AN INVESTIGATION INTO THE IMPACT OF NEUROIMMUNE FUNCTION AFTER PREGNANCY OR STRESS: ARE THERE POTENTIAL LINKS TO THE ONSET OF DEPRESSION?

by

Julie Gomez

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 Neuroscience

Spring 2018

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Approved: _____
Jaclyn M. Schwarz, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Robert F. Simons, Ph.D.
Chair of the Department of Psychological and Brain Sciences

Approved: _____
George Watson, Ph.D.
Dean of the College of Arts and Sciences

Approved: _____
Ann L. Ardis, Ph.D.
Senior Vice Provost for Graduate and Professional Education

ACKNOWLEDGMENTS

I would first like to express my gratitude to my advisor and mentor, Dr. Jaclyn Schwarz for providing me with guidance, support, and opportunities to gain research experience and invaluable tools that will stay with me throughout my career. I am truly thankful for your guidance and encouragement throughout my undergraduate and graduate career. I appreciate your interest and involvement with both my work in lab and my life outside of lab.

I would also like to thank my lab mates for creating a wonderful atmosphere the past three years. Specifically, Morgan Sherer and Laurne Terasaki for being wonderful teachers and showing me proper lab skills and techniques. Brittany Osborne and Lexi Turano, thank you for always supporting me and making long days in the basement enjoyable. I would also like to thank Caitlin Posillico for providing the previous research that I have based my work on. Without your previous findings, I would not have been able to conduct these experiments. I would especially like to thank our lab manager, Nicole Haas, for helping me with these experiments. Through trial and error with trying new techniques, you were always there to lend a helping hand. I am thankful for your support throughout the past year and our time together in lab.

In addition, I would like to thank my undergraduate advisor, Dr. Robert Simons. Thank you for your continued support throughout my time at the University of Delaware. I appreciate our meetings and your guidance as my advisor. If it weren't for your advice and encouragement, I would not be the student or person I am today.

Finally, I would like to thank my family and friends for their love and support throughout my journey at the University of Delaware. To my family, I appreciate your interest in my research, despite not being able to completely understand it. To my friends, I appreciate your support and patience with my long days and nights spent at the library. Your support has helped me earn this degree, I could not have done it without you.

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ABSTRACT

The National Institute of Mental Health has identified postpartum depression as one of several types of depression that affects 10-15% of mothers. It has previously been hypothesized that postpartum depression may be caused by dramatic changes in hormone levels that occur throughout pregnancy and the immediate postpartum period (Harris et al., 1994); however, the exact underlying mechanisms are still unknown. Like the dramatic change in hormone levels that occurs during and after pregnancy, the peripheral immune system is also altered during pregnancy to protect the developing semi-allogenic fetus from being rejected by the maternal immune system (Fallon et al., 2002). Despite this well-known phenomenon, the literature on how the *central* immune system is altered during pregnancy is still very limited.

It is known that women are at a greater risk of developing postpartum depression if they have experienced depression during previous pregnancies (Patel et al., 2012). Thus, our first experiment sought to identify potential differences in postpartum anhedonia between first and second pregnancies. We have seen that female rats show significant anhedonia, measured by a decrease in sucrose preference, immediately after their first pregnancy (Posillico and Schwarz, 2016). In contrast, second-time mothers showed no significant anhedonia.

The aim of the second experiment in this thesis was to identify a potential molecular basis for the onset of depressive-like behaviors caused by a first-time pregnancy and compare this to the effects of sub-chronic stress, also known to induce depressive-like behaviors. Increased circulating cytokines have been associated with numerous types of depression as well as chronic stress (Dantzer, 2009). Recently, we

have found a significant increase in IL-6 expression in the female brain on the day of birth, or following sub-chronic stress (Posillico and Schwarz, 2016). Thus, we sought to determine whether blocking the function of this pro-inflammatory cytokine may prevent the anhedonia observed following a first-time birth or sub-chronic stress. Treatment with an IL-6 receptor antibody effectively attenuated depressive-like behavior immediately postpartum, but had no effect following sub-chronic stress. These results suggest that the molecular mechanisms that underlie the onset of anhedonia following birth and sub chronic stress may be distinct.

Chapter 1

INTRODUCTION

Postpartum depression is one of several types of major depressive disorders outlined by the National Institute of Mental Health (National Institute of Mental Health, 2011). Postpartum depression affects 10-15% of mothers and is characterized by labile mood, increased anxiety, increased irritability, and feelings of depression (Winser et al., 2013). Just prior to birth, a pregnant female has extremely high levels of circulating estrogen and progesterone necessary to sustain pregnancy, but these levels drop down to pre-pregnancy levels rapidly, within the first week after giving birth (Harris et al., 1994). These dramatic changes in hormone levels have previously been thought to be a potential underlying cause of "baby blues", sadness and anxiety that often occurs immediately after birth in many women (Klier et al., 2007). In support of this hypothesis, experiments that have mimicked these dramatic fluctuations in hormones during the last trimester and the postpartum period can induce a depressive-like phenotype in rats (Suda et al., 2008). It is also thought that these dramatic changes in hormone levels during the first week postpartum may precipitate the more persistent and dramatic changes in mood and anxiety, like those associated persistent postpartum depression (Brummelte and Galea, 2016). That said, very little is known about the physiological mechanisms and exact causes of postpartum mood disorders.

It is well known that the function of the peripheral immune system is significantly altered during pregnancy to protect the developing semi-allogenic fetus

from being rejected by the maternal immune system (Trowsdale and Betz, 2006). In the periphery, pro-inflammatory cytokine expression is attenuated, and in contrast, there is generally an increase in alternate cytokines that are less pro-inflammatory, such as IL-4 (Fallon et al., 2002). Because of these dramatic changes in immune function, pregnant women are more likely experience complications or death following serious infections such as influenza or pneumonia, particularly in the later trimesters (Robinson and Klein, 2012). Despite this, the literature is still very limited on how the *central* immune system might be altered during pregnancy. We have recently shown that in addition to robust changes in peripheral immune function, there is a significant change in cytokine production in the brain during pregnancy and the postpartum period (Sherer et al., 2017; Posillico and Schwarz, 2016). Among these changes, cytokine expression is attenuated following an immune challenge across all brain regions examined throughout the duration of pregnancy (Sherer et al., 2017). Many of these changes reverse within the first day or two postpartum. Important for the following experiments, we have consistently found a significant increase in IL-6 expression in the maternal brain immediately postpartum (Posillico and Schwarz, 2016). In particular, IL-6 expression is significantly upregulated in the medial prefrontal cortex (mPFC) and the hippocampus (HP) on the day of birth (**Figure 1AB**). These findings may be important because dysfunction in both the mPFC and HP have been associated with the etiology of depression (Ketter, 1994; Holm et al., 2011; Posillico and Schwarz, 2016). Importantly, we have seen this increase in IL-6 expression in the brain across various experiments. The postpartum increase in IL-6 in the brain can occur independent of additional sub chronic forced swim stress during pregnancy (**Figure 1A**), and it can occur independent of a low-dose immune challenge

with lipopolysaccharide (LPS) during pregnancy (**Figure 1B**). Notably, the timing of this increase in IL-6 expression in the brain immediately after birth correlates with the onset of postpartum depressive-like anhedonia in new moms (**Figure 1C**). Thus, the following experiments sought to examine the role of this increase in IL-6 expression in the onset of postpartum depression using our rat model.

Stress is also a common cause or risk factor for the onset of depression (Sapolsky, 1996). Across numerous species, studies report an increase in depressive behavior or depressive-like symptoms following exposure to chronic stress (Wang et al., 2018; Syed and Nameroff, 2017). Importantly, stress also alters immune function; and thus, perhaps not surprisingly, changes in immune function and circulating cytokines have also been associated with types of depression (Dantzer, 2009). Subsets of patients with major depressive disorder (MDD) have higher levels of several inflammatory markers, including the cytokine interleukin-6 (IL-6; Hodes et al., 201; Maes, 1995; Dowlati et al., 2010). A recent study showed that elevated levels of cytokines in serum correlate with depressive symptoms in humans (Kageyama et al., 2017). Also, it has been documented in that peripheral administration of IL-6 can induce depressive-like behavior in adult male mice (Kurosawa et al., 2015). Inescapable tail shock leads to a significant increase in hippocampal IL-6 in aged rats following surgery (Wang et al., 2016). And moreover, social stress induced a rapid increase in peripheral levels of IL-6 that were significantly correlated with future susceptibility to depressive-like behaviors and social avoidance behaviors in adult male mice (Hodes et al., 2014). In this particular study, bone marrow chimeras were generated to transplant peripheral white blood cells from the stress "resilient" mice to the stress "susceptible" mice to in order significantly decrease levels of peripheral

circulating IL-6; and this alone was sufficient to increase resilience to subsequent stress and depression in the previously vulnerable male mice (Hodes et al., 2014). In contrast to this overwhelming evidence in support of IL-6 as a biomarker or risk factor for depression, our own previous studies found something different. We found that sub chronic stress, just one week of forced swim test for 5 minutes/day was ***not*** sufficient to increase IL-6 expression in the mPFC (**Figure 1A**) or the HP in rats, though it ***was*** sufficient to induce depressive-like anhedonia as measured by a decrease in sucrose preference (Posillico and Schwarz, 2016; **Figure 1C**). Taken together, these studies indicate that IL-6 may play a crucial role in the onset of depression due to severe or chronic stress or, as we hypothesize below, in the onset of postpartum depressive symptoms.

It is also well known that women are at greater risk of developing postpartum depression if they have experienced depression previously, or if they have experienced depression during a previous pregnancy (Patel et al., 2012). Thus, we sought to determine whether a second pregnancy would have the same effect as a first-time pregnancy for inducing postpartum anhedonia in our rat model. Consistent with the epidemiological data in humans, we hypothesized that second-time dams would similarly exhibit postpartum anhedonia following the birth of a second liter.

The implications and significance of this work are of great importance. Depression experienced during the postpartum period can lead to a decrease in attachment or even maltreatment of offspring in both rodent models and humans (Burke 2003; Brummelte and Galea, 2016). In turn, poor maternal care and attachment relationships with the offspring can lead to improper psychosocial development of the offspring themselves. For example, poor maternal care can result in epigenetic

changes, which may lead to cognitive deficits and an increased risk of neuropsychiatric disorders in the offspring later in life (Roth et at., 2009; Roth and Sweatt, 2011). Thus, understanding the mechanisms that underlie postpartum depression and identifying potential treatments is crucial for the benefit of both mothers and offspring.

The findings from these experiments seek to provide a novel line of research with the goal of better understanding the etiology of postpartum depression and depression in general, in order to identify potential therapeutic targets for depression, particularly in postpartum women. The current treatments for postpartum depression do not significantly differ from that of major depressive disorders (Deligiannidis et al., 2014). Specifically, the same antidepressants are prescribed during pregnancy and the postpartum period to prevent major depressive episodes in affected women; however, it is unclear how the central immune changes interact with or predict the effectiveness of these drugs in pregnant and postpartum women. Interestingly, current animal models that have used typical antidepressants have not seen effective treatment of depressive-like behaviors in pregnant or postpartum animals (Bourke et al., 2013; Bourke et al., 2014). These data suggest that postpartum depression may not be adequately treated with typical antidepressants.

Our first experiment sought to identify potential differences in the expression of depressive-like anhedonia following a first and a second pregnancy. As previously stated, it is known that depression during pregnancy can put women at greater risk for developing postpartum depression after a subsequent pregnancy. Our previous work has consistently observed postpartum anhedonia in first-time dams, thus we hypothesized that female rats would similarly exhibit depressive-like behaviors

following a second pregnancy. Our second experiment sought to identify a potential therapeutic target for postpartum depression or the onset of depression following chronic stress, by investigating the role of IL-6 in the brain. We hypothesized that blocking IL-6 receptor activation using an antibody infused directly into the brain would attenuate these depressive-like symptoms seen previously following pregnancy, and perhaps sub chronic stress.

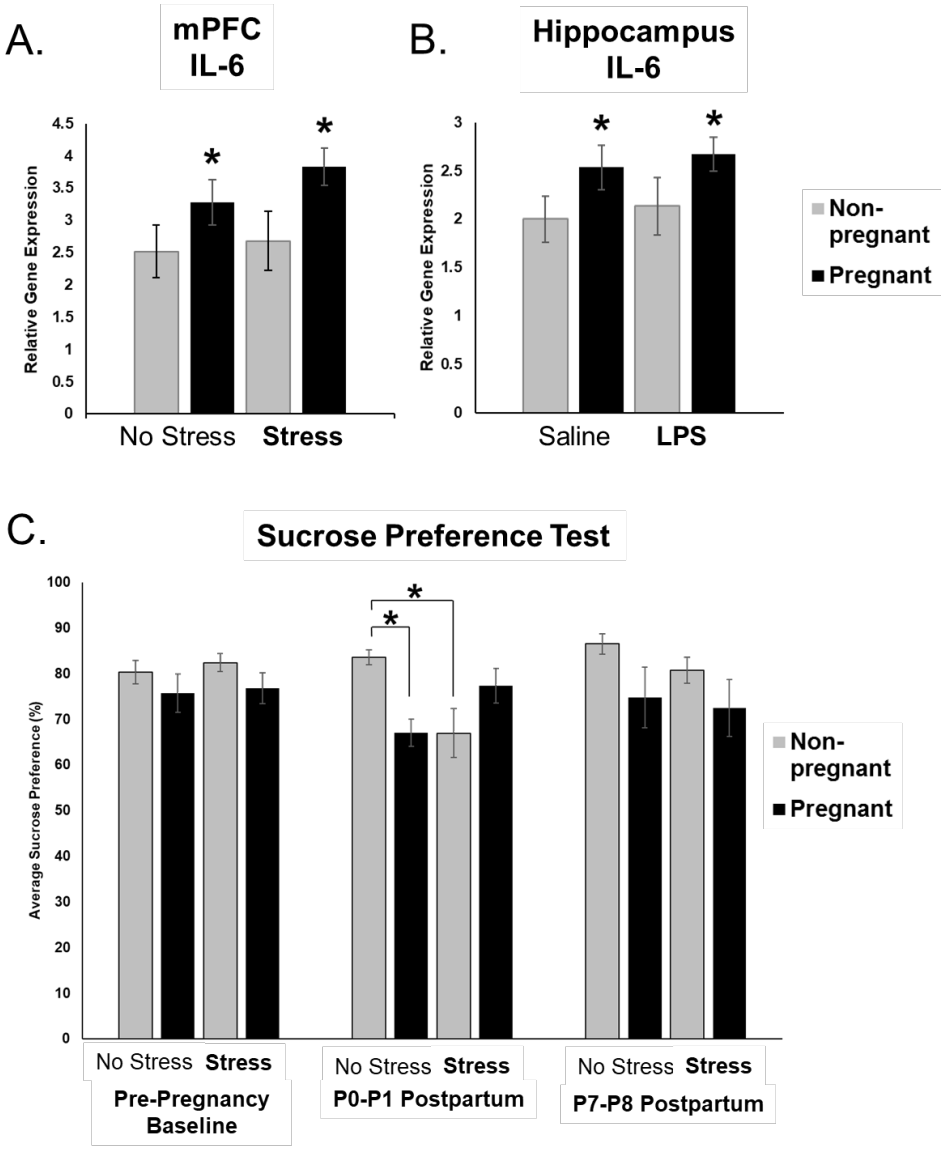

Figure 1 A. Increased expression of IL-6 levels in the medial prefrontal cortex on the day of birth. B. Increased expression of IL-6 levels in the hippocampus on the day of birth. Pregnancy alone increased levels of IL-6 regardless of treatment, stress vs no stress. C. Decreased sucrose preference on the day of birth or following chronic stress. * = $p < 0.05$. *Adapted from Posillico and Schwarz 2016.*

Chapter 2

MATERIALS AND METHODS

2.1 Animals

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

In Experiment 1, we examined the effects of a second pregnancy on postpartum anhedonia. 10 Female rats were bred and allowed to give birth naturally, remaining undisturbed throughout gestation and the first three weeks postpartum. Pups were weaned three weeks after birth. Seven days after the pups were weaned, the mothers were bred a second time. A separate cohort of 10 females that served as non-pregnant, time-matched controls. The control rats remained undisturbed throughout their time-matched period.

In Experiment 2.1, we examined the effects of an intra cisterna magna infusion of an IL-6 receptor antibody on cytokine expression in the brain following an intraperitoneal injection with LPS. The goal of this "preliminary" experiment was to determine whether the antibody could effectively inhibit cytokine signaling associated with this LPS immune challenge, in order to confirm whether it would be an effective "drug" for manipulating IL-6 function in subsequent experiments. A total of 24 rats were assigned to one of the following three treatment groups: **(1)** Control IgG

Antibody infusion (.5µg/10µL) followed by an intraperitoneal injection with Saline (1 ml/kg; n = 4), **(2)** Control IgG Antibody infusion followed by an intraperitoneal injection with LPS (100µg/kg; n = 10), and **(3)** IL-6 receptor Antibody infusion (.5µg/10µL, from RnD systems Cat. No RDAF506) followed by an intraperitoneal injection with LPS (100µg/kg; n = 10). All rats received the intra cisterna magna infusion and intraperitoneal injection while under brief isoflurane anesthesia (see additional procedural details below). Rats were housed separately one day prior to these treatments and remained individually-housed between the infusion/injection and tissue collection that occurred 2 hours later.

In Experiment 2.2, we examined the effects of an IL-6 receptor antibody on postpartum and sub chronic stress-induced anhedonia. 20 female rats were assigned to the postpartum condition, and 20 other females (who were not bred) were assigned to the stress condition. Each group was subdivided into two treatment groups that received either an IL-6 receptor antibody (.5µg/10µL), or a non-specific IgG control antibody (.5µg/10µL). Rats in the postpartum condition were bred, pair housed, and then housed individually three days prior to giving birth, on embryonic day 20, to allow them to give birth individually and undisturbed. The postpartum rats received the antibody infusions (.5µg/10µL) on the day of birth (P0) and again on postnatal day 1 (P1), and then were tested two hours later using the sucrose preference test on both days. Rats in the stress condition were placed in the Forced Swim Test (FST) for 5 minutes each day for 7 days on the "time equivalent" of the last week of gestation (e.g. embryonic day 17-23). These rats received the antibody infusion (.5µg/10µL) the next day after stress (P0 equivalent), and again the next day (P1 equivalent) before being tested two hours later using the sucrose preference test. See Section 2.7 for a detailed

protocol of the sucrose preference testing. Rats in the stress condition were housed individually 24 hours prior to the beginning of stress and throughout the stress paradigm.

In Experiment 2.3, we examined the effects on an IL-6 receptor antibody on postpartum and sub chronic stress-induced cytokine expression in the brain. A separate cohort of 40 females were separated into the same four experimental conditions used in Experiment 2.2, including **(1)** Postpartum + IgG Control Antibody, **(2)** Postpartum + IL-6 receptor Antibody, **(3)** Stress + IgG Control antibody, and **(4)** Stress + IL-6 receptor Antibody. Rats in the stress condition were housed individually 24 hours prior to the beginning of stress and throughout the duration of the stress. Postpartum rats were individually housed three days prior to expected date of birth. These rats received an intra cisterna magna injection on P0 (or the day after stress) and were euthanized two hours later. From each rat, we collected a blood sample, perfused the rats with ice-cold saline to remove peripheral immune cells and cytokines from the brain, and then collected the brain tissue for subsequent analysis of brain cytokine expression.

2.2 Brief Isoflurane Anesthesia

In Experiments 2.1, 2.2, and 2.3 rats were briefly anesthetized using isoflurane for the intra cisterna magna infusions. Before beginning any anesthesia procedures, all oxygen tanks and isoflurane levels were checked to ensure a sufficient amount for the day's experiments. Rats were placed in a chamber with oxygen set constantly to 1L/min and isoflurane set to 3L/min. Once anesthetized, rats were removed from the chamber and placed on a nosecone, and the isoflurane was adjusted down to 2.5L/min. Prior to any injections, complete anesthesia was determined by a toe-pinch that elicits

no response or withdrawal. Rats remained under anesthesia for no longer than 10 minutes for the completion of the entire intra cisterna magna infusions.

2.3 Intra Cisterna Magna Infusions

Rats under isoflurane anesthesia were rested with a head angle of 90º while remaining on the nosecone. A 30-gauge needle was inserted 1cm into the cerebrospinal fluid of the intra cisterna magna between the base of the skull and C1 vertebrae (see **Figure 5**). This vertebral region was accessed by leaning the rat's head off the edge of a Styrofoam box at a 90º angle from the body. 10µL of either the IL-6 receptor antibody or an IgG control antibody was infused over the course of one minute. Antibodies were obtained from RnD systems: (IL-6 Cat. No RDAF506; IgG Cat. No. RD6-001-F). Both antibodies were diluted to a concentration of .5µg/10µL in deionized sterile phosphate buffered saline (DPBS).

2.4 Lipopolysaccharide

Lipopolysaccharide (LPS) derived from Escherichia coli 0111:B4 was obtained from Sigma-Aldrich (Cat. No. L2630). LPS was diluted in DPBS to a concentration of 100µg/mL and administered via an intraperitoneal injection at a dose of 100 µg/kg/mL.

2.5 Intraperitoneal Injections

Rats in Experiment 2.1 were injected using either sterile saline (DPBS) or lipopolysaccharide (LPS) while under isoflurane anesthetic. Female rats were injected with LPS at a dose of 100 µg/kg of body weight or saline in a volume of 1mL/kg as a control. All rats were housed separately post-injection for the two hours prior to euthanasia and tissue collection.

2.6 Forced Swim Test

Female rats in Experiments 2.2 and 2.3 that were assigned to the Stress condition underwent the Forced Swim Test (FST) every day for 7 days. These rats were forced to swim in 20ºC tap water with no option for rest or escape for 5 minutes each day. The FST was performed each day between 12:00 and 1:00 PM. Rats were monitored throughout the duration of the 5-minute test to prevent accidental drowning and the total time spent floating was measured to consider the influence of possible individual differences in the expression of floating behavior as a covariate in subsequent analyses. Stressed rats were housed separately prior to the first day of testing and throughout the duration of the testing.

2.7 Sucrose Preference Test

Experiments 1, and 2.2 used a sucrose preference test on the day of birth (P0) and P1, or time-matched equivalent in stressed rats, to analyze depressive-like behaviors. Females were individually housed in clean testing cages from 3pm to 7pm. Rats were provided two water bottles: one containing tap water, and the other containing a 1% sucrose solution. Both water bottles were weighed prior to testing and immediately after the completion of the test to measure the amount of liquid consumed in grams. The left versus right orientation of the bottles was randomized on the first day of testing and switched on the second day of testing to prevent any possible location preference for the bottles. The test was performed over the course of two days to obtain an average score for sucrose preference for each rat. The average amount of sucrose consumed over the two days converted to a sucrose preference score using the following formula:

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

2.8 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 using toothy forceps. Once anesthetized, rats were perfused with a 0.9% saline solution to remove blood, including peripheral immune cells and cytokines, from the brain tissue. After perfusion, the medial prefrontal cortex (mPFC) and hippocampus (HP) were collected from brain tissue and placed immediately on dry ice. Tissue was stored in a -80º freezer until further processing.

2.9 Real-Time PCR

Messenger RNA (mRNA) was extracted from frozen brain tissue using Ribosol, an RNA Lysis Reagent. 1000 ng of extracted RNA were subjected to DNase treatment to remove any genomic DNA prior to synthesizing cDNA from the mRNA using the QuantiTect Reverse Transcription Kit and its protocol. Relative gene expression was measured using the RealMasterMix Fast SYBR Kit in 10µL reactions on a CFX96Touch Real Time PCR machine.

2.10 ELISA

Serum protein levels were analyzed using RnD Systems IL-6 ELISA kit (Cat. No. R6000B). All samples were stored at -80º C between extraction and analysis. Samples were diluted 1:2 at the time of analysis with the buffer provided. Relative protein levels were analyzed following the ELISA protocol using Gen5 software and a Biotek ELx808 Absorbance Reader for the detection of chemiluminescence.

2.11 Statistical Analyses

A two-way repeated measures ANOVA was used to analyze the differences in sucrose preference in Experiments 1 and 2.2, with baseline sucrose preference and test sucrose preference as a within-subjects factor and the antibody infusion as a between subjects factor. A one-way ANOVA was used to analyze the expression of immune molecules from the three groups (Saline + IgG Antibody, LPS + IgG Antibody, and LPS + IL-6 Antibody) examined in Experiment 2.1. One-tailed t-tests were used to assess statistical significance of cytokine expression between IgG and IL-6 antibodies in each condition, either pregnancy or stress in Experiment 2.3. The conditions of pregnancy and stress were not analyzed compared to each other. A one-tailed t-test was also used to assess the statistical significance of swim time during the Forced Swim Test in Experiments 2.2 and 2.3. Significant main effects and significant interactions of variables are reported using α-level = 0.05. Significant interactions were followed up with Fisher's LSD post hoc comparisons using α-level = 0.05. Data in the graphs represent the mean of each group ± SEM.

Chapter 3

EXPERIMENT ONE: EFFECT OF A SECOND PREGNANCY ON POSTPARTUM ANHEDONIA

It is well-known that women who experience depression before, during or after a previous pregnancy are more likely experience postpartum depression on subsequent pregnancies (Patel et al., 2012). That said, little is known about how a second pregnancy affects postpartum anhedonia and depressive-like behavior in a rodent model. Experiment 1 sought to determine whether a second pregnancy produces postpartum anhedonia similar to a first-time pregnancy (Posillico and Schwarz, 2016). For this experiment, two groups of rats were assigned to either a second pregnancy group (n=10) or a virgin group (n=10). Rats in the second pregnancy group were bred once, remained undisturbed, and bred one week following the weaning of their pups on P21. Baseline measurements for sucrose preference were taken prior to the start of the experiment. No significant differences existed between the groups in the expression of sucrose preference before the experiment. During testing, pups were placed in the testing cage with the mother. See **Figure 3** for a detailed timeline of this experiment. We predicted that a second pregnancy would result in a similar decrease in sucrose preference in the second-time mothers.

Interestingly, we found no significant decrease in sucrose preference following a second pregnancy. A preference for sucrose is indicated when total sucrose consumed is significantly greater than chance. Chance in this paradigm is defined as 50% of total liquid consumed. See **Figure 3** for results. Rats in both groups consumed an average of 32g of total liquid, 85% percent of which was sucrose ($p > 0.05$, indicating sucrose preference and no significant differences between groups).

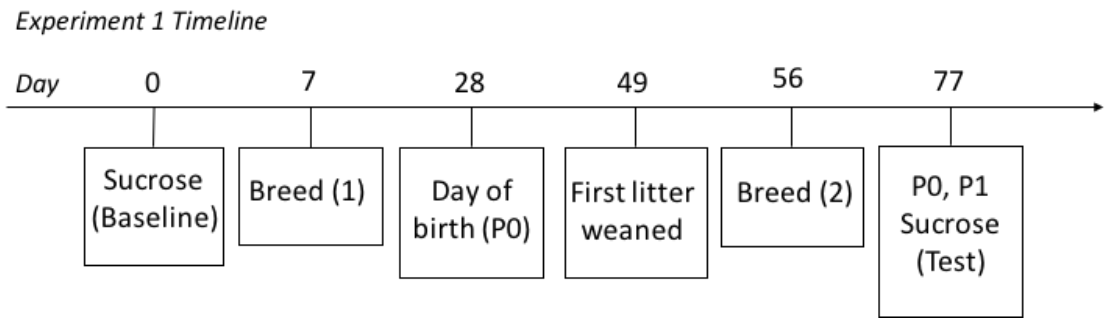

Figure 2 Timeline for Experiment 1. In this experiment, pregnancy was determined by the presence of a sperm plug. Sucrose preference was measured over the course of four hours from 3pm to 7pm during both baseline and testing days. Virgin rats remained undisturbed during the entire timeline between baseline and experimental testing. All rats were euthanized following the last day of sucrose preference testing on Postnatal day 2 (P2).

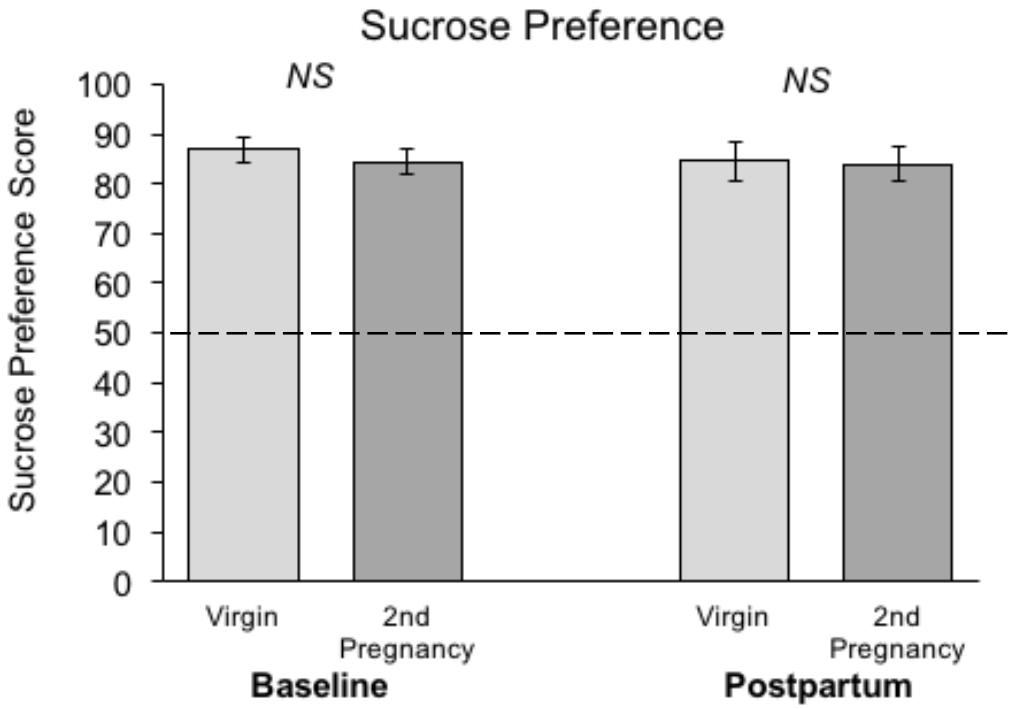

Figure 3 Sucrose preference scores for female rats after a second pregnancy. Baseline testing was performed before any breeding. The non-pregnant rats that were time-matched to the pregnant rats also did not show any decrease in sucrose preference due to age. There was no significant change in sucrose preference following a second pregnancy ($p > .05$).

Chapter 4

EXPERIMENT TWO: EFFECTS OF BLOCKING IL-6 IN THE BRAIN USING AN ANTIBODY

Experiment 2 sought to examine the effects of an IL-6 receptor antibody in the brain following an immune challenge (Experiment 2.1), stress or pregnancy (Experiment 2.2 and 2.3). See **Figure 4** for a detailed timeline of Experiments 2.1, 2.2, and 2.3. We hypothesized that the IL-6 receptor antibody would effectively block the IL-6 receptor within two hours following the injection, based on previous reports using the intra cisterna magna infusions (Bilbo et al, 2005; Proescholdt et al., 2000), and thus the time point would also be effective for testing the behavioral effects of blocking the upregulation of IL-6 in the brain postpartum. To that end, we also predicted that the IL-6 antibody would reverse the effects of pregnancy, and possibly sub chronic stress, on depressive-like behaviors. See **Figure 5** for a diagram of the intra cisterna magna injection procedure.

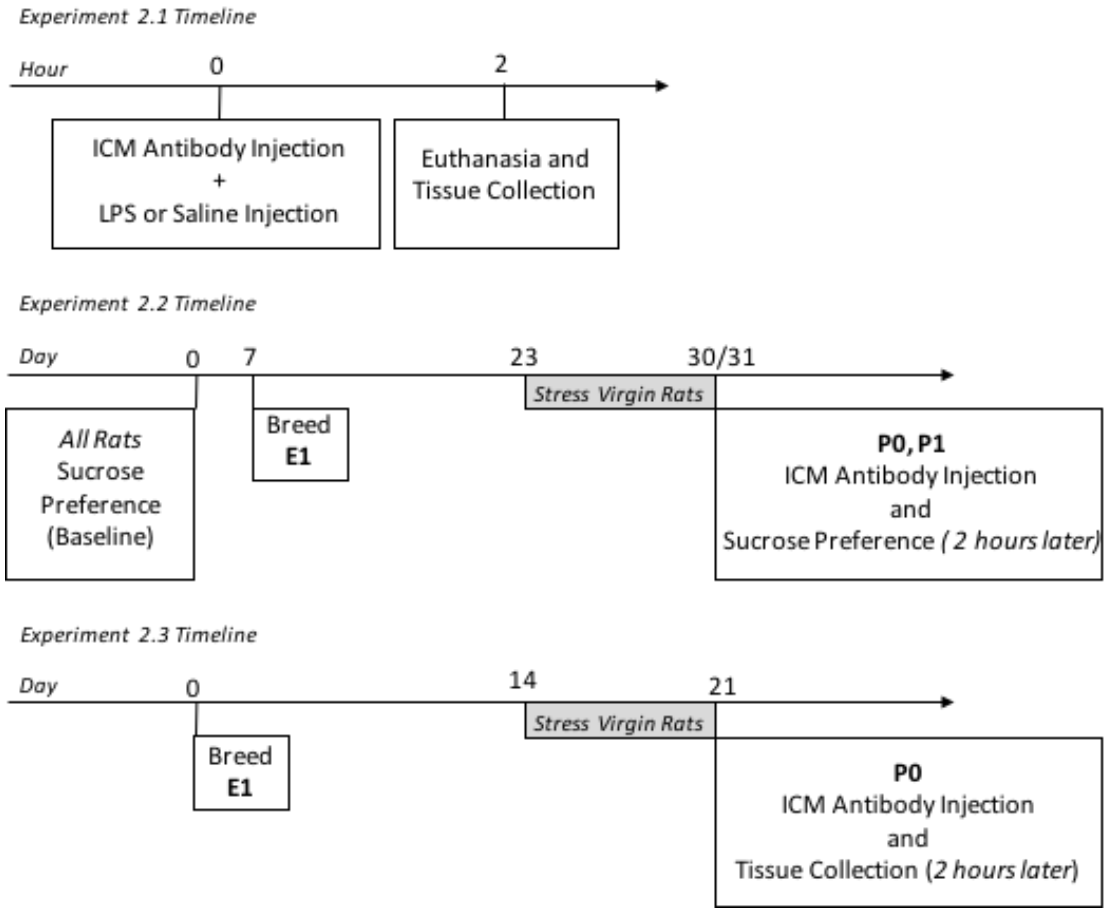

Figure 4 Timeline for Experiments 2.1, 2.2 and 2.3. All rats were single housed 24 hours prior to testing. Baseline sucrose preference was tested over the course of two days prior to the start of the experiments. For Experiment 2.2, rats in the pregnant groups were bred on Day 7 (embryonic day, E1), virgin rats remained undisturbed until forced swim test on Day 23 in-house. The forced swim test lasted the course of seven days in the time equivalent to late gestation in the pregnancy group. For Experiment 2.3, rats in the pregnant groups were bred on Day 0 (embryonic day, E1), virgin rats remained undisturbed until forced swim test on Day 14 in-house. The forced swim test lasted the course of seven days in the time equivalent to late gestation in the pregnancy group.

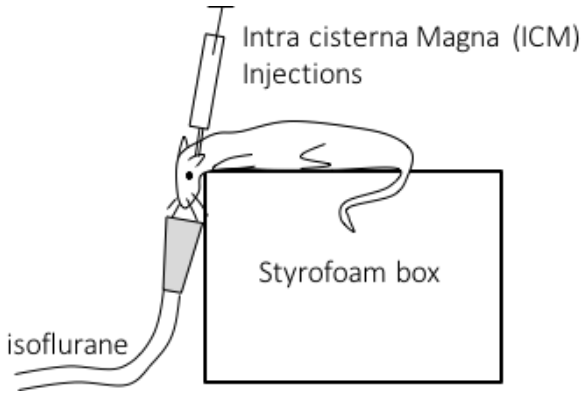

Figure 5 A diagram of the intra cisterna magna injection performed in Experiments 2.1, 2.2, and 2.3. Under brief isoflurane anesthesia, rats were placed on a Styrofoam box with a nosecone of isoflurane. The head was angled at 90º to the body, in order to access the gap between the skull and vertebrae in the neck region. The injection used a 30-gauge needle inserted approximately 1cm under the skin, into the cerebrospinal fluid. 10µL of antibody were infused over the course of one minute.

4.1 Experiment 2.1: Effects of an IL-6 receptor antibody on cytokine expression and signaling following an immune challenge

Experiment 2.1 sought to characterize the response of cytokines in the medial prefrontal cortex and hippocampus following the administration of an IL-6 receptor antibody or non-specific IgG control antibody. Rats received one of the following three treatments: **(1)** Saline injection and IgG antibody infusion, **(2)** LPS injection and IgG antibody infusion, or **(3)** LPS injection and IL-6 antibody infusion. Antibodies were administered (.5μg/10μL) via an intra cisterna magna injection under anesthesia. We examined cytokine expression two hours following the injections. We hypothesized that, if effective, the IL-6 receptor antibody would prevent the increased expression of cytokines, second messengers and signaling molecules induced by the immune challenge. We also predicted that the non-specific IgG antibody would have no effect on the cytokine expression and signaling response produced by the immune challenge (LPS). To that end, we analyzed the expression of both cytokines and secondary messengers including IL-1β, IL-6, IL-6st, Stat3, and NF-κB in the hippocampus and medial prefrontal cortex in order to determine whether the antibody could be effective at spreading from the initial infusion site (ICM) to our brain regions of interest.

In the medial prefrontal cortex (mPFC), we observed a significant main effect of treatment ($F_{2,12} = 4.2$, $p = 0.041$) such that LPS increased IL-1β cytokine expression compared to saline treated controls, and this effect was blocked by the IL-6 receptor antibody. Similarly, we also found a main effect of treatment on IL-6 expression in the mPFC ($F_{2,18} = 3.65$, $p = 0.047$), such that rats that received LPS had significantly elevated levels of IL-6 in the mPFC compared to saline treated controls, an effect that was blocked by the IL-6 receptor antibody. Contrary to expectation, we found no

significant effect of treatment on the expression of downstream signaling molecules IL-6 signal transduction molecule ($F_{2,16} = 1.08$, $p = 0.363$), NF-κB ($F_{2,13} = 1.85$, $p = 0.19$), or Stat 3 ($F_{2,14} = 1.71$, $p = 0.217$). See **Figure 6** below for results. Thus, we were able to conclude that the IL-6 receptor antibody can inhibit the synthesis of important cytokines, including IL-6 itself, in the medial prefrontal cortex. This is important to note for our subsequent experiments investigating behavioral differences between the groups.

In the hippocampus (HP), we observed a significant increase in the expression of IL-6 following an LPS injection compared to rats treated with saline, an effect that was blocked by infusion with the IL-6 receptor antibody (main effect of treatment: $F_{2,13} = 6.38$, $p = 0.012$). Similarly, we also found a notable increase in the expression of two downstream signaling molecules, IL-6 signal transduction molecule ($F_{2,13=9} = 3.28$, $p = 0.060$) and NF-κB ($F_{2,20} = 2.72$, $p = 0.09$). Both of these trends were blocked by infusion of the IL-6 receptor antibody. In contrast, we saw no main effect of treatment on the expression of IL-1β ($F_{2,16} = 2.38$, $p = 0.127$) or the downstream signaling molecule Stat 3 ($F_{2,14} = 1.71$, $p = 0.217$). See **Figure 7** below for results.

Based on these results, we felt confident in using the antibody to prevent the effects or the production of IL-6 in the brain immediately postpartum, in order to determine the effect on postpartum depressive-like behaviors.

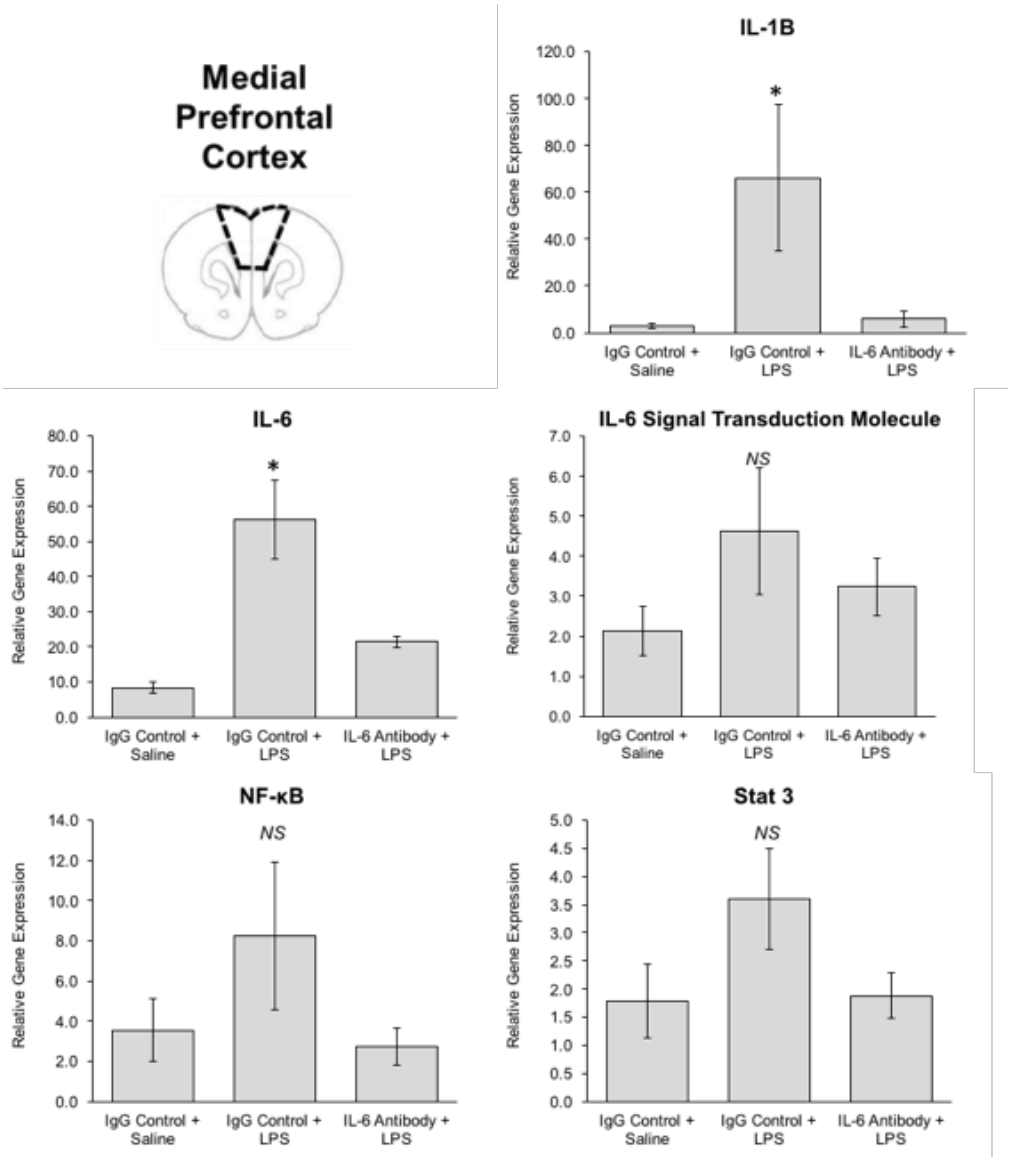

Figure 6 Analysis of relative gene expression in the medial Prefrontal Cortex. Medial Prefrontal Cortex tissue was collected 2 hours post IL-6 receptor antibody infusion or control antibody infusion, and injection with LPS or saline. Analysis of IL-1β and IL-6 expression reveals increased gene expression following an LPS injection. Analysis of IL-6 signal transduction molecule, Stat 3, and NF-κB revealed no significance. *: $p<0.05$

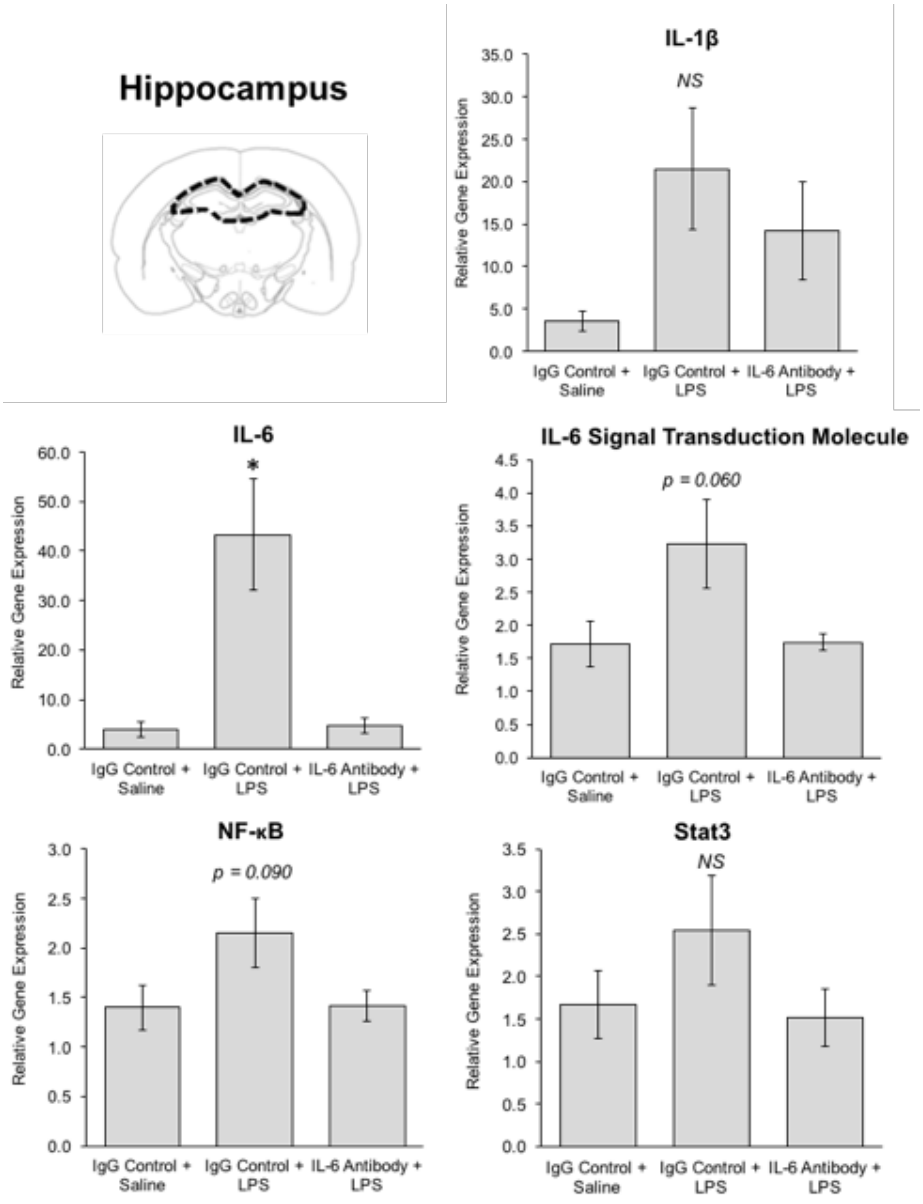

Figure 7 Analysis of relative gene expression in the Hippocampus. The tissue dissected from the Hippocampus two hours post IL-6 receptor antibody or control antibody infusion, and injection with LPS or saline, is shown. Analysis of IL-6, IL-6 signal transduction molecule, and NF-κB expression reveals increased relative gene expression following an LPS injection. Analysis of IL-1β and Stat 3 revealed no significance. *: $p<0.05$

4.2 Experiment 2.2: Effects of an IL-6 receptor antibody on depressive-like behaviors following birth or sub chronic stress

Experiment 2.2 sought to determine the effects of an IL-6 receptor antibody on depressive-like behaviors following birth, and for relative comparison, depressive-like behaviors caused by sub chronic stress. On the day of birth, P0, and again on P1 postpartum rats received an intra cisterna magna injection of an IL-6 receptor antibody or non-specific IgG control antibody. In the stress condition, virgin rats were chronically stressed via the force swim test for seven days. On the first and second days following the sub chronic stress, rats received an intra cisterna magna injection of one of the two antibodies. Two hours following the antibody infusions, the rats were placed into clean testing cages to measure their sucrose preference score.

Postpartum Results:

As expected, there were no significant differences between groups during baseline testing of sucrose preference. Consistent with previous findings from our lab, postpartum rats showed a significant decrease in sucrose preference on P0 and P1 that was not affected by infusion with the control antibody. Rats that received an IL-6 receptor antibody showed no significant decrease in sucrose preference following birth indicating that the antibody could prevent the postpartum anhedonia (Repeated measures two-way ANOVA: $F_{1,35} = 4.68$, $p = 0.046$). See **Figure 8** below for results.

Sub Chronic Stress Results:

Throughout the seven days of the Forced Swim Test, we found no significant differences in the amount of time spent swimming on any day, or between the rats that would be assigned to the IgG control and IL-6 receptor antibody groups. (Repeated measures two-way ANOVA: $F_{3,36} = 0.1$, $p = 0.960$). **Figure 9** below displays swimming time for Days 1 and 7 only.

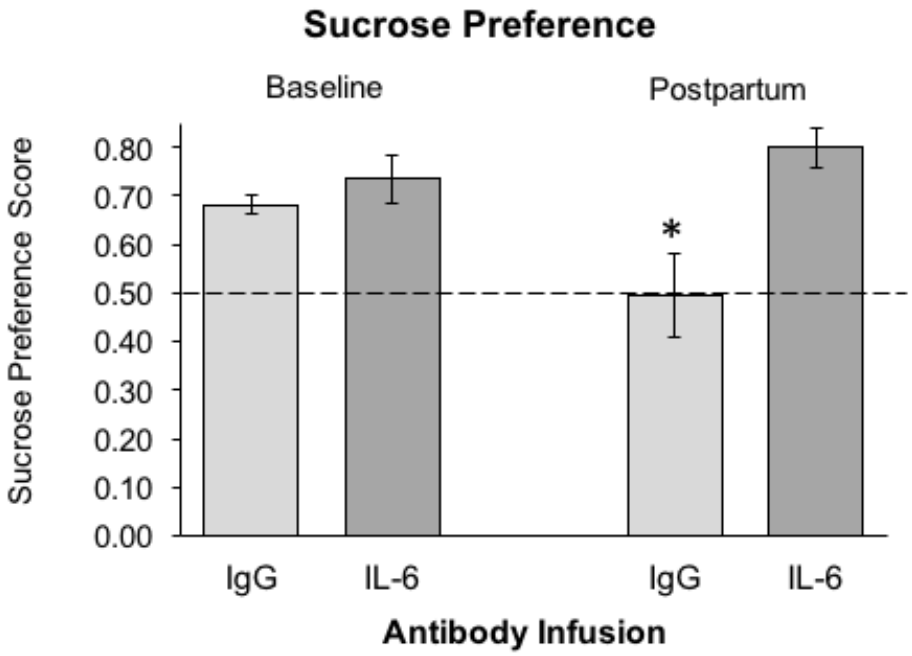

Figure 8 Effects of an IL-6 receptor antibody infusion on Sucrose Preference tested on the day of birth, P0 and P1, two hours following an intra cisterna magna infusion of either an IL-6 receptor antibody or a control IgG antibody each day. Sucrose preference testing was completed prior to the start of the experiment (at Baseline), and immediately postpartum on day of birth (P0) and P1 to measure anhedonia. Statistical analysis revealed a significant decrease in sucrose preference following parturition. The IL-6 receptor antibody infusion prevented this decrease in sucrose preference. *: $p < 0.05$

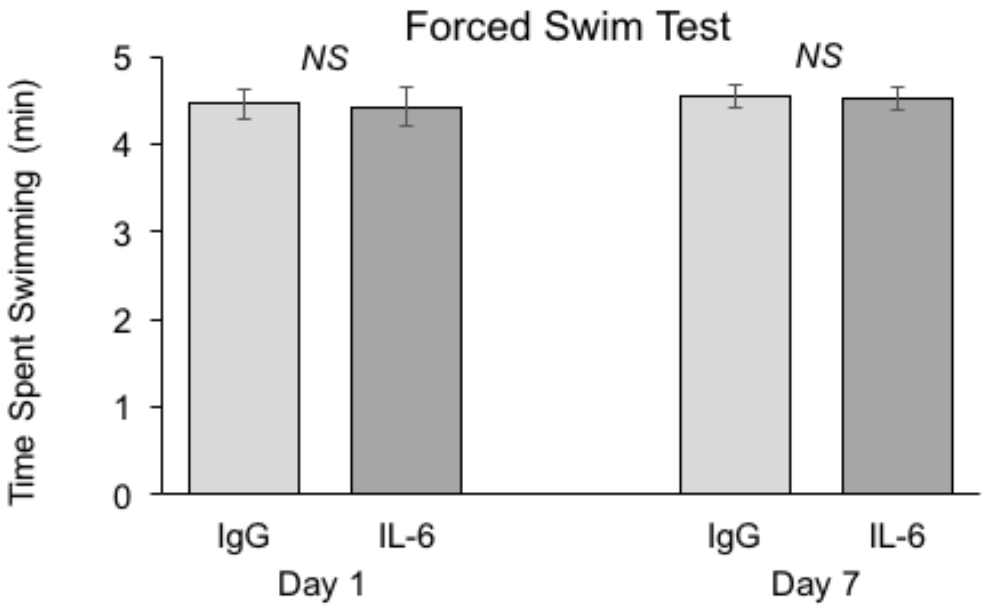

Figure 9 Average time spent swimming on Days 1and 7 of the 1-week forced swim test in the same rats that were subsequently tested for sucrose preference in Experiment 2.2. Time spent swimming for each rat was measured each day and reported, prior to the rats being assigned to the IgG control or IL-6 receptor antibody groups. Despite momentary floating, no rats showed signs of giving up or distress throughout the week of forced swim.

Sub Chronic Stress Results continued:

As expected based on previous results from our lab (Posillico and Schwarz, 2016), sucrose preference decreased significantly following sub chronic stress for seven days in both the IL-6 receptor antibody and IgG control antibody groups. Specifically, there was a main effect of the repeated measure (post-stress effect, $F_{1,39} = 4.92$, $p = 0.039$) indicating that sub chronic stress via forced swim test for seven days is a sufficient method to induce anhedonia, or a decrease in sucrose preference in rats. In contrast to the effects seen in the previous experiment, the IL-6 receptor antibody had no effect on this sub chronic stress-induced anhedonia, suggesting that IL-6 may not play a key role in the early onset of stress-induced depressive-like behaviors. See **Figure 10** below for results.

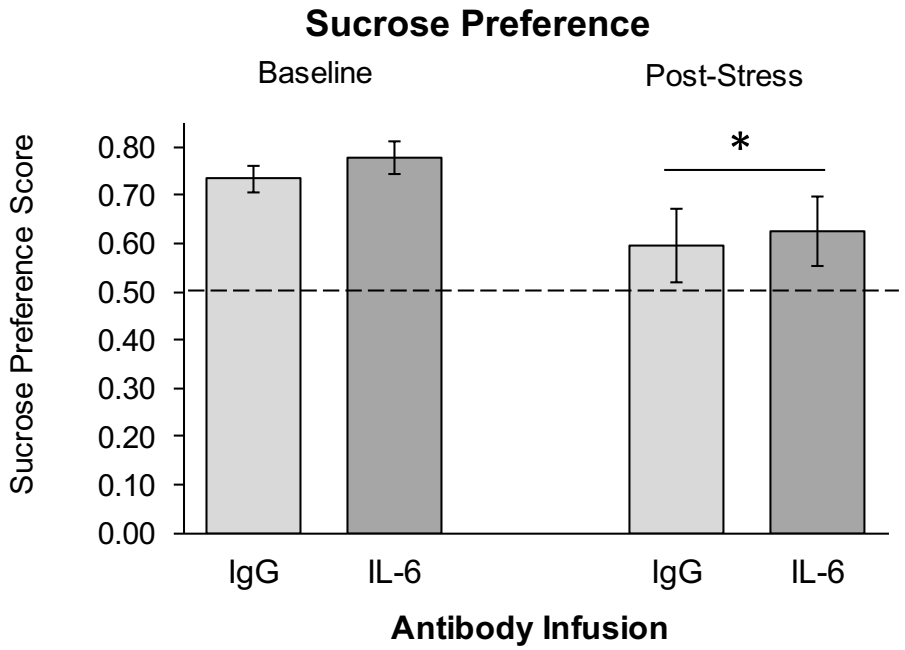

Figure 10 Effects of an IL-6 receptor antibody on Sucrose Preference tested one day following seven days of sub chronic forced swim stress and two hours following an intra cisterna magna infusion of either an IL-6 receptor antibody or a control IgG antibody. Stressed rats received daily forced swim for seven days. Sucrose preference testing was completed prior to the start of the experiment (at Baseline), and over the next two days after the week of forced swim to measure anhedonia. Statistical analysis revealed a main effect of stress on sucrose preference and no effect of the IL-6 receptor antibody infusion. *: $p < 0.05$

4.3 Experiment 2.3: Effects of an IL-6 receptor antibody on cytokine expression following birth or sub chronic stress

Experiment 2.3 sought to determine the effect of the IL-6 receptor antibody on cytokine expression, specifically IL-6, induced following birth or chronic stress. Using the same experimental groups as Experiment 2.2, rats were euthanized two hours after the infusion of the IL-6 receptor antibody or control antibody, on the day of birth or one day post chronic stress for tissue collection and PCR analysis. Similar to Experiment 2.1, both cytokines and second messengers within different pathways associated with the binding of a ligand to the IL-6 receptor including IL-1β, IL-6, IL-6st, Stat3, and NF-κB were analyzed. In addition, we analyzed the expression of Brain Derived Neurotrophic Factor because we have previously observed a robust increase in its expression within the mPFC after birth (Posillico and Schwarz, 2016). Based on the results from Experiment 2.1 and 2.2, we hypothesized that the IL-6 receptor antibody would prevent the increase in postpartum IL-6 and perhaps downstream signaling molecules associated with this particular cytokine. **Figure 11** below displays swimming time for Days 1 and 7 for the rats assigned to the stress condition. Notably, there was no main effects or interactions of day or antibody infusion on swim time across the 7 day Forced Swim Test (RM two-way ANOVA: $F_{3,36} = 0.1$, $p = 0.95$).

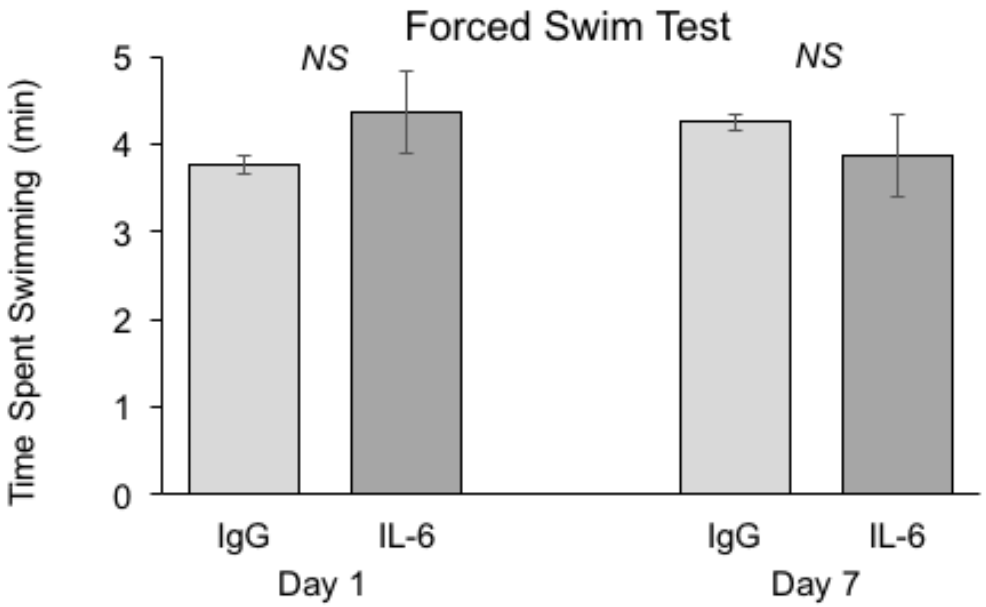

Figure 11 Average time spent swimming on Days 1and 7 of the 1-week forced swim test in the same rats that were subsequently euthanized for cytokine expression in Experiment 2.3. Times spent swimming for each rat was measured each day and reported. Despite momentary floating, no rats showed signs of giving up or distress throughout the week of forced swim.

Gene Expression in the mPFC following IL-6 Receptor Antibody Infusion:

Analysis of gene expression in the mPFC revealed a significant decrease in the expression of IL-6 and BDNF following infusion with the IL-6 receptor antibody on the day of birth (IL-6: $t_{11} = 2.19, p = 0.026$, BDNF: $t_{16} = 1.96, p = 0.034$). Additionally, we found a trending decrease in the expression of both IL-6 signal transduction molecule and NF-κB in the following the infusion of with the IL-6 receptor antibody on the day of birth (IL-6st: $t_{17} = 1.49, p = 0.077$; NF-κB: $t_{16} = 1.56$, $p = 0.069$, respectively). Notably, we saw no significant effect of the IL-6 receptor antibody infusion on cytokine expression in the brains of the stressed females. Though, interestingly, expression of NF-κB displayed an increasing trend in the medial prefrontal cortex following the infusion of an IL-6 antibody after seven days of chronic stress ($t_{15} = 1.42, p = 0.083$). **Figure 12** displays the results from the mPFC gene expression data.

Gene Expression in the Hippocampus following IL-6 Receptor Antibody Infusion:

Analysis of gene expression in the hippocampus revealed no significant changes in the expression of IL-6, IL-6 signal transduction molecule, Stat3, NF-κB, or BDNF following infusion of the IL-6 receptor antibody in the postpartum females. Similarly, no significant differences were observed following IL-6 receptor antibody following sub chronic stress; however, there was a decreasing trend in BDNF following the infusion of the IL-6 antibody after seven days of chronic stress in the hippocampus ($t_{18} = 1.46, p = 0.081$). **Figure 13** displays the results from the HP gene expression data.

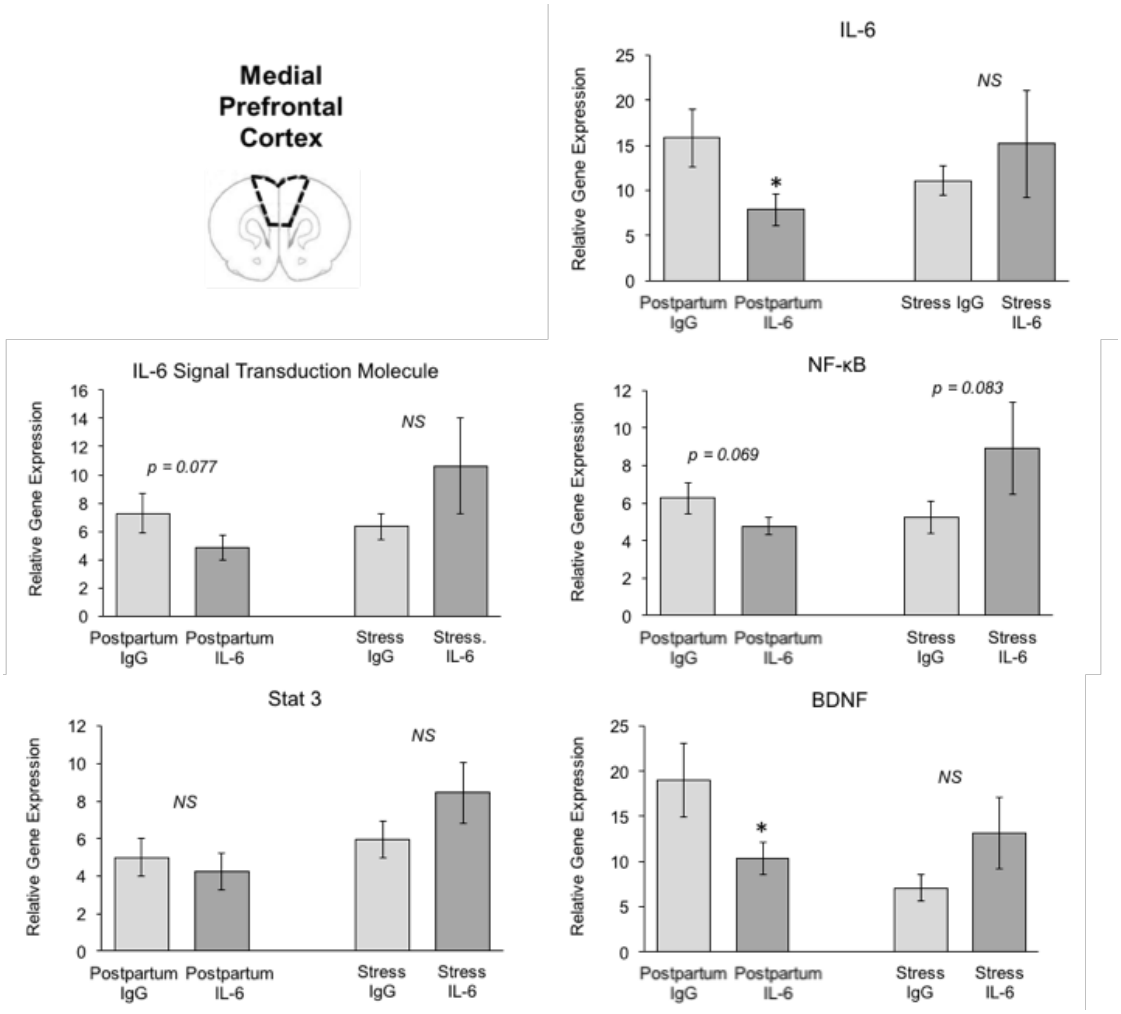

Figure 12 Analysis of relative gene expression in the medial Prefrontal Cortex. The medial Prefrontal Cortex was dissected two hours post IL-6 receptor antibody or control antibody infusion, in postpartum and stressed females. Analysis of IL-1β and IL-6 shows increased relative gene expression following an LPS injection. Analysis of IL-6 signal transduction molecule, Stat 3, and NF-κB showed no significant differences. *: $p<0.05$

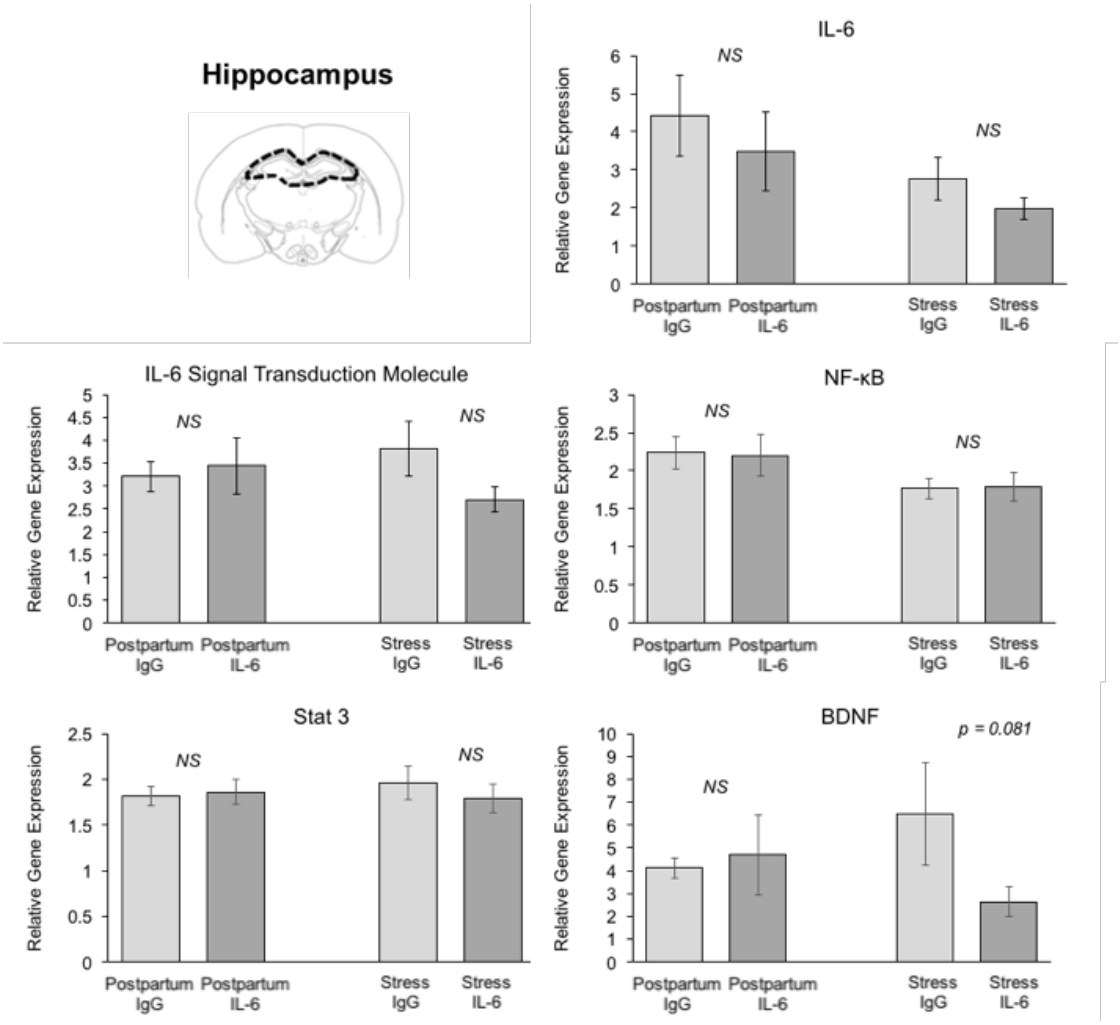

Figure 13 Analysis of relative gene expression in the Hippocampus. Tissue dissected from the Hippocampus two hours post IL-6 receptor antibody or control antibody infusion, in postpartum and stressed females. We found no significant differences in gene expression as a result of IL-6 receptor antibody infusion. There was a trending decrease in the expression of BDNF following IL-6 receptor antibody infusion, but only in the stressed females ($p = 0.081$).

Given the significant decrease in IL-6 expression that we saw in the postpartum brain following intra cisterna magna infusion of the IL-6 receptor antibody, we sought to determine whether peripheral levels of circulating IL-6 protein were either increased postpartum or inhibited by the brain-specific infusion of the IL-6 receptor antibody. Analysis of circulating IL-6 in serum revealed no significant difference between groups that received the IL-6 antibody or control antibody in the postpartum groups ($t_{19} = +0.07$, $p = 0.47$). Similarly, we saw no significant differences in IL-6 protein levels in the sub chronic stress groups ($t_{15} = +0.25$, $p = 0.40$). These results allowed us to conclude that the administration of the antibodies into cerebrospinal fluid did not affect circulating levels of IL-6 in the periphery. **Figure 14** displays the results from the IL-6 ELISA of serum samples.

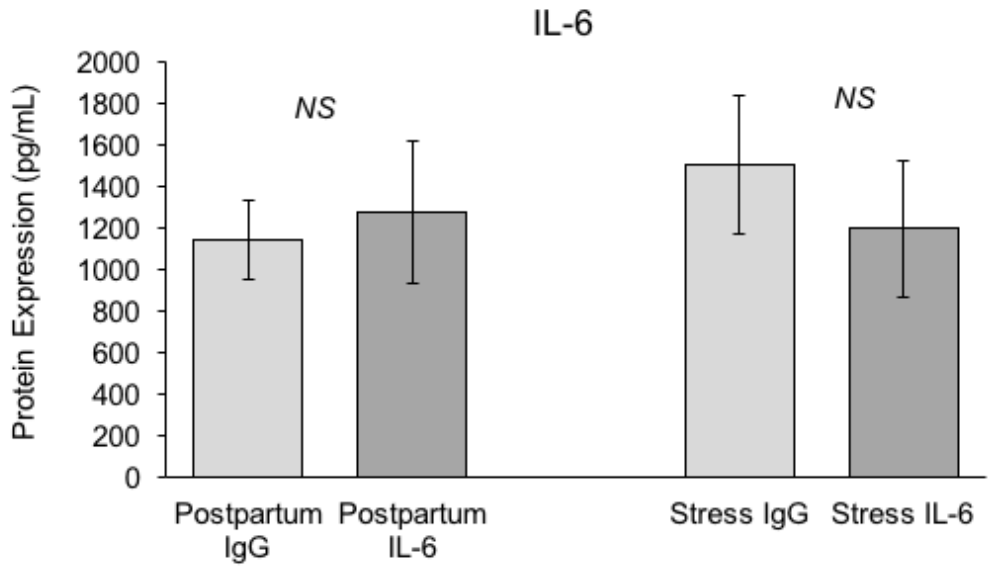

Figure 14 Analysis of circulating protein levels of IL-6 in serum. Serum samples were collected two hours following the intra cisterna magna infusion of either an IL-6 receptor antibody or control antibody on the day of birth or the day after seven days of sub chronic stress. The circulating IL-6 levels were also not significantly affected by the intra cisterna magna infusion of the IL-6 receptor antibody in either the postpartum or stressed females.

Chapter 5

DISCUSSION

The first aim of these experiments was to assess postpartum depressive-like anhedonia following a second pregnancy in order to determine whether it was expressed in second-time moms in a manner similar to our previous reports of postpartum anhedonia in first-time moms (Posillico and Schwarz, 2016). The second aim of this study was to identify a potential therapeutic target for the onset of postpartum depressive-like behavior and compare the effectiveness of this target on the depressive-like behavior induced following sub chronic stress. The overall goal of this second experiment was to determine whether both types of anhedonia (postpartum and stress-induced) may be induced via similar mechanisms or not.

First, we found that a second pregnancy did not significantly affect sucrose preference. Specifically, we saw no significant decrease in sucrose preference in female rats following the birth of their second litter. Previous studies suggest that separation of mother from pups for more than 3 hours may induce maternal depressive-like behaviors (Boccia et al., 2007). In this particular experiment, and in contrast with our previous experiments in first time mothers, we allowed the pups to remain with the second-time mothers, in the sucrose preference testing cages, which may be a possible explanation as to why our second-time mothers did not express any anhedonia or depressive-like symptoms in contrast to our previous experiments. That said, the females in the current experiment still drank from the sucrose bottle (and the water bottle) during the test, indicating that they were not too busy or preoccupied with caring for the pups to consume the liquids during the testing session. Moreover, the findings in Experiment 2 argue against the idea that the postpartum anhedonia we

have observed in first time mothers (tested without their pups) may have been the result of maternal separation during the sucrose preference test, and this will be discussed below. Given the different behavioral findings in first-time and second-time mothers, one might also hypothesize there are differences in immune function or brain function following a first and second pregnancy. To that end, it would be interesting to measure cytokine expression in the brains of first- vs. second-time mothers in future experiments. Based on these findings from Experiment 1, however, and potential concerns about this caveat, we tested sucrose preference for three hours in subsequent experiments in postpartum females in the *absence* of their pups for consistency with our previous experiments (Posillico and Schwarz, 2016).

In our second experiment, we found that administration of an IL-6 receptor antibody to the brain via an intra cisterna magna infusion was a sufficient method to block the significant increase in IL-6 expression (and even IL-1β expression) in the medial prefrontal cortex and hippocampus following immune activation using LPS. These data from Experiment 2.1 gave us confidence that the IL-6 receptor antibody could effectively spread throughout the brain, to our brain regions of interest, and inhibit the function or production of specific cytokines, in particular IL-6. Given that we had consistently seen an increase in IL-6 expression in the postpartum brain, we sought to determine whether blocking the increased expression of IL-6 in the postpartum brain, using the antibody, could prevent the anhedonia that we see in these first-time moms. In fact, blocking the IL-6 receptor with the antibody was sufficient to decrease IL-6 expression in the postpartum mPFC, and effectively prevent the postpartum anhedonia typically observed in these new moms. In contrast, the IL-6 receptor antibody had no effect on the anhedonia produced by sub chronic stress in the

current experiments, and the IL-6 receptor antibody had few effects on cytokine expression and signaling in the hippocampus. These contrasting between postpartum and stress-induced anhedonia results suggest that the mechanisms that may initiate the onset of depressive-like behavior following pregnancy or depressive-like behaviors following sub chronic stress may be different.

These results also provide some evidence against the caveat described above for Experiment 1. Specifically, we have consistently seen an increase in IL-6 expression in the postpartum brain, an effect that is observed even when pups are with the mom, up until the moment of euthanasia. This suggests, of course, that the upregulation of IL-6 in the postpartum brain is not the result of unrelated stressors, but rather an effect specific to having just given birth. In addition, we found that inhibiting the expression of IL-6 using the IL-6 receptor antibody was able to block postpartum anhedonia specifically. This provides support for the idea that the IL-6 expression seen in the postpartum brain is a possible underlying mechanism by which the depressive-like behavior is expressed, and is not dependent on the method by which the sucrose preference test was run. Specifically, these data argue *against* the idea that the postpartum anhedonia is a non-specific effect observed by removing the pups from the dam during the duration of the sucrose preference test.

In support of our original hypothesis that IL-6 expression in the postpartum brain may be an important molecular initiator of postpartum depressive-like symptoms, we found that the infusion of an IL-6 antibody could sufficiently blocked the increase expression of IL-6 in the medial prefrontal cortex and hippocampus of the postpartum brain within two hours. We had also predicted that the IL-6 antibody would also inhibit the expression of secondary messengers and signal transduction

factors typically induced by IL-6 receptor activation; however, the IL-6 receptor antibody ***did not significantly*** affect the expression of the second messengers measured here (IL-6 signal transduction molecule, NFκB and Stat3) in the medial prefrontal cortex. In fact, we only found a trending decrease in the expression of IL-6 signal transduction molecule and NF-κB in the hippocampus following the IL-6 antibody infusion. In combination with our results from Experiment 2, these results suggest one of two things, **(1)** the IL-6 receptor antibody may be effectively reducing the signaling of IL-6 via other secondary messengers not measured in these experiments; or **(2)** that the IL-6 receptor antibody can downregulate the synthesis of IL-6 *first* (within 2 hours), and then have a subsequent impact on the downstream signaling pathways at a later time point (a time point not measured here). The latter explanation is possible given that we measured gene expression in Experiment 2.3 at two hours post infusion with the antibody, but we measured behavior in Experiment 2.2 just *beginning* at two hours post infusion with the antibody. In fact, our sucrose preference testing in Experiment 2.2 continued for an additional four hours after that point, a full 6 hours after the initial antibody was infused into the brain. Thus, it is possible that the IL-6 receptor antibody may have effectively decreased the expression of IL-6 ***as well as*** its associated downstream signaling pathways during that 6- hour timeframe, though it wasn't measured in the current experiments.

As an alternate explanation, previous studies suggest that the IL-6 receptor antibody can only affect membrane IL-6 receptor signaling, and not trans-signaling. Trans-signaling occurs when IL-6 and its soluble IL-6R (sIL-6R) bind to proteins in cells that **do not** express the classical IL-6R, and thus would not typically respond to IL-6 otherwise. (Maes et al., 2014; Rose-John, 2012). We did not observe any

significant changes in the expression of Stat3 in the prefrontal cortex, which is signaled via IL-6 receptor activation by trans-signaling. Taken together, these data suggest that the IL-6 receptor antibody used here may be effective at only modulating specific pathways or molecules of IL-6R membrane signaling, and thus inhibit depressive-like behaviors. Perhaps different inhibitors of IL-6 receptor signaling, such as inhibitors of IL-6 trans-signaling, may be more effective in treating other types or symptoms of depression.

In the current studies, the IL-6 antibody was effective at preventing postpartum anhedonia, but had no effect on stress-induced anhedonia. The notable differences between the postpartum and stress groups were decreases in expression IL-6, IL-6 signal transduction molecule, and BDNF in only the pregnancy group following administration of the IL-6 antibody. In contrast, in the hippocampus, the IL-6 antibody decreased the expression of BDNF in the stress group only. Thus, suggesting that levels of IL-6, IL-6 signal transduction molecule, and BDNF in the mPFC may play a role in the onset of postpartum anhedonia or depressive-like behaviors following birth. The fact these cytokines and signaling molecules were not affected by the infusion of the IL-6 antibody in the mPFC of the stress group may explain why we did not see behavioral changes in these groups. However, in the hippocampus of the stress group, the expression of BDNF displayed a decreasing trend following chronic stress with the infusion of the IL-6 antibody. Hippocampal BDNF is thought to be responsible for synaptic plasticity and cognition (Vaynman et at., 2004). It is possible that the antibody has the potential to affect synaptic plasticity and cognitive functions such as anhedonia as well.

IL-6 receptor antibodies are currently used as an approved treatment for Rheumatoid Arthritis, however, in the treatment of this peripheral inflammatory disorder, the antibody cannot cross the blood brain barrier. The key for using this antibody as a treatment for depression would be to administer it in a way that it could cross the blood brain barrier to affect the IL-6 receptors in the brain. Interestingly, we found that administration of the IL-6 receptor antibody intra cisterna magna was unable to affect peripheral circulating IL-6 levels. These data highlight that the central and peripheral immune systems often have different roles in the induction of the immune response and subsequent effects on behavior, and that the antibody is likely unable to pass across the blood brain barrier from (in this case) the brain to the periphery. In particular, these data suggest that peripheral IL-6 levels may *not* influence depressive-like behaviors postpartum, or depressive-like behavior following sub chronic stress. However, it is possible that these peripheral levels may impact depressive-like behavior following more severe or chronic stress, an idea which is supported by the significant evidence linking chronic stress and increased IL-6 in the periphery to major depression (described above in the Introduction). For example, depressed patients have elevated levels of circulating IL-6 in their serum (Chourbaji et al., 2006). In support of our original hypothesis, the infusion of an IL-6 antibody into the central nervous system was sufficient to prevent depressive-like behaviors in rats following parturition. Despite preventing anhedonia immediately postpartum, the IL-6 antibody had no significant effect on anhedonia following chronic stress. Taken together, these data may suggest that the ***initial onset*** of anhedonia following birth versus stress is mediated via different mechanisms.

Microglia are the resident immune cells in the brain. The results of this study have shown differences in cytokine expression, and microglia play a key role in the expression of cytokines and additional immune molecules specifically in the brain. In the future, it would be interesting to analyze microglia morphology and densitometry to see how the IL-6 receptor antibody affects microglia number of function more specifically. Previous results have shown that microglia density is significantly decreased during pregnancy, compared to non-pregnant controls, on the day of birth in the hippocampus, specifically in CA1 and the dentate gyrus. (Posillico and Schwarz, 2016). Thus, in future experiments, microglia density should be analyzed to see if the IL-6 antibody can prevent this decrease of microglia in the hippocampus on the day of birth. It is possible that we do not see an increase in cytokines on the day of birth in the hippocampus because of this decreased number in microglia and would be interesting to confirm this theory with further experiments.

Maternal behavior was not measured in these experiments; however, it is also important for future experiments to include this factor to understand if the expression of appropriate maternal behavior is affected by the IL-6 receptor antibody in first time mothers. Moreover, the effects of the brief isoflurane anesthesia would be important to control for in terms of maternal care to ensure that the anesthesia is not affecting behavior as well. In humans, behavioral effects following anesthesia have been widely studied, and results indicate that anesthesia can have adversely negative behavioral effects following anesthesia at a young age (Frick et al., 2011). Thus, investigating behavioral effects in mothers following anesthesia would be very important to understand the effects of this procedure fully.

This work demonstrates that the onset of postpartum anhedonia may be mediated differently than other types of depression. The successful attenuation of postpartum anhedonia following the infusion of an IL-6 receptor antibody suggests that this antibody, or other drugs that affect IL-6 signaling, may be good potential targets for the relief of postpartum depression and its associated symptoms that may not be fully alleviated by typical antidepressants.

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Appendix

APPROVAL FOR THE USE OF ANIMAL SUBJECTS

UNIVERSITY OF DELAWARE | Institutional Animal Care and Use Committee (IACUC)

Newark, DE 19716-1561
Phone: 302-831-2616
Fax: 302-831-0154
Email: [illegible]

To: Office of Graduate and Professional Education

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

Subject: IACUC approval for Julie Gomez

Date: 4/26/2018

Julie Gomez 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 with any additional questions.