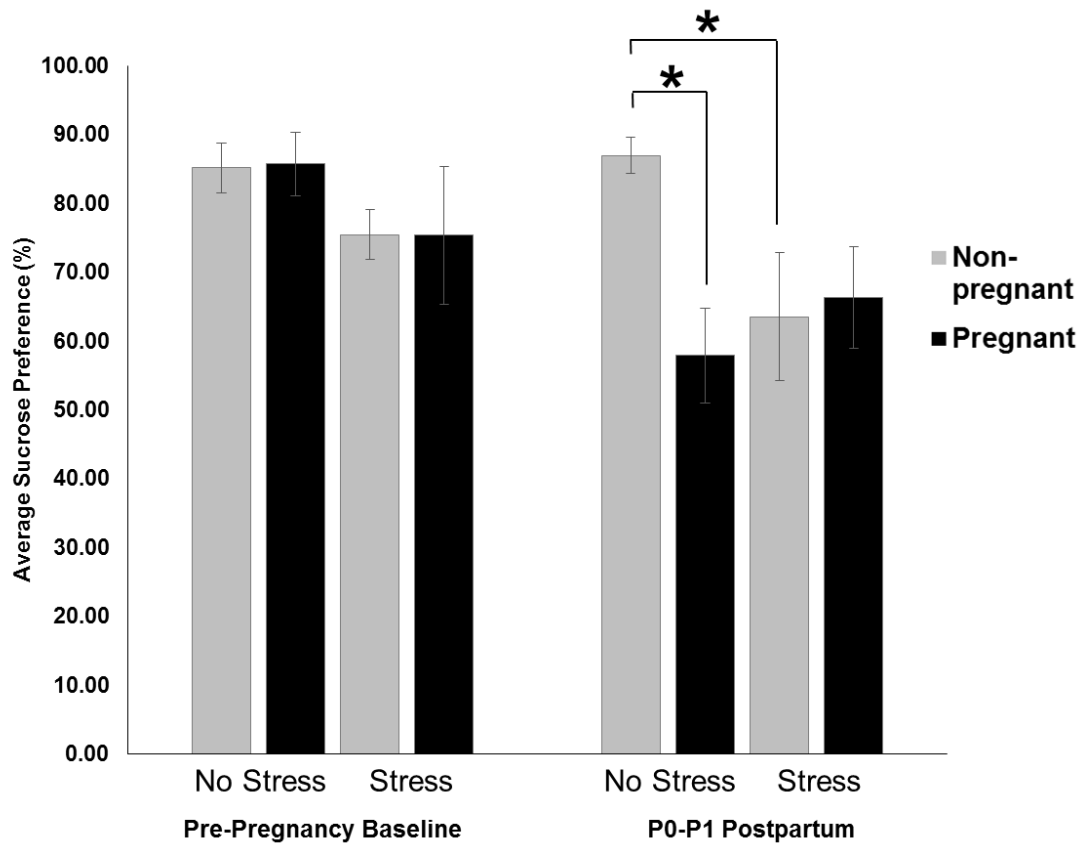


Figure 7. Effects of Pregnancy or Parturition and Forced Swim Test on Sucrose Preference Test Immediately Postpartum. Stressed rats received daily forced swim test from Embryonic (E) day 16 through E21 while rats in the No Stress groups remained undisturbed. Sucrose preference testing was completed prior to the start of the experiment (Pre-Pregnancy Baseline), and immediately postpartum on day of birth (P0) and P1 to measure anhedonia. Analysis showed an interaction of pregnancy and stress postpartum ( $p < 0.05$ ). Post hoc analysis revealed Pregnant, No Stress and Non-pregnant, Stress groups had significantly lower sucrose preference than Non-pregnant, No Stress controls. \*:  $p < 0.05$

EPM baseline measurements were also taken prior to the start of the experiments. We found no significant main effects or interactions of pregnancy and stress. Interestingly, however, when we performed just a pair-wise comparison of the groups, a baseline difference was observed such that the Pregnant, Stress group spent significantly more time in the open arms of the maze compared to the Non-pregnant, Stress groups ( $p = 0.024$ ; **Figure 8**). However, postpartum, we found that pregnancy or parturition induced the opposite effect such that animals postpartum spent significantly *less* time in the open arms compared to the non-pregnant animals ( $F_{3,30} = 5.547$ ,  $p = 0.025$ ; **Figure 8**). Despite expressing less anxiety-like behavior prior to the start of the experiment, both postpartum groups displayed increased anxiety compared to non-pregnant animals (Main effect of pregnancy;  $F_{3,30} = 5.547$ ,  $p = 0.025$ ). We found no effect of the forced swimming stressor on immediate postpartum or post-stress measures of anxiety. Thus we can conclude that pregnancy increases anxiety immediately postpartum, while stress itself (or in combination with pregnancy) had no effect on the expression of anxiety.

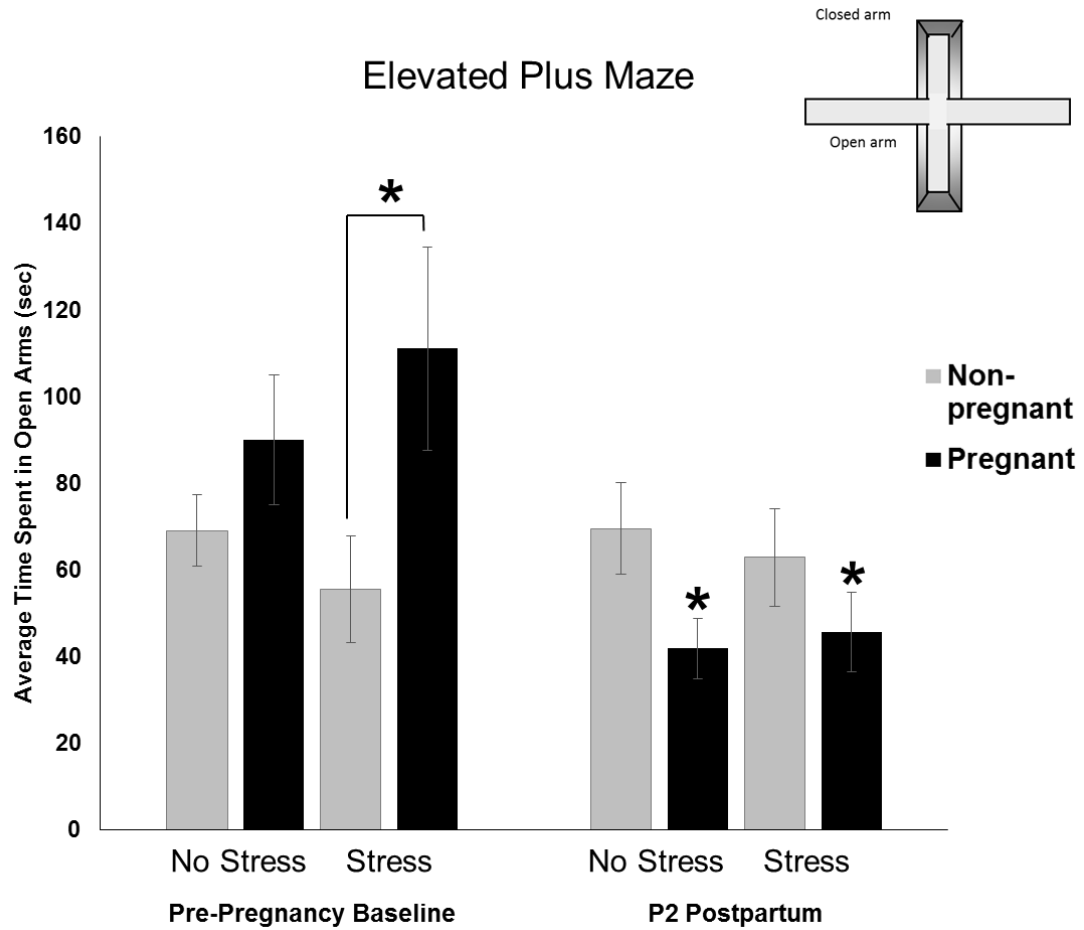


Figure 8. Effects of Pregnancy or Parturition and Forced Swim Test on Elevated Plus Maze Immediately Postpartum. Stressed rats received daily forced swim test from Embryonic (E) day 16 through E21 while rats in the No Stress groups remained undisturbed. A top-down view of the Elevated Plus Maze (EPM) is shown. EPM testing was completed prior to the start of the experiment (Pre-Pregnancy Baseline) and immediately postpartum on Postnatal (P) day 2 to measure anxiety-like behavior via average time spent in the open arms of the maze in seconds. Pairwise comparison of groups for baseline measurements showed the Pregnant, Stress group spent significantly more time in the open arms than the Non-pregnant, Stress group. Analysis of postpartum behavior showed a significant main effect of pregnancy. \*:  $p < 0.05$

### 4.3 Experiment 2.3: Impact of pregnancy and stress on long-term postpartum depressive-like behaviors

In Experiment 2.2, we found that pregnancy/parturition could induce both depressive-like anhedonia and an increase in anxiety immediately postpartum. Thus in the next experiment, we sought to determine what the time course was for the expression of these behaviors postpartum either alone or following stress. Therefore, we repeated Experiment 2.2 with a separate cohort of rats in order to add a second point of behavioral analysis one week postpartum (**Figure 4**). As before, baseline measurements of sucrose preference and EPM were taken prior to breeding. After pregnancy and/or stress, females received sucrose preference and EPM testing immediately postpartum (P0-1 and P2, respectively – see **Figure 4** for timeline), and these tests were repeated one week after giving birth or the time matched equivalent (P7-8, and P9, respectively – see **Figure 4** for timeline). Findings from this experiment sought to determine if the behavioral findings from the previous experiment extended beyond the immediate postpartum period.

As before, baseline sucrose preference testing was completed prior to animals being bred and divided into the experimental groups. No significant differences existed in sucrose preference at baseline (**Figure 9**). Immediately postpartum at P0-1, we replicated our findings from Experiment 2.2 such that we found a significant interaction of pregnancy and stress ( $F_{3,25} = 11.519$ ,  $p = 0.002$ ; see **Figure 7** for comparison). As before, the Pregnant, No Stress group and the Non-pregnant, Stress groups displayed a significantly lower preference for sucrose compared to Non-pregnant, No Stress controls ( $p = 0.008$  and  $p = 0.006$ , respectively; **Figure 9**). The Pregnant, Stress group did not significantly differ from control animals or any other treatment group, indicating that the effects of pregnancy and stress were not additive;

however, analysis revealed a trend ( $p < 0.08$ ) of significant difference in this group relative to the Non-pregnant, No Stress controls ( $p = 0.072$ ; **Figure 9**). Replicating our findings from Experiment 2.2, these findings also suggest that pregnancy induces anhedonia immediately postpartum, similar to the effects of stress. They also suggest that pregnancy may modulate the effects of stress on this immediate postpartum behavior. Interestingly, the groups did not significantly differ from one another on P7-8 testing. Thus, the impact of pregnancy or parturition is no longer affecting the expression of this behavior one week postpartum.

### Sucrose Preference Test

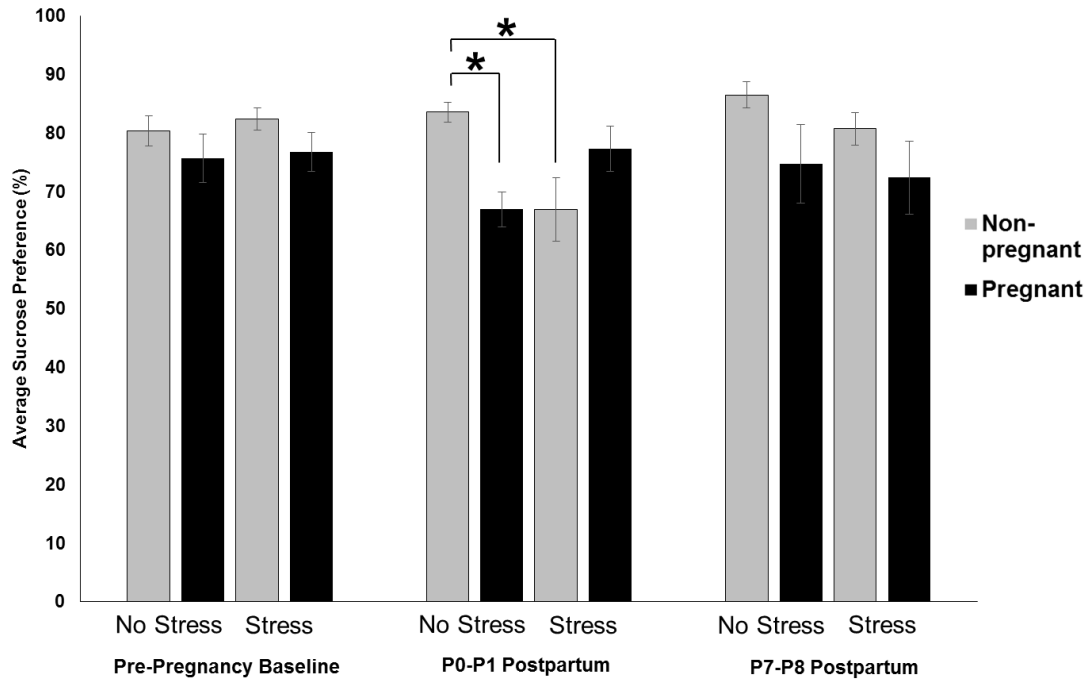


Figure 9. Effects of Pregnancy or Parturition and Forced Swim Test on Long-Term Sucrose Preference Test Postpartum. Stressed rats received daily forced swim test from Embryonic (E) day 16 through E21 while rats in the No Stress groups remained undisturbed. Sucrose preference testing was completed prior to the start of the experiment (Pre-Pregnancy Baseline), immediately postpartum on day of birth (P0) and P1, and one week later (P7 and P8) to measure anhedonia. Analysis showed an interaction of pregnancy and stress immediately (P0 and P1) postpartum ( $p < 0.05$ ). Post hoc analysis revealed Pregnant, No Stress and Non-pregnant, Stress groups had significantly lower sucrose preference than Non-pregnant, No Stress controls. There were no significant differences one week later. \*:  $p < 0.05$

Identical to the previous experiment, EPM performance was tested prior to the start of breeding and manipulations. Surprisingly, we find a similar significant difference across groups at baseline as we did in Experiment 2.2. Specifically, we found a significant main effect of pregnancy such that there was an increase in amount of time spent in the open arms of the Elevated Plus Maze in all animals that would later become pregnant after breeding as compared to the females that remained non-pregnant ( $F_{3,27} = 5.157$ ,  $p = 0.031$ ; **Figure 10**). Similar to the findings in the Experiment 2.2, these findings suggest that female rats that will later become pregnant exhibit decreased anxiety-like behaviors prior to the start of testing, prior to breeding.

Immediately postpartum (P2), we find a significant effect of the forced swimming stress on EPM behaviors such that stressed animals spent significantly less time in the open arms than their non-stressed counterparts ( $F_{3,25} = 7.833$ ,  $p = 0.010$ ; **Figure 10**). Contrary to the findings in Experiment 2.2, we found no significant effect of pregnancy on anxiety immediately postpartum (**Figure 10**; see **Figure 8** for comparison). Instead, we found a significant main effect of pregnancy one week later (P9) such that postpartum females spent significantly less time in the open arms compared to the non-pregnant animals ( $F_{3,27} = 4.938$ ,  $p = 0.035$ ; **Figure 10**). This is particularly interesting given the fact that prior to the start of the experiment, the animals in pregnant groups appeared significantly less anxious. After pregnancy or parturition, the same animals appear to be much more anxious compared to their non-pregnant counterparts, similar to the data observed in Experiment 2.2. These findings suggest that both pregnancy and forced swimming stress are significantly altering anxiety-like behaviors, and that anxiety presents itself in a time-dependent manner.

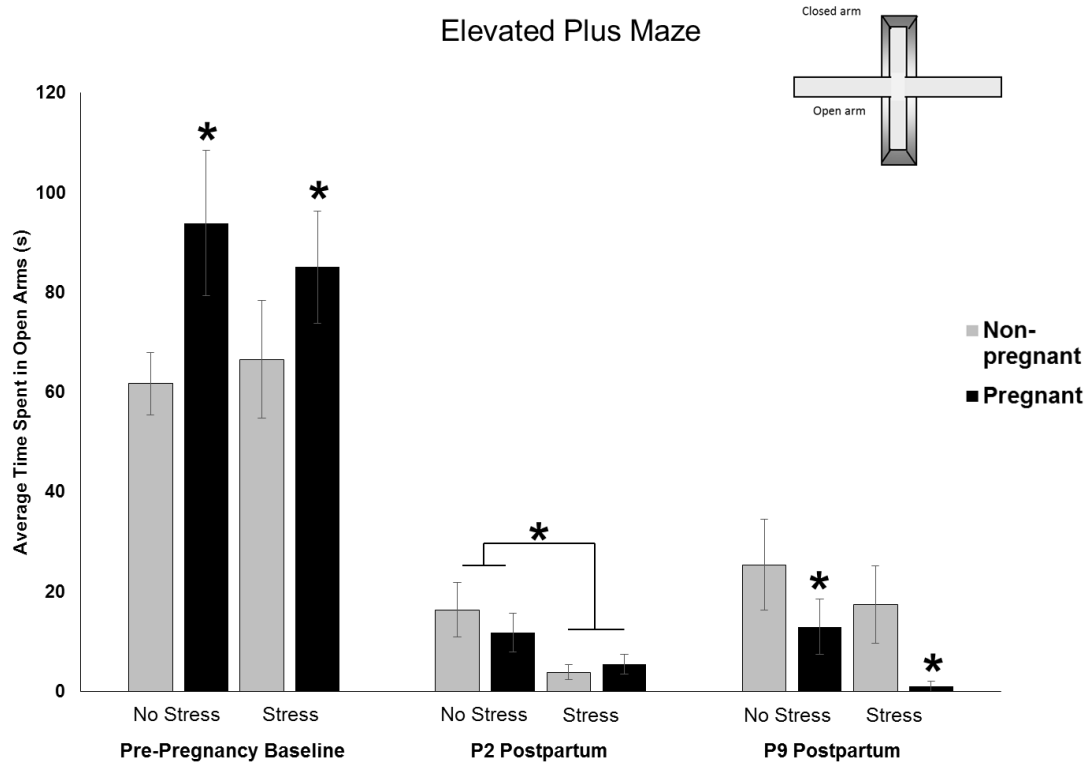


Figure 10. Effects of Pregnancy or Parturition and Forced Swim Test on Long-Term Elevated Plus Maze Postpartum. Stressed rats received daily forced swim test from Embryonic (E) day 16 through E21 while rats in the No Stress groups remained undisturbed. A top-down view of the Elevated Plus Maze (EPM) is shown. EPM testing was completed prior to the start of the experiment (Pre-Pregnancy Baseline), immediately postpartum on Postnatal (P) day 2, and one week later on P9 to measure anxiety-like behavior via average time spent in the open arms of the maze in seconds. Analysis of baseline measurements showed a main effect of pregnancy where animals that would later become pregnant spent significantly more time in the open arms compared to non-pregnant groups. Behavior on P2 showed a main effect of forced swim test such that stressed groups spent significantly less time in the open arms compared to no stress groups. One week later on P9, analysis revealed a significant main effect of pregnancy in that animals that had given birth spent significantly less time in the open arms compared to non-pregnant rats. \*:  $p < 0.05$

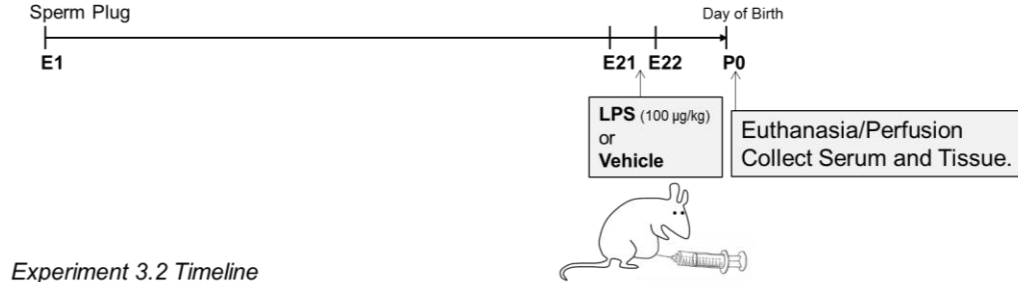
The results of Experiment 2.3 indicate that pregnancy/parturition results in significant anhedonia immediately postpartum, but that these depressive-like behaviors are gone one week postpartum. Stress alone also produces similar anhedonia, consistent with other models of depression; however, after one week without stress, this depressive-like behavior is no longer expressed. Notably, contrary to our initial predictions, stress *during* pregnancy does not produce exaggerated anhedonia or anxiety in females immediately or one week postpartum.

## Chapter 5

### EXPERIMENT THREE

Previous research has shown neuroimmune and behavioral changes take place in response to systemic viral or bacterial infection (Miller et al., 2009; Anisman and Merali, 2002). For example, the cold virus can produce activation of the immune system and altered behavior, including decreased appetite, increased sleepiness, increased agitation, and increased withdrawal. This experiment sought to determine the effects acute immune activation, via systemic lipopolysaccharide (LPS) injection, during late gestation and examine subsequent changes in neuroimmune function (Experiment 3.1) and postpartum depressive-like behaviors (Experiment 3.2). See **Figure 11** for a timeline of events for Experiments 3.1 and 3.2. While the effects of LPS are known to induce increases in the expression of classical pro-inflammatory cytokines and the expression of depressive-like or sickness behaviors (Dantzer et al., 2008), the time points at which we examined these endpoints was after these effects would have ended in rats that are not pregnant (more than 24 hours post-injection; Biesmans et al., 2013). Thus, we hypothesized that effects of acute immune activation with LPS may interact with the effects of pregnancy to instead induce a long-term change in neuroimmune function and depressive-like behaviors postpartum.

### Experiment 3.1 Timeline



### Experiment 3.2 Timeline

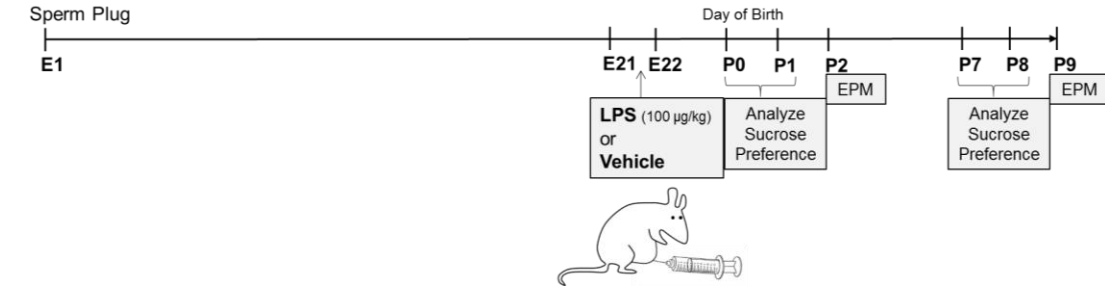


Figure 11. Timeline of Experiments 3.1 and 3.2. This figure depicts the timeline of events for pregnant rats used for Experiments 3.1 and 3.2. In both experiments, pregnancy was determined by the presence of a sperm plug at Embryonic (E) day 1. Between E21 and E22, rats were injected with 100µg/kg of lipopolysaccharide (LPS) or the saline vehicle at equal volume. In Experiment 3.1, rats were euthanized on day of birth (P0) to collect tissue and serum for further processing. In Experiment 3.2, rats completed sucrose preference testing immediately postpartum on day of birth and P1 and Elevated Plus Maze testing on P2. These behavioral tests were repeated one week later. Sucrose preference testing was repeated on P7 and P8, and Elevated Plus Maze testing was repeated on P9. Virgin rats in both experiments followed a time-matched schedule.

### 5.1 Experiment 3.1: Impact of pregnancy and acute immune activation on postpartum neuroimmune function

Pregnant and non-pregnant rats were injected with LPS (100 µg/kg) between 24 and 48 hours prior to giving birth in the pregnant groups and on a matched timeline for non-pregnant animals. Rats were euthanized on the day of birth (P0) or equivalent (**Figure 11**), and their brains were harvested for RNA extraction and subsequent analysis of gene expression for microglial activation markers, inflammatory cytokines, and neurotrophic factors, many of which we had seen significant effects in previous experiments.

In the hippocampus, expression of the pro-inflammatory cytokine IL-1 $\beta$  showed a significant main effect of LPS treatment ( $F_{3,23} = 6.406$ ,  $p = 0.019$ ; **Figure 12**). However, only the Non-pregnant, LPS group showed a significant increase in expression of IL-1 $\beta$  compared to the other three groups ( $p < 0.05$ ), suggesting that pregnancy may be modulating the immune response to LPS immediately postpartum such that pregnancy prevents the same increase in IL-1 $\beta$  expression that occurs in the hippocampus of non-pregnant females treated with LPS (**Figure 12**).

Analysis of the inflammatory cytokine IL-6 revealed a significant main effect of pregnancy ( $F_{3,25} = 4.627$ ,  $p = 0.041$ ; **Figure 12**). Both postpartum groups showed significantly increased expression compared to their non-pregnant counterparts, similar to the findings from Experiment 1 and 2.1 However, we did not see any effects of LPS immune activation and no interaction of pregnancy and immune activation.

Additionally, there were no statistical effects of pregnancy or parturition and/or injection treatment in the microglial activation marker CD11b or in BDNF in the HP.

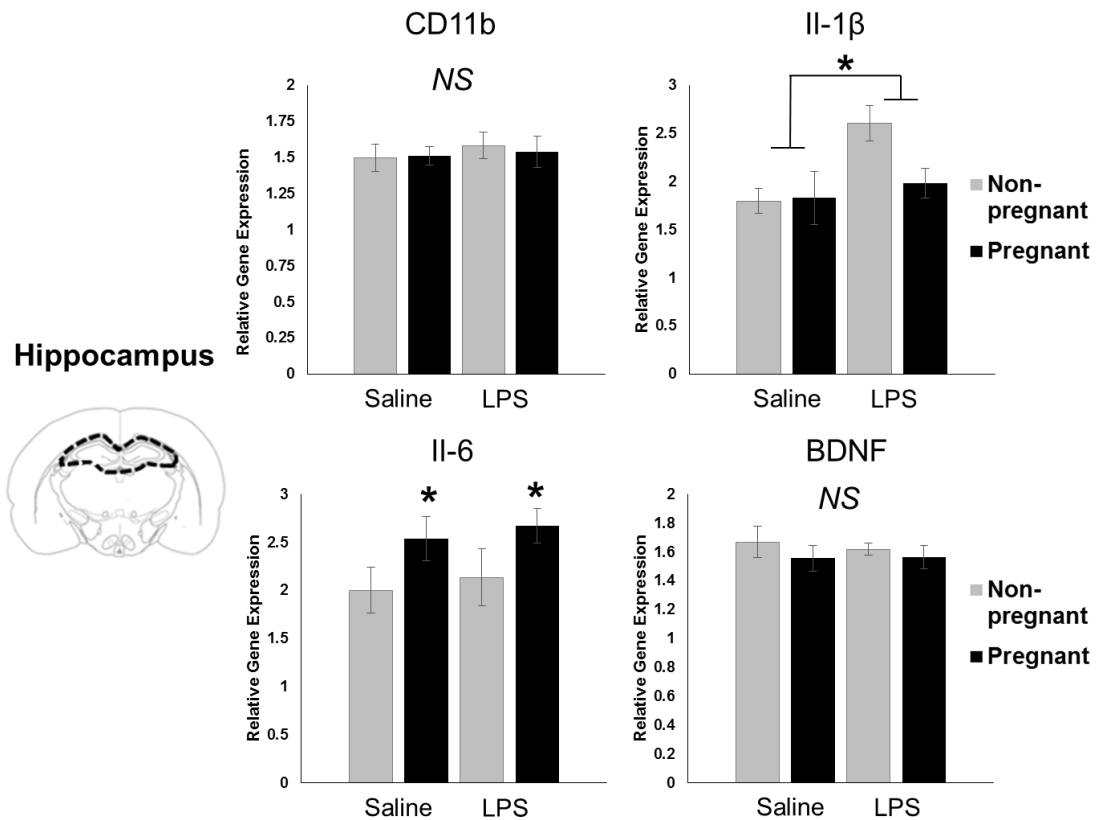


Figure 12. Effects of Pregnancy or Parturition and Acute Immune Activation on Relative Inflammatory Gene Expression in the Hippocampus Postpartum. Between Embryonic (E) day 21 and 22, rats received an injection of either lipopolysaccharide (LPS) (100 $\mu$ g/kg) or its saline vehicle at equal volume. Rats were euthanized on day of birth or at least 24 hours after the injection for non-pregnant animals. The hippocampal tissue dissected from their brains is shown. IL-1 $\beta$  showed a main effect of treatment. Only the Non-pregnant, LPS group showed significantly higher gene expression compared to the other three groups ( $p < 0.05$ ). IL-6 showed a main effect of pregnancy such that rats that had just given birth showed higher gene expression relative to the non-pregnant groups. No significant differences were found for CD11b or BDNF. \*:  $p < 0.05$

Similar to Experiment 2.1, we saw a greater number of changes in gene expression within the mPFC of these rats (**Figure 13**). Analysis of the pro-inflammatory cytokine IL-1 $\beta$  revealed a significant interaction of pregnancy or parturition and immune activation ( $F_{3,26} = 5.324$ ,  $p = 0.029$ ; **Figure 13**). Although there were no statistically significant differences across groups, we found trends ( $p < 0.08$ ) that the Pregnant, Stress group had decreased expression of IL-1 $\beta$  compared to the Pregnant, No Stress ( $p = 0.066$ ) and Non-pregnant, Stress ( $p = 0.077$ ; **Figure 13**) groups. The significant interaction of pregnancy and treatment suggests that pregnancy may be modulating the typical expression of this pro-inflammatory cytokine in response to LPS infection.

Analysis of IL-6 expression in the mPFC revealed a significant interaction of pregnancy and/or parturition and LPS immune activation ( $F_{1,25} = 10.281$ ,  $p = 0.004$ ). Non-pregnant LPS treated females expressed higher levels of IL-6 compared to the non-pregnant saline-injected controls, as expected ( $p = 0.013$ ). Both pregnancy and LPS immune activation induced increased expression of the cytokine; however, the effects were not additive. A trend ( $p < 0.08$ ) suggested decreased expression levels in Pregnant, LPS females compared to the Non-pregnant, Saline animals. These data imply that, again, pregnancy may be modulating the effects of acute immune activation to blunt the expression of pro-inflammatory cytokines in the brain.

Analysis of the microglial activation marker, CD11b, revealed an interaction of pregnancy and immune activation ( $F_{3,26} = 5.745$ ,  $p = 0.024$ ; **Figure 13**). Pregnant females injected with LPS had significantly lower expression compared to the non-pregnant animals with LPS treatment ( $p = 0.022$ ). Similar to IL-6, CD11b showed a trend ( $p < 0.08$ ) that Pregnant, LPS animals had lower expression compared to the

Pregnant, Saline animals, suggesting that the effects of pregnancy may be modulating the gene expression of CD11b following an acute immune challenge.

Lastly, we examined the BDNF gene implicated in depression and necessary for neurogenesis, learning and memory, and neuroplasticity. As in previous experiments, analysis of BDNF expression revealed significantly robust increases within the mPFC of postpartum females relative to the levels expressed in non-pregnant females ( $F_{3,27} = 107.211$ ,  $p < 0.001$ ; **Figure 13**). This finding replicates what was found in Experiment 2.1, and the trends found in Experiment 1, indicating that BDNF is significantly increased in the mPFC following pregnancy or parturition. We did not see any effects of acute immune activation on the expression of BDNF in mPFC.

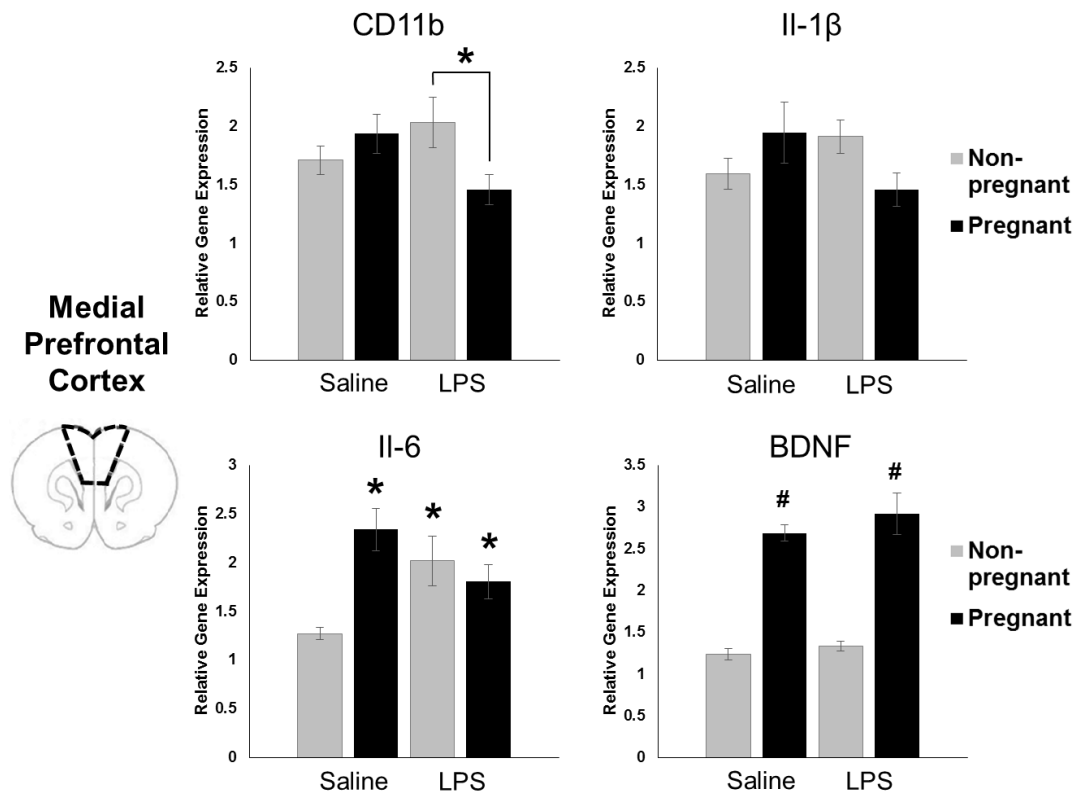


Figure 13. Effects of Pregnancy or Parturition and Acute Immune Activation on Relative Inflammatory Gene Expression in the Medial Prefrontal Cortex Postpartum. Between Embryonic (E) day 21 and 22, rats received an injection of either lipopolysaccharide (LPS) (100 $\mu$ g/kg) or its saline vehicle at equal volume. Rats were euthanized on day of birth or at least 24 hours after the injection for non-pregnant animals. The medial prefrontal cortex tissue dissected from their brains is shown. Analysis of CD11b expression showed an interaction of pregnancy and immune activation. Post hoc analysis showed the Pregnant, LPS group had significantly lower expression than the Non-pregnant, LPS group. IL-1 $\beta$  analysis showed a significant interaction of pregnancy and immune activation. Post hoc analysis showed trends that the Pregnant, LPS group had lower expression than the Non-pregnant, LPS and Pregnant, Saline groups ( $p < 0.08$ , not shown). Analysis of IL-6 showed a significant main effect of pregnancy and an interaction of pregnancy and immune activation such that both Pregnant groups and the Non-pregnant, LPS group all had significantly higher expression compared to Non-pregnant, Saline controls. BDNF analysis revealed a significant main effect of pregnancy in that rats that had just given birth showed higher gene expression relative to non-pregnant rats. \*:  $p < 0.05$ ; #:  $p < 0.001$

## 5.2 Experiment 3.2: Impact of pregnancy and acute immune activation on postpartum depressive-like behaviors

We hypothesized that the classical sickness and depressive-like behaviors that are induced by acute immune activation would no longer be present in non-pregnant females 24 hours after LPS injection. However, we predicted that LPS would induce selective changes in depressive-like or anxiety behaviors in postpartum females and that these depressive-like and anxiety behaviors may continue to be expressed nearly one week after parturition. Similar to Experiment 2, baseline measures of sucrose preference and Elevated Plus Maze behavior were taken prior to breeding and the start of the experiments. Pregnant and non-pregnant rats were injected with either saline or LPS (100  $\mu\text{g}/\text{kg}$ ) between 24 and 48 hours prior to giving birth, or the time-matched equivalent in non-pregnant rats. Anhedonia was measured using the sucrose preference test immediately postpartum and again one week later (P0-1, and P7-8), and anxiety-like behaviors were tested using EPM immediately postpartum (P2) and one week later (P9) in order to obtain a time course of depressive-like and anxiety behaviors postpartum (see **Figure 11** for timeline).

Baseline sucrose preference analysis did not reveal any significant differences between groups. However, immediately postpartum, rats that had just given birth revealed significant anhedonia in decreased sucrose preference scores compared to the non-pregnant females ( $F_{3,28} = 8.181$ ,  $p = 0.008$ ; **Figure 14**), similar to the findings from Experiment 2.2 and 2.3. The effects of pregnancy did not continue one week postpartum, similar to the findings from Experiment 2.3. Interestingly, we found a significant main effect of the acute immune activation at this second time point such that females treated with LPS more than one week earlier had a higher preference for sucrose and thus much less anhedonia in comparison to saline-treated females ( $F_{3,30} =$

5.944,  $p = 0.021$ ; **Figure 14**). These data suggest that the effects of pregnancy or parturition influence anhedonia immediate postpartum while the effects of LPS may influence the expression of reward-seeking behaviors later than the immediate window during which typical sickness and depressive-like behaviors are expressed following acute immune activation.

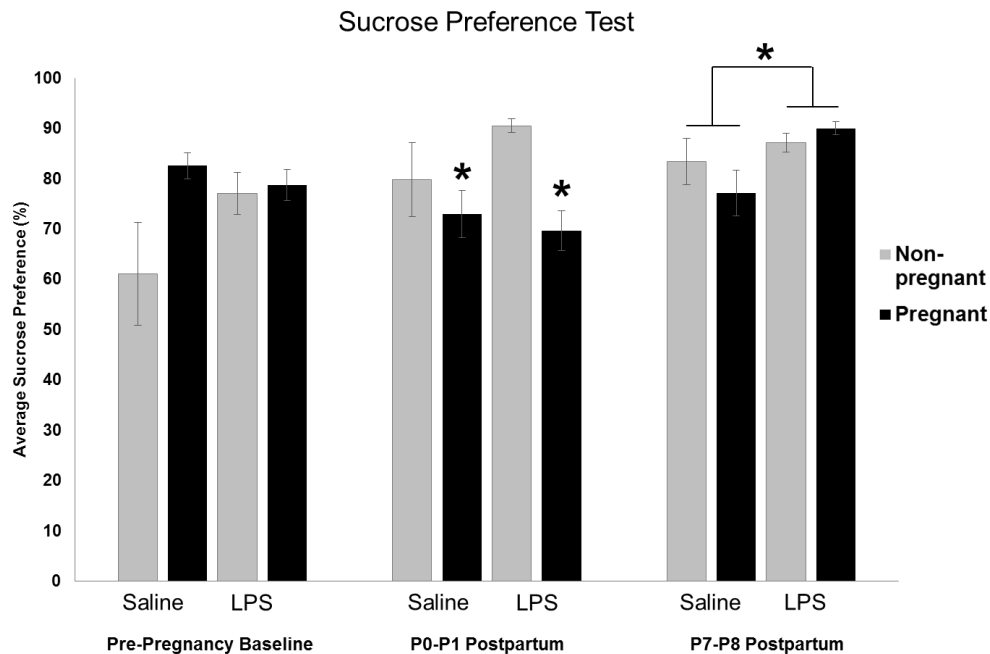


Figure 14. Effects of Pregnancy or Parturition and Acute Immune Activation on Long-Term Sucrose Preference Test Postpartum. Between Embryonic (E) day 21 and 22, rats received an injection of either lipopolysaccharide (LPS) (100 $\mu$ g/kg) or its saline vehicle at equal volume. Sucrose preference testing was completed prior to the start of the experiment (Pre-Pregnancy Baseline), immediately postpartum on day of birth (P0) and P1, and one week later (P7 and P8) to measure anhedonia. Analysis showed a significant main effect of pregnancy immediately postpartum (P0 and P1) such that rats that had just given birth showed lower preference for sucrose compared to non-pregnant rats. One week later, analysis showed a main effect of treatment such that LPS rats had greater sucrose preference compared to their saline counterparts. \*:  $p < 0.05$

EPM testing at baseline, prior to the start of the experiment, revealed no significant differences between groups. Remarkably, we see a significant and striking decrease in the amount of time spent in the open arms of the maze in all groups compared to the baseline numbers. However, rats that had just given birth showed significantly more time spent in the open arms, and thus possibly less anxiety, as compared to their non-pregnant counterparts ( $F_{3,30} = 13.745$ ,  $p = 0.001$ ; **Figure 15**). These effects are not maintained one week later; however, we do see a trend ( $p < 0.08$ ) such that postpartum animals are still showing less anxiety-like behaviors relative to the non-pregnant females ( $p = 0.066$ ; **Figure 15**). Interestingly, there are no significant effects of acute immune activation on this anxiety behavior postpartum.

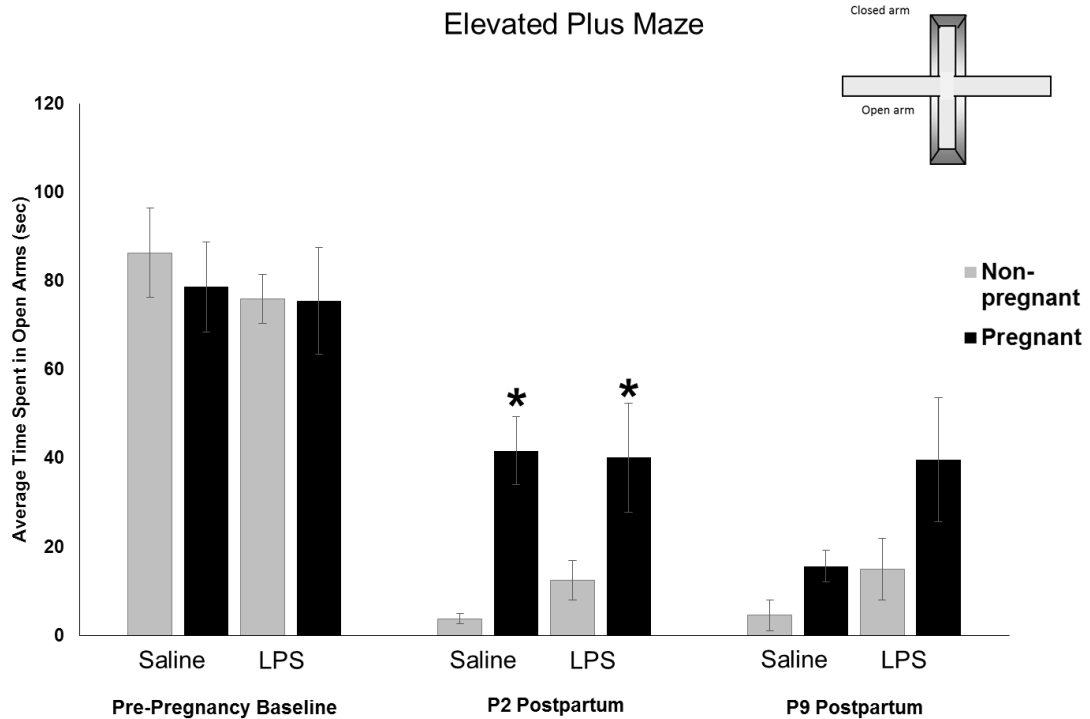


Figure 15. Effects of Pregnancy or Parturition and Acute Immune Activation on Long-Term Elevated Plus Maze Test Postpartum. Between Embryonic (E) day 21 and 22, rats received an injection of either lipopolysaccharide (LPS) (100 $\mu$ g/kg) or its saline vehicle at equal volume. A top-down view of the Elevated Plus Maze (EPM) is shown. EPM testing was completed prior to the start of the experiment (Pre-Pregnancy Baseline), immediately postpartum on Postnatal (P) day 2, and one week later on P9 to measure anxiety-like behavior via average time spent in the open arms of the maze in seconds. Analysis of the immediate postpartum period (P2) showed a main effect of pregnancy such that postpartum rats exhibited less anxiety with more time spent in the open arms compared to their non-pregnant counterparts. One week later, analysis showed a trend that Pregnant groups spent more time in the open arms compared to the non-pregnant animals ( $p < 0.08$ , not shown). \*:  $p < 0.05$

In conclusion, pregnancy and/or parturition and acute immune activation caused by LPS exposure significantly alters depressive-like and anxiety behaviors in different ways. Exposure to LPS increases the expression of reward-seeking behaviors (sucrose preference) but only one week after the immune activation. On the other hand, similar to our conclusions from previous experiments, the experience of pregnancy and/or giving birth for these females results in significant anhedonia immediately postpartum that is not maintained into the later postpartum period. In contrast, we found that anxiety was decreased in the pregnant females immediately postpartum in this experiment. Thus, although anhedonia and anxiety are two behaviors often comorbid with one another, they are clearly different and may be affected separately by different physiological stressors as well as under different time courses.

## **Chapter 6**

### **DISCUSSION**

The experiments of this study sought to determine how pregnancy, parturition, and varying stressors affects neuroimmune function and depressive-like and anxiety behaviors of rats postpartum in order to gain a better understanding of how the central immune system responds to such robust physiological changes and whether changes in neuroimmune function that result from pregnancy, stress and/or immune activation may help to understand the expression of different depressive-like behaviors as a result. First, we found a number of significant changes in the gene expression of inflammatory molecules within the hippocampus and medial prefrontal cortex during normal pregnancy and postpartum that, to our knowledge, have not been previously identified. Next, we found that a chronic stressor, such as forced swim for just 7 days, did not induce a significant number of changes in gene expression, contrary to our initial prediction. However, systemic LPS exposure during pregnancy resulted in novel changes in neuroimmune gene expression, suggesting that pregnancy may be modulating or blunting the typical immune response produced by LPS. Finally, we found that both stress and pregnancy or parturition induced significant anhedonia and anxiety-like behaviors immediately postpartum that were fortunately reversed one week later. Thus, these findings suggest that pregnancy and/or parturition is inducing significant changes in brain regions implicated in cases of depression which may be further modulating the impact of additional stress or immune activation during gestation.

In support of our original hypothesis, we observed a number of changes in the expression of inflammatory genes during pregnancy that were seen in the postpartum period as well. In the hippocampus, we found a significant decrease in IL-1 $\beta$  during late gestation which persisted two days after parturition. IL-1 $\beta$  is a typical pro-inflammatory cytokine that is expressed in response to stress and infections. Pregnancy induces an alternate immune state in the periphery that may be part of the necessary changes that take place in the mother to allow for growth and development of the fetus. We hypothesized that we would see similar effects of pregnancy in the brain, particularly given the constant communication between the peripheral and central or neuroimmune systems. Thus, we expected that classical cytokine expression, such as IL-1 $\beta$ , would be dampened during late gestation. The decreased expression of IL-1 $\beta$  aligns with this hypothesis; however, we were surprised to see that expression remained depressed two days postpartum. This suggests that the immune system had not yet returned to a pre-pregnancy state, and that the expression of an alternate system may still be in effect during the immediate postpartum period. In subsequent experiments, we were also able to replicate the decreased expression of IL-1 $\beta$  in the mPFC on day of birth (P0), indicating that typical neuroimmune function is being altered in more than one brain region.

In contrast to the decrease in IL-1 $\beta$  expression that we saw in our experiments, we found an interesting *increase* in the expression of the cytokine IL-6 that was only seen post-parturition in the hippocampus. Our next two experiments were able to replicate this finding in tissue collected on day of birth in postpartum females. Interestingly, IL-6 has been found to act in both a pro- and anti-inflammatory manner via very different mechanisms of action (Scheller et al., 2011; Wong et al., 2003; Xing

et al., 1998). We cannot be sure how IL-6 is acting in these brain regions at this time. However, this is an important piece of information to add to the study of pregnancy and parturition given the fact that we did not see an increase in expression during gestation. Ongoing experiments will also determine whether IL-6 is similarly increased in the periphery immediately postpartum, which will allow us to determine that this effect is specific to the mPFC. Knowing the manner in which IL-6 is acting postpartum, particularly within the brain, may allow us to distinguish between changes that take place in response to pregnancy and those in response to parturition and new motherhood, and ultimately how these changes in immune function may result in depressive-like behaviors postpartum.

In our next experiments, we observed a marked decrease in the expression of CD11b in mPFC of postpartum females. CD11b is generally used as a marker for microglial activation. As the resident immune cells of the brain, microglia may become activated by stress and infections in order to survey surrounding cells and activate signaling cascades to induce a pro-inflammatory response for detected insults and/or cellular debris. Decreased expression of CD11b may suggest that microglia are less activated during pregnancy or post-parturition. This would lend support for an additional mechanism of action of the attenuated immune function seen in pregnant women (Brunton and Russell, 2008) such that less active microglia at baseline could make it more difficult for additional stress or immune activation to cause the typically robust response by the immune system in the brain. However, a recent study using a chronic unpredictable stress procedure in mice may provide an alternative explanation (Kreisel et al., 2014). After five weeks of chronic stress, microglia were significantly decreased in number, and there was also an increase in the number of damaged or

dying microglia compared to control animals. In a subsequent experiment, the mice received microglial activators, and researchers saw a reversal in depressive-like behaviors and an increase in neurogenesis. This study suggests the possibility that pregnancy may be replicating aspects of a chronic stressor so as to damage the number of existing microglia, resulting in a decrease in activated microglia seen in our experiments with decreased CD11b expression specifically in the mPFC. Thus, our ongoing and future studies are required to characterize the number and morphology of microglia in response to pregnancy in order to better understand these findings of CD11b gene expression in the brain.

In addition to these neuroimmune changes, pregnancy or parturition induced a robust increase in the expression of brain derived neurotrophic factor, or BDNF, in mPFC that was replicated in our later experiments. The BDNF gene is important for synaptic plasticity, learning and memory, and neurogenesis. More specifically, patients suffering from depression are seen to have decreased expression of this gene (Lee and Kim, 2010). Thus, we hypothesized that our model of pregnancy and stress would induce depressive-like symptoms neurologically and behaviorally such that we would see a decrease in BDNF expression. However, our data showed the opposite effect, specifically in the mPFC, and this was unaffected by our added stressors. We did not see any changes in BDNF expression in our hippocampal tissue following pregnancy or stress; therefore, the affected brain region may be an important aspect in understanding this unexpected result.

It is important to note that motherhood in rodents greatly differs from motherhood in humans. Naïve female rats that have never had a litter will not immediately care for a pup from another dam unless there is continuous re-exposure to

this foreign pup (Moltz et al., 1970). However, once a female has her first litter, she will instantly develop these maternal instincts and properly care for her own pups as well as those from other dams (Rosenblatt et al., 1988; Moltz et al., 1970). Knowing that BDNF is involved in learning and memory, plasticity, and neurogenesis, the increase in BDNF could be underlying the expression of new maternal behaviors and the learning involved with new motherhood. Thus, we cannot be sure if the change in BDNF expression is as a result of experiencing the pregnancy, the birthing process, or the existence of a new litter of pups in the cage. We did not explicitly examine maternal care in this study; however, future studies intend to characterize those behaviors which may help us to understand the action of BDNF in this case.

In addition to the effects of pregnancy alone, we sought to determine if stress during late gestation would interact with pregnancy and/or parturition that would cause altered neuroimmune functioning that may present with depressive-like behaviors postpartum. Our second experiment used a chronic stressor of forced swimming during the last week of pregnancy in order to analyze changes in gene expression and behavior. These data showed a significant decrease in the mPFC expression of IL-1 $\beta$  of our stressed animals in comparison to their non-stressed counterparts. We predicted that the expression of typical pro-inflammatory cytokines would be decreased in response to pregnancy and increased in response to the stress; however, even the stressed non-pregnant animals exhibited decreased IL-1 $\beta$  expression. These data pose additional interesting questions. Although the chronic stressor is eliciting changes in neuroimmune function separate from pregnancy, we hypothesized that stress would induce a pro-inflammatory response. Decreased IL-1 $\beta$  expression may suggest that either the forced swimming stressor does not produce

typical pro-inflammatory changes or that IL-1 $\beta$  is acting in a separate manner than we originally predicted.

Taking these data into account, our third experiment sought to explore the impact of an acute immune challenge of systemic LPS injection 1-2 days prior to giving birth. This type of immune activation is known to induce a typical pro-inflammatory immune response in addition to depressive-like behaviors for about 24 hours post-injection (Biesmans et al., 2013). Our tissue was collected after this time period had passed. Thus, we hypothesized that pregnancy or parturition would modulate the classical immune response such that longer-lasting changes in gene expression may be seen specifically in pregnant rats.

In the hippocampus, the stress of LPS injection only affected the expression of IL-1 $\beta$  such that LPS-injected animals showed higher expression compared to saline-injected controls. Contrary to the forced swimming stressor, LPS induced the predicted pro-inflammatory immune response in the hippocampus of control (non-pregnant) females. Most interestingly, only the non-pregnant group treated with LPS had a significantly higher expression of IL-1 $\beta$  than the other three groups. This suggests that experiencing pregnancy and/or parturition modulated the way in which the central immune system responded to immune activation. This blunted IL-1 $\beta$  response in pregnant rats could either be a protective effect of pregnancy or a blunted immune response, indicating that the immune response is not properly responding to the challenge. Given the alternate immune changes produced by pregnancy that we are also seeing in the brain, we predict that this is, in fact, a blunted or inappropriate immune response to the LPS challenge, the full consequences of which we have yet to understand.

Within the mPFC, we found a significant interaction of pregnancy and/or parturition and immune activation on the expression of IL-6. Females in both pregnant groups as well as non-pregnant females treated with LPS showed significant increases in IL-6 expression compared to non-pregnant animals injected with saline as controls. However, the effects of both pregnancy and infection were not additive suggesting that, again, pregnancy or parturition may be modulating the response of the immune system to infection so as to prevent robust changes. Given the typical pro-inflammatory nature of LPS injections, the data suggest IL-6 may be acting in a more pro-inflammatory manner, but considering that the time of analysis is past the window of typical pro-inflammatory effect of LPS, more research is necessary to determine whether LPS does interact with pregnancy to affect IL-6 expression within the hippocampus. Notably, our time point of analysis does coincide with our time point of behavioral analysis, suggesting that IL-6 may influence the expression of postpartum anhedonia, independent of the late gestation immune challenge.

The expression of microglial activation marker CD11b also showed an interaction of pregnancy or parturition and LPS treatment in the mPFC. These data show that only the pregnant females injected with LPS showed a significantly lower level of expression compared to the non-pregnant animals with LPS infection. This supports our previous claim that pregnancy or parturition may be modulating the neuroimmune response to infections so as to blunt any robust changes from baseline levels.

In addition to changes observed in gene expression, we also wanted to know how pregnancy or parturition with added stressors may influence behaviors of the dam postpartum. In response to forced swimming stress, we found increased anhedonia in

our non-pregnant rats with our sucrose preference test. We also found that pregnancy or parturition alone induced anhedonia immediately postpartum. However, the two effects did not produce an additive effect of significant anhedonia in the pregnant females exposed to the chronic stressor. These effects of stress and pregnancy on anhedonia were not present one week later suggesting that the immediate effects of these factors on depressive-like behavior are reversible. Given that we did not see an additive effect of pregnancy and stress, it may be the case that pregnancy is acting in a protective manner to prevent significant effects of additional stressors immediately postpartum; however, this remains to be determined.

Interestingly, when we analyzed baseline anxiety-like behavior, we found a significant difference between the females that would go on to become pregnant later that week and the females that did not get pregnant and thus remained non-pregnant for the duration of the study. Our results showed that females that would later become pregnant exhibited decreased anxiety-like behavior as measured by more time spent in the open arms of the Elevated Plus Maze compared to their non-pregnant counterparts. This finding in Experiment 2.2 replicated itself in our second cohort of animals, in Experiment 2.3. Potentially, measures of anxiety-like behavior may be affected by the estrous cycle and thus a predictor for successful pregnancy during breeding just a few days later. It would be interesting to analyze the sex behaviors of these females when exposed to males to determine the validity of this claim in another line of study.

Nonetheless, postpartum or post-stress anxiety-like behaviors showed effects of the stressor and pregnancy similarly to what we had hypothesized. Immediately postpartum, the effects of the stressor induced significant anxiety compared to our non-stressed animals, findings that correspond to observed depressive behaviors in

response to a longer model of forced swimming stress of a previous study (Pan et al., 2013). One week later, however, we see an effect of pregnancy such that only the pregnant animals exhibited the anxiety behaviors. These results outline an interesting time course for the expression of different behaviors in response to stress and pregnancy. It appears that the stress did not have long-lasting effects on its own regarding anxiety behaviors, but the effects of pregnancy or parturition on anxiety were still present one week after giving birth.

In response to the acute immune challenge from the LPS injection, we saw changes in sucrose preference one week later. We hypothesized that LPS may induce a long-lasting decrease in sucrose preference, increased anhedonia, when combined with the stress of pregnancy that would present itself in postpartum females. First, we saw increased anhedonia in females that just given birth which supports the data from the previous experiments. However, one week later, we saw that the females exposed to LPS showed increased sucrose preference compared to females treated with saline one week earlier. This was in contrast to what we initially predicted. In this measure of behavior, the experience of pregnancy or parturition did not appear to impact the effects of LPS. Furthermore, observing effects of the LPS exposure at a later time point suggests that the stress of infection and experiencing sickness behaviors may induce changes in either reward behavior or calorie consumption later on.

Next, we tested anxiety-like behaviors in response to LPS or saline injections during late pregnancy and found interesting results. First, we saw that pregnant animals appeared to be less anxious than non-pregnant animals immediately postpartum. This does not align with our initial hypothesis, nor does it align with what we previously observed in this behavior during Experiment 2.1. However, it is

important to also compare the change in anxiety behaviors between the baseline time point and the next time point examined. Here, we see that the non-pregnant, non-stressed animals significantly decrease the amount of time spent in the open arms of the maze. This suggests that there was a potential confound of an additional stressor that was not controlled for in the experiment to cause our control animals to exhibit high levels of anxiety. Possibly, the intraperitoneal injection was stressful enough for the non-pregnant animals that caused anxiety behaviors to present 3-4 days later. However, in our Experiment 2.3, we also see a significant decrease in the amount of time spent in the open arms from baseline to the next time point of analysis, and these virgin females (in the No Stress group) did not receive any manipulations that would be considered stressful. Thus, it is impossible to interpret the anxiety behaviors analyzed on the Elevated Plus Maze in these experiments. It is possible that as the female rats habituate to the animal facility (following transport from Harlan Laboratories) there is a significant change in their anxiety behaviors that we are analyzing using our repeated EPM analysis. Given that we did not see this significant change in anxiety across time in our first behavioral experiment, Experiment 2.1, this explanation may also not be sufficient. Further experiments could examine some of these proposed factors in an attempt to uncover a better explanation for the change in EPM behavior upon repeated analysis.

We found that, in some cases, pregnancy induced decreased expression of microglial activation markers and typical pro-inflammatory molecules postpartum. In other cases, pregnancy caused an increase in the expression of the cytokine IL-6 that has dual pro- and anti-inflammatory properties, and we saw an increase in the expression of a gene critical for neurogenesis and plasticity. These results provide

novel findings of neuroimmune changes that occur during pregnancy and that continue into the postpartum period which may help to explain the significant behavioral changes we are examining here at the same time points, and in mothers that develop baby blues or postpartum depression. When female rats were exposed to the chronic forced swim stress, we did not see many changes in inflammatory gene expression suggesting that the depressive-like behaviors produced by stress and pregnancy may not be the result of similar changes in the brain.

In addition, when female rats were exposed to LPS, it appeared that pregnancy modulated the neuroimmune response to prevent additional robust changes in gene expression from the pregnancy-induced baseline. Furthermore, effects of pregnancy, chronic stress, and immune activation caused interesting changes in two measures of behavior seen in patients with depression. Anhedonia was only expressed immediately postpartum, and it was influenced by both pregnancy and the chronic stress of forced swimming. It appeared as though pregnancy prevented increased anhedonia when combined with stress in a protective manner. Anxiety-like behaviors were induced by forced swimming stress and pregnancy immediately postpartum as well. However, anxiety continued to present itself later into the postpartum period, particularly in Experiment 2.3, which suggests that different behaviors associated with depression have varying time courses in response to pregnancy or stress.

It is not clear whether the “pregnancy” effects seen across all experiments in both a neurological and behavioral sense were due to experiencing pregnancy, the process of parturition, or the stress of caring for a litter of pups. However, these findings outline the postpartum period as a significant and stressful period for the mother. It may be the case that the postpartum period is adding a significant amount

of stress to the mother that the effects of pregnancy can no longer be protective against. Combined with the stress of pregnancy and additional gestational stress of outside insults, the risks for developing postpartum depression may greatly increase when incorporating the stress of immediate postpartum motherhood. Outlining the postpartum period as the critical factor for developing postpartum depression may also provide an explanation for why it takes up to four weeks to be initially diagnosed with the disorder. Future experiments may examine mothers that have had more than one litter to determine whether these changes in anhedonia are also experienced by mothers that have “done it before” and know what to do with the new litter they are caring for.

Postpartum depression is a very serious mental health condition for which the causes and risk factors have not yet been well identified in humans using animal research. An additional gap in our current knowledge is the mechanism by which this specific type of major depression presents itself in the brain, body, and behaviors. A proper understanding for these aspects of the disease is critical for developing the safest and most effective treatments for new mothers. The results of this study have outlined several interesting changes in neuroimmune gene expression within areas of the brain implicated in depression. While we cannot venture to say these findings lend a complete mechanism of action for postpartum depression, they provide an important foundation for further research. Additionally, various studies have addressed the impact of maternal stress and maternal maltreatment on the development and cognitive abilities of offspring. An overwhelming amount of evidence has shown that unhappy, unhealthy, and stressed mothers do not adequately care for their young and in turn negatively impact the offspring such that cognitive deficits are seen during

adolescence and adulthood. We did not observe maternal behaviors in this study; however, future studies will include these measures during the postpartum period to determine if the results of anhedonia and anxiety-like behaviors present themselves in poor maternal care for the pups as well. It would be interesting to determine how the pups continue to develop as a result, and whether the pups also exhibit significant changes in neuroimmune function as a result of this maternal stress. Thus, it is not only important that postpartum depression be understood for the sake of the mother's health, but also for the sake of the proper development of the babies of suffering mothers. Our study only begins to fill in the gaps in our knowledge of postpartum depression and provides a critical foundation upon which future studies can build.

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**Appendix**

**APPROVAL FOR THE USE OF ANIMAL SUBJECTS**



Office of Laboratory Animal Medicine

Life Science Research Facility  
79 E. Delaware Avenue  
Newark, DE 19711  
Phone: 302-831-2616  
Fax: 302-831-0154

To: Office of Graduate and Professional Education

From: Gwen Talham, DVM, Director, Animal Care Program

A handwritten signature in black ink that reads 'Gwen Talham'.

Subject: IACUC approval for Caitlin Posillico

Date: 4/27/2015

Caitlin Posillico was approved by the IACUC to work with animals on Jaclyn Schwarz's protocol #1263 "Inflammation and Postpartum Depression". Please contact me at 831-2980 or [gtalham@udel.edu](mailto:gtalham@udel.edu) with any additional questions.