

**EXAMINING THE IMPACT OF NEUROIMMUNE DYSREGULATION
ON SOCIAL PLAY BEHAVIOR OF MALE AND FEMALE JUVENILE RATS**

by

Elizabeth McAuley

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ON SOCIAL PLAY BEHAVIOR OF MALE AND FEMALE JUVENILE RATS**

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Elizabeth McAuley

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

Approved: _____
Tania L. Roth, Ph.D.
Chair of the Department of Psychological and Brain Sciences

Approved: _____
John A. Pelesko, Ph.D.
Dean of the College of Arts & Sciences

Approved: _____
Louis F. Rossi, Ph.D.
Vice Provost for Graduate and Professional Education and
Dean of the Graduate College

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TABLE OF CONTENTS

LIST OF TABLES	vi
LIST OF FIGURES	vii
ABSTRACT	viii
1 INTRODUCTION.....	1
1.1 Social Behavior and Neurodevelopmental Disorders.....	1
1.2 Modeling Social Behavior in Rats.....	2
1.3 Immune Activation and Neurodevelopmental Disorders	5
1.3.1 Dopamine and the Two-Hit Hypothesis of Neuroinflammation ...	6
1.4 Questions, Hypothesis, and Predictions	7
2 MATERIALS AND METHODS	9
2.1 Experimental Subjects	9
2.2 Experimental Treatments.....	10
2.2.1 P4 Subcutaneous Injections with <i>E. coli</i>	10
2.2.2 P25 Intraperitoneal Injections with LPS.....	11
2.3 Behavioral Testing: Social Play	11
2.4 Brain and Tissue Collection	13
2.5 RNA Extractions and Real-Time PCR	14
2.6 Immunohistochemistry	15
2.6.1 Image Acquisition and Analysis.....	16
2.7 Statistical Analysis	17
2.7.1 Statistical Analysis for Behavior	17
2.7.2 Statistical Analysis for PCR	17
2.7.3 Statistical Analysis for IHC.....	18
3 RESULTS.....	19

3.1	Behavioral Testing.....	19
3.1.1	Social Play Behavior	19
3.2	Real-Time PCR	22
3.2.1	D1R Relative Gene Expression	22
3.2.2	D2R Relative Gene Expression	27
3.3	Immunohistochemistry	30
3.3.1	D1R Expression.....	30
3.3.2	Iba1 Expression	33
4	DISCUSSION.....	36
4.1	Brief Summary of Findings	36
4.2	Summary of Behavioral Results	36
4.2.1	Neonatal Infection Significantly Increased Social Play Behaviors in Test Juvenile Male and Female Rats	37
4.3	Summary of PCR Results	40
4.4	Summary of IHC Results.....	42
4.5	Conclusions	43
4.6	Limitations and Future Directions	45
	REFERENCES	47
	Appendix APPROVAL FOR THE USE OF ANIMAL SUBJECTS	51

LIST OF TABLES

Table 1: IDT rat primers used for quantitative real-time PCR	15
Table 2: Summary of collective results from the current study	45

LIST OF FIGURES

Figure 1: Impact of neonatal infection and/or juvenile LPS administration on social play behavior (instances of behavior) of individual test animals within the home cage across a fifteen-minute interval	22
Figure 2: Impact of neonatal infection and/or juvenile LPS treatment on D1r relative gene expression in the amygdala of male and female juvenile rats at four, twenty-four, and seventy-two hours post-LPS treatment.	24
Figure 3: Impact of neonatal infection and/or juvenile LPS treatment on D1r relative gene expression in the mPFC of male and female juvenile rats at four, twenty-four, and seventy-two hours post-LPS treatment	26
Figure 4: Impact of neonatal infection and/or juvenile LPS treatment on D2r relative gene expression in the amygdala of male and female juvenile rats at four, twenty-four, and seventy-two hours post-LPS treatment	28
Figure 5: Impact of neonatal infection and/or juvenile LPS treatment on D2r relative gene expression in the mPFC of male and female juvenile rats at four, twenty-four, and seventy-two hours post-LPS treatment	29
Figure 6: Impact of neonatal infection and/or juvenile LPS treatment on expression of dopamine receptor (D1r) density in the amygdala of male and female juvenile rats at P28	31
Figure 7: Impact of neonatal infection and/or juvenile LPS treatment on expression of dopamine receptor (D1r) density in the mPFC of male and female juvenile rats at P28	33
Figure 8: Impact of neonatal infection and/or juvenile LPS treatment on expression of microglial density in the amygdala of male and female juvenile rats at P28	34
Figure 9: Impact of neonatal infection and/or juvenile LPS treatment on expression of microglial density in the mPFC of male and female juvenile rats at P28	35

ABSTRACT

There are many neurodevelopmental disorders associated with altered social behavior in humans. Current literature indicates that along with many genetic risk factors there are also environmental factors that contribute to the risk of developing neurodevelopmental disorders, including those with disordered social behavior. These environmental factors are known to activate the immune system. Various environmental factors (infection, pollution, drugs) and their associated dysregulation of the immune system in early life may in fact have negative consequences on neural function and social behavior. In the current project, we examined the impact of neonatal infection, with or without juvenile immune activation, on the expression of social behavior, and dopamine receptor expression in male and female test juvenile rats. We modeled disordered social behavior in a test animal and scored a typical social interaction (play behavior) from the point of view of the test animal. This was done by coding for behaviors initiating a play bout by test as well as behaviors that indicated participating in a play bout.

The outcomes of these experiments revealed that neonatal infection significantly increased juvenile play behavior in male and female test rats. Moreover, we saw that the test rats showed increased instances of initiating a play bout rather than participating. According to the two-hit hypothesis of neuroinflammation,

increases in dopamine receptors and changes in behavior should be most robust in rats exposed to *both* an early-life and later-life immune challenge. Within the current project this hypothesis was sometimes true, however, the molecular data indicates that neonatal infection alone led to significant differences in dopamine receptor expression within the juvenile brain. The outcomes of these experiments revealed that neonatal infection significantly decreased dopamine relative gene expression, but significantly increased dopamine receptor density. Taken together, the behavioral and molecular data support the sensitivity of the juvenile brain to immune activation (both with and without neonatal infection), especially in the expression of species typical social behavior. Thus, environmental factors that activate the immune system during development are likely significant risk factors for neurodevelopmental disorders in human children.

Chapter 1

INTRODUCTION

1.1 Social Behavior and Neurodevelopmental Disorders

Both humans and animals express a wide variety of social behaviors that are important for the survival of the individual as well as the survival of the species. For this reason, it is rewarding for an individual to engage in pro-social behaviors (Vanderschuren, 2016). In the human population, however, there are individuals for whom such social behaviors are impaired. These individuals are often diagnosed with one of many disorders, most notably Autism Spectrum Disorders (ASDs), based upon criteria in the Diagnostic and Statistical Manual of Mental Disorders-V that indicate deficits in social behavior (Cotter et al., 2018). We can utilize animal models to examine deficits in species-typical social behaviors as a way to better understand disorders with associated deficits in social behavior, including their various origins and causes of these disorders. The neural correlates implicated in rodent social behaviors specifically, including social play behavior include the amygdala and the medial prefrontal cortex among others. These brain regions are part of a larger “social network” within the brain (Gheusi, Bluthé, Goodall, & Dantzer, 1994; Ko, 2017; E. Lee et al., 2016; Rudebeck et al., 2007; Tzakis & Holahan, 2019). We propose here,

that if there are variations in the development of these brain structures, it could significantly impact the ontogeny and expression of age-appropriate social behaviors.

Recent literature indicates that in addition to genetic risk factors, there are a variety of perinatal environmental factors that contribute to the risk of developing ASD (Chaste & Leboyer, 2012). Many of these environmental risk factors are known to activate the immune system, and a general working hypothesis in the field of neurodevelopmental disorders is that dysregulation of the immune system early in life may in turn have negative consequences on neural function as well as social behaviors later in life. The goal of our current studies was to model early life activation of the immune system to understand the social behavior deficits seen in many neurodevelopmental disorders, including ASD and schizophrenia. An important additional goal of this data analysis has been to further investigate the differences present between the sexes, and whether one sex is more at risk of deficits in social behaviors caused by early life immune activation.

1.2 Modeling Social Behavior in Rats

Play behavior is an important social behavior of many species and is considered an ideal behavioral measure to test as it occurs naturally, and thus requires no training. Moreover, many animal species, including rats, also display robust social play behavior early in life, prior to puberty. This makes it particularly unique, as we can examine the impact of early-life immune activation on the development of a social behavior that emerges early in life. The now well-known, stereotyped play behaviors

exhibited by juvenile rats were first characterized in 1981 (Meaney & Stewart, 1981). These behaviors can include pouncing, pinning, boxing, chasing, social exploration and are the foundation for the rat dyads play bout. Moreover, many neurodevelopmental and neuropsychiatric disorders are associated with deficits in social behavior that are detected or emerge early in life, manifested as social deficits in childhood and into adolescence. Thus, examining the effect of early-life immune activation social play behaviors in rodents, makes for a particularly unique model to examine the consequences of early-life immune activation on the development of age-appropriate juvenile social behavior.

Previous work done by Dr. Alexandra Turano in our lab examined play behavior of juvenile pairs, meaning the instances of various play behaviors were recorded when either animal of the pair initiated a play bout. In this case, the pairs included a test rat and an age-matched stimulus (untreated control) rat. With that in mind, we are moving forward in the current set of experiments by scoring the same pairs as before, but this time, only scoring the behaviors of the test rat to determine whether significant increases in play behaviors caused by early-life immune activation were, in fact, the result of increased expression of play behaviors from the test rat. Scoring in this case will consist of instances during which the test animals are pouncing, pinning, and chasing. We have defined these as test initiated behaviors. Additionally, we scored when the test rat was being pounced, being pinned, and being chased, we consider these behaviors to be when test was participating in a play bout. Differentiating between initiation and participation in a play bout has been quantified

and qualified by various investigators, and pouncing is typically described as *initiating* a playful encounter while pinning is seen as *contributing* to the *continuation* of a playful encounter (Meaney & Stewart, 1981; Vanderschuren et al., 2016). The play behaviors like pouncing and pinning are referred to as “rough-and-tumble play” and are known to vary with the level of familiarity with their play partner and vary based on the sex and age of their partners. Literature has shown that within a male-male dyad there are more instances of rough-and-tumble play behaviors than within a female-female dyad (Meaney & Stewart, 1981; Vanderschuren, Niesink, & Van Ree, 1997), and experiments across various species indicate that exposure to testosterone during prenatal development is the cause for increased expression of rough-and-tumble play behaviors in juvenile males.

Our goal is to provide better understanding of immune activation of dysregulation during early brain development affects the expression of social play behaviors from the perspective of the test rat. We can then compare the data collected for the pair and the data collected from the test animal only in order to determine whether differences in play behavior expressed by the test animal are affecting the behaviors exhibited by the pair. This comparison will allow us to determine how early-life immune activation affects the expression of appropriate play behavior in an individual that, in turn, affect interactions with others. It is by analyzing the play behaviors described above that we can provide more insight into the social behaviors of juvenile rats. It also provides an opportunity to examine how this play behavior can be altered following experimental manipulation.

1.3 Immune Activation and Neurodevelopmental Disorders

Microglia are the primary immune cells of the brain, and they release inflammatory cytokines in response to immune activation. Neurons are particularly sensitive to production of pro-inflammatory cytokines, and increased inflammation and associated cytokine production in the brain can significantly impact neuronal function and behavior. Various models of early-life immune activation have shown that exposure to inflammatory cytokines during development can induce long-term changes in the function of microglial cells. Specifically, immune activation early in life can “prime” the function of microglial cells (Bilbo and Schwarz, 2009), and as a result of this “priming”, a “second hit” or subsequent immune challenge later in life can result in the release of exaggerated levels of cytokines from microglia in the brain, which can alter neuronal function and lead to persistent behavioral disorders (Bilbo and Schwarz, 2009). This is important considering that most neurodevelopmental and behavioral disorders have their origins in early development and are associated with immune activation as a primary risk factor. Thus, neuroinflammation during early-brain development (stimulated by environmental factors) has been implicated in the emergence of social cognitive deficits observed in both autism and early-onset schizophrenia (Lewis & Levitt, 2002; Nakagawa & Chiba, 2016; Patterson, 2009).

Our lab has utilized a model of early-life immune activation, which introduces an immune challenge of *E. coli* bacteria to rats on day 4 of life (P4) (our “first hit”)

and then challenges the immune system again with a low dose of lipopolysaccharide (LPS) on day 25 (P25) (our “second hit”). This model has been referred to as a “two-hit model of neuroinflammation” (Turano et al.), and has been shown to induce cognitive and behavioral deficits later in life (Williamson et al., 2011). Using this model, we recently examined a variety of play behaviors in rats, including pouncing, pinning, chasing, and social exploration. Our goal has been to understand the impact the two-hit model of neuroinflammation has on the development and expression of species-typical social play behaviors in male and female juvenile rats as it relates to the neural mechanisms associated with it.

1.3.1 Dopamine and the Two-Hit Hypothesis of Neuroinflammation

Dopamine and its associations with reward and play are well known in the literature and are still a focus of continued research. The dopaminergic mesolimbic system has connections within the nucleus accumbens, amygdala, and medial prefrontal cortex that play an essential role in broader cognitive, social, and motor functions. Recent studies have implicated the expression of D1 Dopamine receptors (D1r’s) of the nucleus accumbens in modulating social play behaviors in adolescent rats (Kopec et al, 2017). The downregulation of cellular material like D1r’s can be accomplished by complement C3 tagging of the receptors or their synapses for microglia to subsequently recognize and phagocytize for degradation (Presumey, Bialas, & Carroll, 2017; Stephan, Barres, & Stevens, 2012). Further implicating the complement system in typical neurodevelopment, Kopec et al. (2018) found that the

microglial sculpting of neural circuits regulating social behavior is mediated by the *sex-specific* C3 tagging of dopamine receptors (D1rs) for downregulation in the nucleus accumbens (NAc) of developing rats. While male rats show a peak (P30) followed by a decline (P38) in D1r expression in the NAc at mid-adolescence, female rats undergo D1r downregulation a bit earlier than males, at P30. Importantly, male (but not female) rats show an associated increase and decrease in play behavior that corresponds with the peak (P30) and decline (P38) in D1r expression in the NAc. This research helps further detail the important role of microglia in a developing brain. It also provides clear evidence for microglia's functional role in maintaining species typical play behaviors through D1r's.

Early-life immune activation can *prime* microglial cells, resulting in exaggerated neuroinflammation and prominent behavioral deficits upon subsequent immune activation (Bilbo & Schwarz, 2012). This is known as the “two-hit hypothesis of neuroinflammation” and is the basis for the current project.

1.4 Questions, Hypothesis, and Predictions

The current study examined the impact of neonatal immune activation, with or without juvenile immune activation, on the expression of social behavior in male and female juvenile test rats. In addition, the study examined how these immune challenges impacted relative gene expression of dopamine protein receptors (D1r & D2r), as well as dopamine receptor and microglia cell density within the amygdala and medial prefrontal cortex.

The previous research mentioned looking at the play behaviors of the play pair provide insight into the intensity of play we can expect with test experiencing experimental manipulation. Using what we saw from this previous experiment we are able to hypothesis how play behavior would look when coded in the point of view of the test animal alone. Thus, we predicted that neonatal infection and/or juvenile LPS treatment of the test animal would significantly increase the instances initiation and participation of play behaviors observed for the test animal. Additionally, we predicted that relative gene expression of dopamine receptors would be significantly increased by a neonatal and or juvenile immune challenge, and that this expression would change across the timepoints examined (P25, P26, P28). Lastly, we also predicted that early-life and or later-life immune activation would result in an increase in dopamine receptor expression density in the amygdala and medial prefrontal cortex, with an associated increase in the activation (density) of microglia in those same regions. We anticipated sex-specific effects at each level of this investigation, as sex differences are known to exist in rodent social play behavior (Argue & McCarthy, 2015a; Vanderschuren et al., 1997). Our two-hit hypothesis of neuroinflammation is as follows, increases in dopamine receptors associated with changes in social play behavior should be most robust in rats that are exposed to *both* early-life and later-life immune activation.

Chapter 2

MATERIALS AND METHODS

2.1 Experimental Subjects

Adult male and female Sprague Dawley rats were obtained from either Envigo Laboratories (Indianapolis, IN) or Charles River Laboratories (Wilmington, MA) and housed in same sex pairs in clear, polyethylene cages (45 cm x 20.5 cm x 24 cm). Once assimilated to the environment, mature males and females were paired for 48 hours and cages were checked for the presence of a sperm plug which was used to determine embryonic day 0. Pups were randomly assigned to experimental groups (see Section 2.2 below), and subsequent behavioral testing or tissue collection. All pups were subject to injections on P4 and again on P25; however, those rats used as stimulus animals for behavioral testing were not subject to any injections. In order to reduce handling anxiety on the day of testing all pups were handled for five minutes by experimenters on P14 and P17. Once the pups reached P21 they were weaned into same sex pairs or trios and housed in one colony room. The colony room was maintained on a 12:12 light/dark cycle with lights on from 7:00 to 18:00 hours, and kept at 22°C. Sentinel rats were housed in the same colony room and were regularly tested for common rodent diseases. All experiments were approved by the University of Delaware Institutional Animal Care and Use Committee.

2.2 Experimental Treatments

Juvenile rats assigned to either behavioral testing or tissue collection were subject to injections of sterile, pyrogen free phosphate buffered saline or *E. coli* [1×10^6 colony forming units (CFU) / 0.1 mL phosphate buffered saline] on P4 as well as either sterile saline or Lipopolysaccharide (LPS, 100 μ g/kg) injections on P25. As a result, the experiments consisted of four experimental groups, including both male and female rats. The experimental groups are as follows: **control** = P4 saline treatment + P25 saline treatment; **LPS treatment alone** = P4 saline treatment + P25 LPS treatment; **neonatally infected** = P4 *E. coli* infection + P25 saline treatment; and **neonatally infected plus LPS-treated** = P4 *E. coli* infection + P25 LPS treatment, the two-hit group.

2.2.1 P4 Subcutaneous Injections with *E. coli*

E. coli cultures (obtained from ATCC 1547) were hydrated and grown overnight in 30 mL of brain–heart infusion (BHI) broth at 37°C. Re-hydrated cultures were aliquoted in 1 mL stock vials, supplemented with 10 percent glycerol, and stored at –20°C for later use. Approximately 24 hours prior to *E. coli* injection, stock cultures were thawed and incubated in 40 mL of BHI at 37°C. Following incubation, the number of bacteria present within the culture was assessed via a microplate reader (BioTek; model ELx808), and the number of colony forming units (CFU) was quantified via previously determined growth curves. Cultures were centrifuged and re-suspended in the appropriate volume of sterile, pyrogen-free, Dulbecco’s phosphate

buffered saline (dPBS) for a final concentration of 1×10^6 CFU of live bacterial *E. coli*. On the day of injection (P4), pups were removed from the dam, sexed, and given an injection of 0.1 mL of 1×10^6 CFU *E. coli* or 0.1 mL of sterile dPBS subcutaneously. These injections always took place between the hours of 8:30am and 10:30am, and the litter was away from the dam for no more than 5 minutes at the time of injection. All pups from one litter received the same P4 injection, in order to reduce the potential of cross-contamination of bacterial infection.

2.2.2 P25 Intraperitoneal Injections with LPS

On P25 the juvenile rats were already separated from the dam and socially housed in a colony room. Prior to injection, a stock concentration of LPS (2,500 ug/mL; obtained from Sigma Aldrich; Cat. No. L2630) was diluted with sterile, pyrogen-free dPBS to a final concentration of 100 ug/mL. They were weighed, labeled, and given an intraperitoneal injection of either 1 mL/kg of LPS (100 ug/mL) or an equivalent volume of dPBS. All cage mates received the same treatments to avoid any possibility of cross-contamination between rats. P25 LPS injections always took place between 8:30am and 10:30am.

2.3 Behavioral Testing: Social Play

Social play behavior testing was used as a means to assess species-typical social play of male and female juvenile rats when paired with a novel stimulus playmate. Male and female test rats (P28) (1 rat/litter/sex/treatment group) were

isolated into clean home cages (45 cm x 20.5 cm x 24 cm), within their normal colony room, three hours prior to their first social encounter to increase sociability during testing. Both test and stimulus rats were weighed prior to being placed in the home cage and the stimulus rat's tail was marked to allow for identification during testing and scoring. Testing began at 15:30 when a stimulus rat (novel, untreated, sex-matched, age-matched, weight-matched, non-littermate) was placed into home cage containing the test rat, still within the same colony room. Video recording software (UniFi® Video) was pre-scheduled to record rat pairs from 15:30 to 16:30 hours, during the light phase.

The play dyad's testing session was hand scored by one experimenter who was blind to sex and treatment group. The first fifteen minutes of the recorded play session was the window of time when the most robust play behavior occurred, and thus was used for the analyses presented here. The instances of pouncing, pinning, and chasing were quantified, as these stereotyped behaviors have been commonly used to assess sociability and playfulness in rats (Meaney & Stewart, 1981; Vanderschuren et al., 2016). These behaviors were observed and scored in terms of the test animals' initiation as well as participation in a play bout. This created six separate play behaviors measured. The breakdown of these behaviors is as follows; **pouncing** = when a *test* rat made intentional contact with the nape of *stim* rat; **pinning** = when *stim* rat to the supine position in response to a pounce from *test*; **chasing** = *test* rat chasing *stim* for more than two seconds; **being pounced** = when *stim* made intentional contact with the nape of *test* rat; **being pinned** = when *test* rat to the supine position in

response to a pounce from *stim*; **being chased** = *stim* rat chasing *test* for more than two seconds.

2.4 Brain and Tissue Collection

In a separate cohort of rats from those used for behavioral testing, brain and peripheral tissues were collected at four (P25), twenty-four (P26), and seventy-two (P28) hours following LPS injection on P25 (De Simoni, Del Bo, De Luigi, Simard, & Forloni, 1995; Dinarello, 2018), male and female juvenile rats were euthanized following an intraperitoneal injection of Euthasol (ANADA 200-071). Once completely anesthetized, rats were perfused via cardiac puncture with an ice-cold, 0.9% saline solution to remove blood and peripheral immune cells from the brain (Kvichansky et al., 2019). Whole brains were collected and preserved using the following steps: day 1 (collection) stored in 4% paraformaldehyde at 4°C, day 2 fresh 4% paraformaldehyde at 4°C, day 3 30% sucrose-PBS solution at 4°C, day 4 fresh 30% sucrose in which it is stored at 4°C until slicing for subsequent histology. Then finally, a separate cohort of experimental rats was used for the collection of peripheral and brain tissues for quantitative PCR analysis. From these animals, the amygdala (AMYG) and medial prefrontal cortex (mPFC) were microdissected from both hemispheres and immediately flash frozen in dry ice. Tissue samples were stored at -80°C and whole brains were stored at -4°C. Animals that did not complete behavioral testing were cage-mates of those that underwent behavioral testing. These animals underwent identical protocols and were used as brain and tissue collection. To control

for litter effects, no more than one male and one female per treatment group was used in any given experiment, either behavioral testing, histology, or PCR analysis.

2.5 RNA Extractions and Real-Time PCR

RNA was extracted from frozen brain tissue using an Isol-RNA Lysis Reagent. Genomic DNA was removed from the tissue and cDNA was synthesized from the extracted RNA (1,000 ng/uL per sample) using the QuantiTect® Reverse Transcription Kit (Qiagen). qPCR was performed using the RealMasterMix™ Fast SYBR Kit (VWR) in 10 µL reactions on a CFX96Touch real-time PCR machine in order to quantify relative gene expression. D1r and D2r primers were ordered from Integrated DNA Technologies (IDT) and diluted to a final concentration of 0.13 µM for qPCR reactions. See **Table 1** for a list of IDT primer sequences. Samples were numbered, blinded to experimental group, and then run in duplicate on either white or green real-time PCR plates. For each reaction, the average quantitative threshold amplification cycle number (C_q) from the duplicate reactions was used to determine the relative gene expression using the $2^{\Delta\Delta C_q}$ method. RPLP1 was used as the housekeeping gene for all experimental groups.

Table 1: Rat Dopamine Receptor primers used for quantitative real-time PCR

Gene NCBI Sequence Forward and Reverse Primers

Gene	NCBI Sequence	Forward and Reverse Primers
D1R	NC_051352.1	F: ACAGATGCATTGTTGATGAC R: TGCTAGTACAAATGGAGAGG
D2R	NC_051343.1	F: TTAACATCGTCTCTCTTCCA R: GCAGGTATAGTGATGTTACA

2.6 Immunohistochemistry

Cryoprotected brains were flash frozen in 2-methylbutane on dry ice and then sectioned at 20µm on a Leica cryostat at -25°C. Sliced brains were stored in wells containing 0.01% Sodium Azide Solution at 4° C until staining. Sections were later selected from the series for subsequent immunohistochemical staining based on the brain regions of analysis and their respective coordinates. Eight representative sections of amygdala (Bregma = -2.0 to -3.6) and five representative sections of mPFC (Bregma = +2.8 to +1.8) were used. Ionized calcium-binding adaptor molecule (Iba)-1 (Schwarz et al., 2012) was chosen as the target protein for staining of microglial cells (Wako, Cat No: 019-19741) . Dopamine receptors were stained using the mouse dopamine receptor D1 (DRD1) polyclonal antibody (MyBioSource, Cat No: MBS2112099) at a concentration of 1:5000. Sections were washed 3 times (for at least 5 minutes each) with phosphate-buffered saline (PBS) and then incubated for 1 hour with a buffer solution containing PBS, normal goat serum (Vector Laboratories), 30% Triton X (Fisher Scientific), and 30% H2O2 (in PBS) to block and permeabilize the tissue, and quench endogenous peroxidase. Sections were again washed three times in PBS and incubated in the buffer solution with primary antibody (goat anti-rabbit IgG, 1:5000; Vector

Laboratories) overnight at room temperature. The next day, sections were washed 3 times with PBS and then incubated in the buffer solution with biotinylated secondary antibody (anti-rabbit IgG) at a concentration of 1:1000 for 2 hours at room temperature for both stains. After a final wash, immunostaining was identified by the streptavidin / horseradish peroxidase technique (Vectastain ABC kit; Cat. No. PK6100 Standard; Vector Laboratories), with diaminobenzidine (DAB) as the chromagen (Schwarz et al., 2012). Finally, sections were mounted on Superfrost++ Micro Slides (Fisher Scientific), dehydrated, coverslipped (Fisher Scientific) with Permount (Fisher Scientific), and stored at room temperature until analysis.

2.6.1 Image Acquisition and Analysis

Images of mPFC and amygdala were acquired with a Zeiss Axio Imager M2 microscope (10X objective) using the StereoInvestigator software (MBF Bioscience). Images were acquired for the mPFC (5 images/rat) and amygdala (8 images/rat). Densitometry of Iba-1 and D1r staining in these brain regions were analyzed in ImageJ (NIH; Schneider et al., 2012) using the digital image analysis (DIA) method (Donnelly et al., 2009; Nelson & Lenz, 2017). Since individual users are able to determine the threshold level for selecting labeled cells using this method, an interrater reliability analysis was performed to validate the DIA method of analysis within our lab (average Pearson's $r = 0.653$ across all brain regions). We found that when analyzing images obtained for dopamine receptor expression that an alternative MACRO was required to accurately assess densitometry within the amygdala. The same MACRO was used to

analyze all of the Iba1 stain as well as the images of mPFC from the dopamine stain. Images of Iba-1 and D1r staining from 6 and 5 sections per brain were used to calculate the percent area and integrated density in each treatment group and brain region.

2.7 Statistical Analysis

All data were analyzed using either the statistical software program SPSS (IBM) or the online statistical computation tool: VassarStats © 1998-2020. All data are presented as the mean +/- the SEM. For all statistical analyses, α -level = 0.05.

2.7.1 Statistical Analysis for Behavior

2x2x2 ANOVAs (between-subject's factors: sex (Males vs. Females, neonatal infection (Saline vs. *E. coli*), and LPS treatment (Saline vs. LPS)) were used to assess statistical differences in social play behavior data. When appropriate, Tukey's post-hoc comparisons were used to analyze individual group differences ($\alpha=0.05$). Independent samples t-tests (two-tailed) were used to assess significant sex differences in the expression of social play behaviors between male and female control rats.

2.7.2 Statistical Analysis for PCR

2x2x2 ANOVAs (between-subject's factors: sex (Males vs. Females, neonatal infection (Saline vs. *E. coli*), and P25 treatment (Saline vs. LPS)) were used to assess statistical differences in relative gene expression for each target gene *within* each time

point (4 hrs., 24 hrs., 72 hrs.). When appropriate, Tukey's post-hoc comparisons were used to analyze individual group differences ($\alpha=0.05$).

2.7.3 Statistical Analysis for IHC

2x2x2 ANOVAs (between-subject's factors: sex (Males vs. Females, neonatal infection (Saline vs. E. coli), and P25 treatment (Saline vs. LPS)) were used to assess statistical differences in dopamine receptor density and microglial density within the mPFC and amygdala. Five images of mPFC and six of amygdala for each brain were analyzed for integrated density and percent area. The images for the regions of interest were acquired from one hemisphere of a whole brain slice. The sum of the integrated density and percent area was calculated for analysis. When appropriate, Tukey's post-hoc comparisons were used to analyze individual group differences ($\alpha=0.05$). Integrated density is equal to the product of the mean gray value of all pixels in the selection and the area of the selected region of interest. Percent area is equal to the percent of pixels highlighted (to distinguish cell from background) within the selected region of interest. The highlighted pixels are the dopamine receptors or microglia selected by the experimenter within the region of interest.

Chapter 3

RESULTS

3.1 Behavioral Testing

Previous data from our lab evaluated pouncing, pinning, and chasing of the play dyad and indicated that neonatal infection significantly increased certain social play behavior on P28. These increases were seen in the instances of pouncing as well as boxing behaviors. In the current study, we first sought to determine whether the increase in play behavior that was measured within the play dyad was indicative of changes in play behaviors exhibited specifically by the test rat.

3.1.1 Social Play Behavior

Social play behavior was conducted on P28 for both male and female juvenile rats of four treatment groups: control, LPS alone, E-coli alone, and E-coli plus LPS. The N per group, per sex was 8 rats. Animals used for social play were unique from those used for tissue and brain collection experiments described below.

3.1.1.1 Neonatal Infection of E-coli Significantly Increased Social Play Behavior of the Test Rat Alone in Both Sexes of Juvenile Rats

Analysis of play behavior in the test animal alone revealed a significant main effect of neonatal infection in play behaviors. Test animals within the juvenile play pairs who were infected neonatally exhibited a significant increase in the instances of initiated play bouts compared to test animals within a pair that were not neonatally infected (**Figure 1**). These test-initiated play behaviors include pouncing, pinning, and chasing. Each of these behaviors were scored and analyzed individually as follows. Statistical analysis revealed the presence of a main effect of neonatal infection on pouncing ($F(1,56) = 7.761, p = 0.007, \eta^2 = 0.122$; **Figure 1A**). There was a main effect of neonatal infection such that both males and females infected with E. coli on P4 ($M=34.65$ $SEM=4.85$) showed significantly more pouncing behavior than both males and females given saline on P4, no matter the P25 treatment ($M=24.35$ $SEM=4.11$). There were also a significant main effect of neonatal infection on pinning behaviors ($F(1,55) = 10.196, p = 0.002, \eta^2 = 0.156$; **Figure 1C**). This main effect indicated that both males and females infected with E. coli on P4 exhibited more instances of pinning behavior than both males and females given saline on P4 ($M=11.56$ $SEM=2.93$), no matter the P25 treatment ($M=6.5$ $SEM=1.89$). We also measured a significant effect of neonatal infection on chasing behaviors exhibited by the test animal during play behavior ($F(1,53) = 6.833, p = 0.012, \eta^2 = 0.114$; **Figure 1E**). There was a main effect such that both sexes infected with E. coli as neonates

($M=16.53$ $SEM=3.86$) had significantly more instances of chasing no matter the P25 treatment ($M=17.12$ $SEM=3.8$). There were no significant main effects or interactions in the instances of test animals' participation in play behaviors; Being Pounced, Being Pinned, or Being Chased (**Figures 1B, 1D, 1F**).

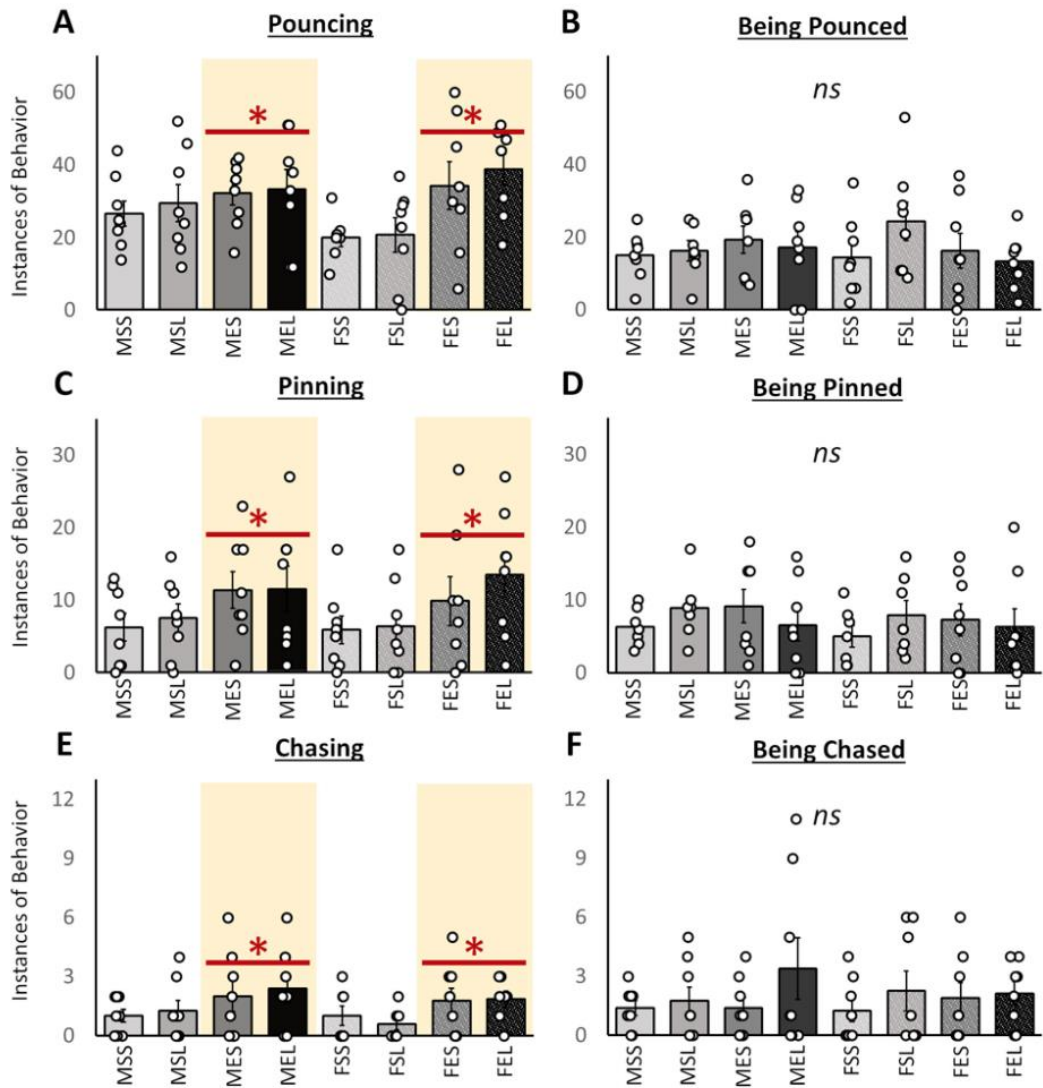


Figure 1: Impact of neonatal infection and/or juvenile LPS administration on social play behavior (instances of behavior) of individual test animals within the home cage across a fifteen-minute interval. **(A)** Neonatal infection significantly increased the total instances in which male and female test animals pounced on their stimulus partners. **(B)** Experimental manipulation had no significant impact on how often test animals were pounced upon. **(C)** Neonatal infection significantly increased the total instances in which male and female test animals pinned their stimulus partners. **(D)** Experimental manipulation had no significant impact on how often test animals succumbed to being pinned. **(E & F)** Experimental manipulations had no significant impact on how often test animals chased or were chased by their stimulus partners. $N = 8/\text{group}$. Error bars represent the $\pm\text{SEM}$. $*p < 0.05$ indicates a main effect of neonatal infection. Treatment groups are as follows: MSS = male, P4 saline, P25 saline; MSL = male, P4 saline, P25 LPS; MES = male, P4 *E. coli*, P25 saline; MEL = male, P4 *E. coli*, P25 LPS; FSS = female, P4 saline, P25 saline; FSL = female, P4 saline, P25 LPS; FES = female, P4 *E. coli*, P25 saline; FEL = female, P4 *E. coli*, P25 LPS.

3.2 Real-Time PCR

Brain tissue used was collected from treated male and female rats at three time points (4, 24, 72 hours) post LPS injection. Specifically, whole amygdala and mPFC was extracted from perfused brains within both hemispheres. The tissue was put through RNA extraction and cDNA generation, then qPCR was conducted for D1r and D2r. RPLP1 was used as the housekeeping gene for the quantification of relative gene expression. The N per treatment group, per sex was between 9-10 tissue samples.

3.2.1 D1R Relative Gene Expression

3.2.1.1 Amygdala D1R

Four hours post LPS injection, there was a sex x P4 treatment interaction for D1r relative gene expression ($F(1,68)=4.001, p=0.049$). However, post-hoc comparisons revealed that females with both P4 saline P25 saline ($M=14.91$,

SEM=4.24) and P4 saline P25 LPS (M=10.85, SEM=3.25) had more robust relative gene expression than males treated with saline on P4 and saline on P25 (M=10.04, SEM=3.27), and males treated with saline on P4 and LPS on P25 (M=7.93, SEM=3.12) ($p=0.030$; **Figure 2A**). On P26, D1r relative gene expression was significantly decreased in both sexes as a result of the neonatal infection ($F(1,67)=7.478$, $p=0.008$; **Figure 2B**). There was a main effect of P4 treatment such that e-coli treated males and females with a P25 injection of saline (M=9.98, SEM=3.10; M= 11.05, SEM=2.71) as well as males and females with a P4 injection of e-coli and P25 of LPS (M=8.51, SEM=2.42; M=5.99, SEM=1.51) expressed less D1 receptors than their saline P4 treated counterparts (MSS; M=10.48 SEM=2.82, MSL; M=11.80 SEM=3.08, FSS; M=14.84 SEM=5.05, FSL; M=16.22 SEM=3.61). 72 hours post LPS injection, on P28, there were no significant differences of sex, treatment, or timepoint found in the amygdala for expression of D1r (**Figure 2C**).

Amygdala: D1R Relative Gene Expression

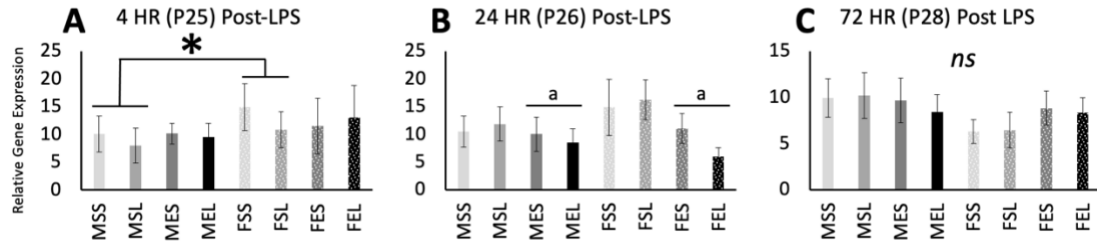


Figure 2: Impact of neonatal infection and/or juvenile LPS treatment on D1r relative gene expression in the amygdala of male and female juvenile rats at four, twenty-four, and seventy-two hours post-LPS treatment. **A)** At four hours post LPS injection a sex by P4 interaction had females who received saline on P4 had significantly more D1r relative gene expression than males who also received P4 saline, $p=0.049$. **B)** Twenty-four hours post P25 injection a main effect of P4 treatment e-coli animals had significantly less D1r relative gene expression than saline treated counterparts no matter the P25 treatment, $p=0.008$. **C)** At seventy-two hours post-LPS treatment, there were no significant differences in D1r relative gene expression. Error bars represent the \pm SEM. N = 9-10/group. “ns” indicates no significant differences. * indicates significant interaction of sex x P4 injection, $p < 0.05$; ^a indicates main effect of P4 treatment, $p < 0.05$. Treatment groups are as follows: MSS = male, P4 saline, P25 saline; MSL = male, P4 saline, P25 LPS; MES = male, P4 *E. coli*, P25 saline; MEL = male, P4 *E. coli*, P25 LPS; FSS = female, P4 saline, P25 saline; FSL = female, P4 saline, P25 LPS; FES = female, P4 *E. coli*, P25 saline; FEL = female, P4 *E. coli*, P25 LPS.

3.2.1.2 mPFC D1R

At P25, 4 hours post LPS injection, there was a significant interaction of sex x P4 treatment ($F(1,68)=4.267$, $p=0.043$; **Figure 3A**). Post-hoc comparisons indicated that neonatally infected males administered saline on P25 ($M=5.41$ $SEM=1.22$) or LPS on P25 ($M=5.41$ $SEM=1.28$) had significantly less D1r relative gene expression ($p=0.027$) than male rats given saline on P4 and saline on P25 ($M=10.05$ $SEM=2.73$) as well as males given saline on P4 and LPS on P25 ($M=6.34$ $SEM=0.93$); however, there were no significant differences in D1r relative gene expression between saline (P4) and saline (P25) treated females ($M=6.9$ $SEM=1.5$), saline and LPS females ($M=6.19$ $SEM=0.87$), e-coli and saline females ($M=5.65$ $SEM=0.81$), and e-coli and LPS treated females ($M=6.35$ $SEM=1.19$). There were no significant main effects or interactions of sex, treatment, or time point post LPS administration present in the expression of D1r in the medial prefrontal cortex at P26 and P28 (**Figure 3B&C**).

mPFC: D1R Relative Gene Expression

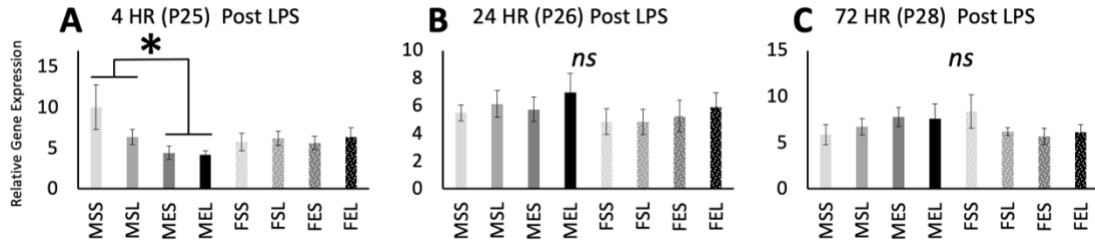


Figure 3 Impact of neonatal infection and/or juvenile LPS treatment on D1r relative gene expression in the mPFC of male and female juvenile rats at four, twenty-four, and seventy-two hours post-LPS treatment. **A)** At four hours post P25 injection a sex by P4 interaction had males who were neonatally infected had significantly less D1r relative gene expression than males who received a P4 saline injection no matter the P25 treatment, $p=0.027$. **B)** At twenty-four hours post-LPS treatment, there were no significant differences in D1r relative gene expression. **C)** At seventy-two hours post-LPS treatment, there were no significant differences in D1r relative gene expression. Error bars represent the \pm SEM. $N = 9-10$ /group. “ns” indicates no significant differences. * indicates significant interaction of sex x P4 injection, $p < 0.05$. Treatment groups are as follows: MSS = male, P4 saline, P25 saline; MSL = male, P4 saline, P25 LPS; MES = male, P4 *E. coli*, P25 saline; MEL = male, P4 *E. coli*, P25 LPS; FSS = female, P4 saline, P25 saline; FSL = female, P4 saline, P25 LPS; FES = female, P4 *E. coli*, P25 saline; FEL = female, P4 *E. coli*, P25 LPS.

3.2.2 D2R Relative Gene Expression

3.2.2.1 Amygdala D2R

At 4 and 24 hours post LPS injection there were no significant differences in expression of D2r within the amygdala for sex, treatment, or timepoint (**Figure 4A&B**). 72 hours post LPS injection, on P28, D2r relative gene expression was significantly increased for male rats ($F(1,69)=5.457, p=0.022$; **Figure 4C**). A main effect of sex such that males given P4 of saline and P25 saline (M=6.52 SEM=1.43), P4 saline and P25 LPS (M=6.4 SEM=1.81), P4 e-coli and P25 saline (M=8.68 SEM=2.44), and P4 e-coli with P25 LPS (M=6.7 SEM=2.21) showed robust relative gene expression compared to female groups of a P4 injection with saline and P25 with saline (M=3.96 SEM=0.98), P4 saline and P25 LPS (M=4.55 SEM=1.67), P4 e-coli and P25 saline (M=6.27 SEM=1.78), and P4 e-coli with P25 LPS (M=5.14 SEM=1.24).

Amygdala: D2R Relative Gene Expression

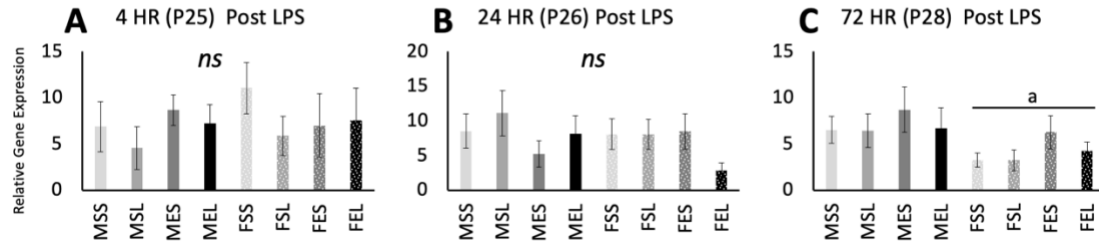


Figure 4 Impact of neonatal infection and/or juvenile LPS treatment on D2r relative gene expression in the amygdala of male and female juvenile rats at four, twenty-four, and seventy-two hours post-LPS treatment. **A)** At four hours post-LPS treatment, there were no significant differences in D2r relative gene expression. **B)** At twenty-four hours post-LPS treatment, there were no significant differences in D2r relative gene expression. **C)** Seventy-two hours post P25, D2r relative gene expression was most robust for males of any treatment group compared to females, $p=0.022$. Error bars represent the \pm SEM. $N = 9-10/\text{group}$. “ns” indicates no significant differences. ^a indicates main effect of sex, $p < 0.05$. Treatment groups are as follows: MSS = male, P4 saline, P25 saline; MSL = male, P4 saline, P25 LPS; MES = male, P4 *E. coli*, P25 saline; MEL = male, P4 *E. coli*, P25 LPS; FSS = female, P4 saline, P25 saline; FSL = female, P4 saline, P25 LPS; FES = female, P4 *E. coli*, P25 saline; FEL = female, P4 *E. coli*, P25 LPS.

3.2.2.2 mPFC D2R

Relative gene expression of D2r in the mPFC post LPS injection showed no significant main effects or interactions of sex, treatment, or timepoint (Figure 5A&B&C).

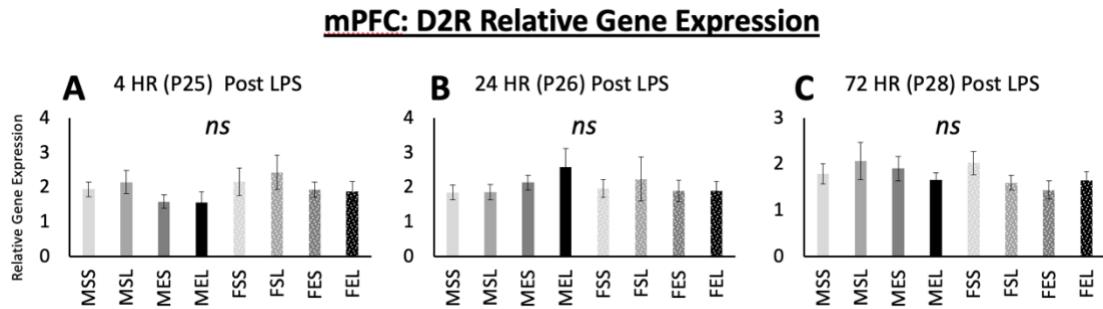


Figure 5 Impact of neonatal infection and/or juvenile LPS treatment on D2r relative gene expression in the mPFC of male and female juvenile rats at four, twenty-four, and seventy-two hours post-LPS treatment. **A)** At four hours post-LPS treatment, there were no significant differences in D2r relative gene expression. **B)** At twenty-four hours post-LPS treatment, there were no significant differences in D2r relative gene expression. **C)** At seventy-two hours post-LPS treatment, there were no significant differences in D2r relative gene expression. Error bars represent the \pm SEM. N = 9-10/group. “ns” indicates no significant differences. Treatment groups are as follows: MSS = male, P4 saline, P25 saline; MSL = male, P4 saline, P25 LPS; MES = male, P4 *E. coli*, P25 saline; MEL = male, P4 *E. coli*, P25 LPS; FSS = female, P4 saline, P25 saline; FSL = female, P4 saline, P25 LPS; FES = female, P4 *E. coli*, P25 saline; FEL = female, P4 *E. coli*, P25 LPS.

3.3 Immunohistochemistry

Whole brains were collected at P28, preserved, and sliced according to the methods described above. Two separate stains were run on selected whole brain slices and were mounted then cover slipped following a standard protocol. Five images of the mPFC and six of the amygdala were analyzed and we used the sum of each of the measures (both integrated density and the percent area) for statistical analysis. The N per treatment group, per sex was between 2-7 samples.

3.3.1 D1R Expression

3.3.1.1 Amygdala D1R

Analysis of D1r density within the amygdala of P28 males and females revealed a main effect of P25 treatment. Specifically, LPS immune activation on P25 significantly decreased D1r density in the amygdala three days later ($F(1,31)=4.907$, $p=0.034$) (**Figure 6B**). The main effect was such that males who received P4 saline and P25 saline ($M=2492.38$ $SEM=246.89$), males of P4 e-coli and P25 saline ($M=3339.58$ $SEM=1315.78$), females of P4 saline and P25 saline ($M=3261.27$ $SEM=571.79$), and females of P4 e-coli and P25 saline ($M=2904.71$ $SEM=386.99$) showed an *increased density* of dopamine receptors compared to males who received P4 saline and P25 LPS ($M=2550.45$ $SEM=170.88$), males of P4 e-coli and P25 LPS ($M=2370.92$ $SEM=294.83$), females who received P4 saline and P25 LPS

(M=2526.89 SEM=194.03), and females of P4 e-coli and P25 LPS (M=2198.88 SEM=281.52). There were no significant differences found for the percent area of dopamine receptors for sex or treatment (**Figure 6A**).

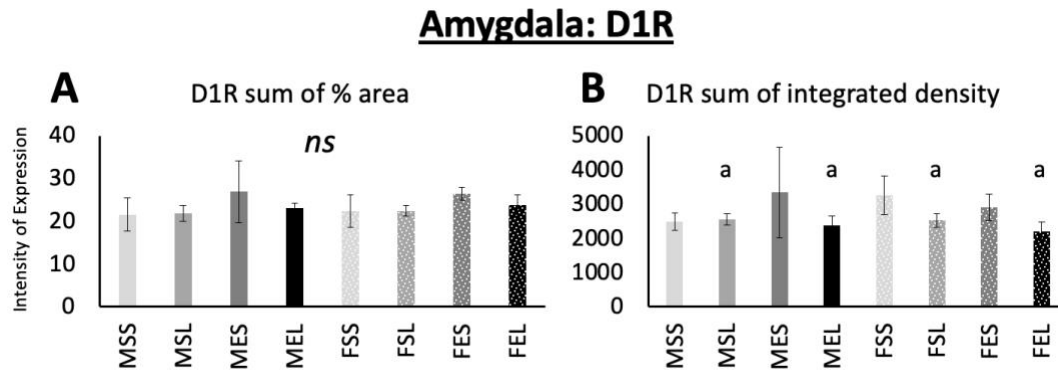


Figure 6 Impact of neonatal infection and/or juvenile LPS treatment on expression of dopamine receptor (D1r) density in the amygdala of male and female juvenile rats at P28. **A**) There were no significant differences in the percent area of D1r staining. **B**) Both males and females who received an injection of LPS on P25 no matter the P4 treatment showed a decrease in integrated density of dopamine receptors, $p=0.034$. Error bars represent the \pm SEM. $N = 2-7/\text{group}$. “ns” indicates no significant differences. ^a indicates main effect of P25 treatment, $p < 0.05$. Treatment groups are as follows: MSS = male, P4 saline, P25 saline; MSL = male, P4 saline, P25 LPS; MES = male, P4 *E. coli*, P25 saline; MEL = male, P4 *E. coli*, P25 LPS; FSS = female, P4 saline, P25 saline; FSL = female, P4 saline, P25 LPS; FES = female, P4 *E. coli*, P25 saline; FEL = female, P4 *E. coli*, P25 LPS.

3.3.1.2 mPFC D1R

Statistical analysis of D1 receptor density within the mPFC yielded a main effect of P4 treatment for both sum of percent area as well as sum of integrated density. Those rats that were neonatally infected ($F(1,35)=8.177$), $p=0.007$) had a significantly greater percent of dopamine receptors per area than their saline treated counterparts (**Figure 7A**). The effect was such that males that received e-coli on P4

and saline on P25 (M=35.24 SEM=3.85), males that received e-coli on P4 and LPS on P25 (M=32.76 SEM=2.09), as well as females that received e-coli on P4 and saline on P25 (M=35.85 SEM=2.34), and females that received e-coli on P4 and LPS on P25 (M=31.63 SEM=1.73) had a *greater percent area* of dopamine receptors than males that received saline on P4 and saline on P25 (M=30.81 SEM=2.14), males that received saline on P4 and LPS on P25 (M=29.63 SEM=1.52), as well as females that received saline on P4 and saline on P25 (M=29.81 SEM=0.83), and females that received saline on P4 and LPS on P25 (M=31.63 SEM=1.73). Additionally, a similar main effect of P4 treatment occurred for the sum of integrated density of D1 dopamine receptors. Animals who received a neonatal infection ($F(1,35)=7.258, p=0.011$) had a higher integrated density of receptors than those that did not receive a neonatal infection (**Figure 7B**). The main effect was such that males given e-coli on P4 and saline on P25 (M=5791.48 SEM=631.27), males given e-coli on P4 and LPS on P25 (M=5111.92 SEM=570.43), as well as females given e-coli on P4 and saline on P25 (M=5510.73 SEM=322.57), and females given e-coli on P4 and LPS on P25 (M=4665.26 SEM=224.60) had *significantly higher integrated density* of dopamine receptors than males given saline on P4 and saline on P25 (M=4552.38 SEM=313.22), males given saline on P4 and LPS on P25 (M=4507.75 SEM=221.83), females given saline on P4 and saline on P25 (M=4495.03 SEM=333.40), as well as females given saline on P4 and LPS on P25 (M=4878.36 SEM=141.07).

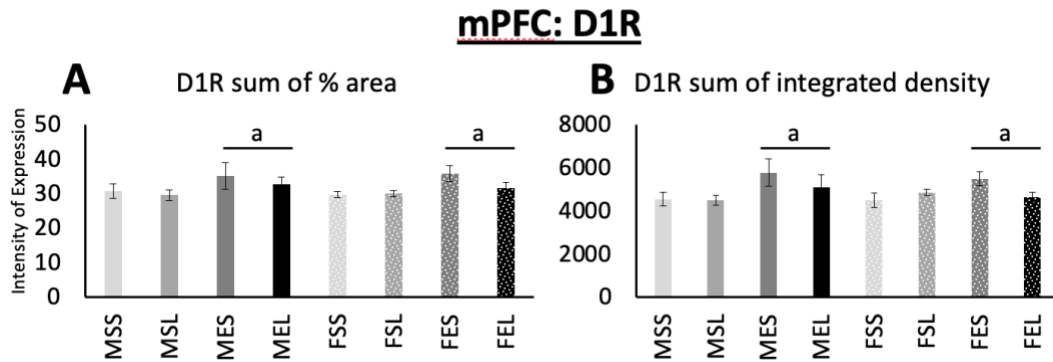


Figure 7 Impact of neonatal infection and/or juvenile LPS treatment on expression of dopamine receptor (D1r) density in the mPFC of male and female juvenile rats at P28. **A)** Those that were neonatally infected, no matter the P25 treatment, had a significant increase in present area of dopamine receptors than those that received P4 saline, $p=0.007$. **B)** Both males and females who received a neonatal infection, no matter the P25 treatment, had a higher integrated density of dopamine receptors than their saline treated counterparts, $p=0.011$. Error bars represent the \pm SEM. $N = 2-7/\text{group}$. ^a indicates main effect of P4 treatment, $p < 0.05$. Treatment groups are as follows: MSS = male, P4 saline, P25 saline; MSL = male, P4 saline, P25 LPS; MES = male, P4 *E. coli*, P25 saline; MEL = male, P4 *E. coli*, P25 LPS; FSS = female, P4 saline, P25 saline; FSL = female, P4 saline, P25 LPS; FES = female, P4 *E. coli*, P25 saline; FEL = female, P4 *E. coli*, P25 LPS.

3.3.2 Iba1 Expression

3.3.2.1 Amygdala Iba1

Statistical analysis of the percent area and integrated density of microglia in the amygdala indicated there were no significant interactions or main effects of sex or treatment (**Figure 8A&B**).

Amygdala: Iba1

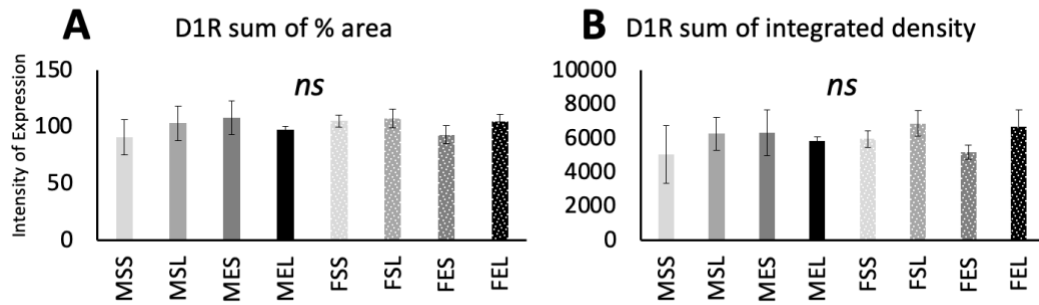


Figure 8 Impact of neonatal infection and/or juvenile LPS treatment on expression of microglial density in the amygdala of male and female juvenile rats at P28. **A)** There were no significant differences in the percent area of Iba1 staining. **B)** There were no significant differences in the integrated density of Iba1 staining. Error bars represent the \pm SEM. N = 2-7/group. “ns” indicates no significant differences. Treatment groups are as follows: MSS = male, P4 saline, P25 saline; MSL = male, P4 saline, P25 LPS; MES = male, P4 *E. coli*, P25 saline; MEL = male, P4 *E. coli*, P25 LPS; FSS = female, P4 saline, P25 saline; FSL = female, P4 saline, P25 LPS; FES = female, P4 *E. coli*, P25 saline; FEL = female, P4 *E. coli*, P25 LPS.

3.3.2.2 mPFC Iba1

Statistical analysis of the percent area and integrated density of microglia in the mPFC indicated there were no significant interactions or main effects of sex or treatment (**Figure 9A&B**).

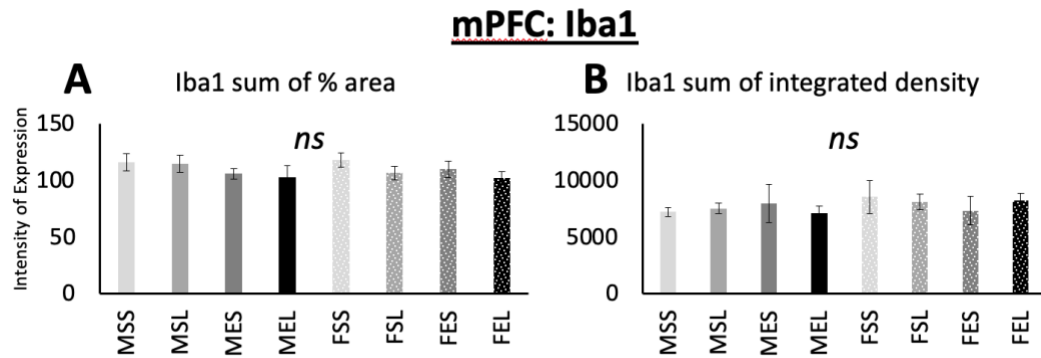


Figure 9 Impact of neonatal infection and/or juvenile LPS treatment on expression of microglial density in the mPFC of male and female juvenile rats at P28. **A)** There were no significant differences in the percent area of Iba1 staining. **B)** There were no significant differences in the integrated density of Iba1 staining. Error bars represent the \pm SEM. N = 2-7/group. “ns” indicates no significant differences. Treatment groups are as follows: MSS = male, P4 saline, P25 saline; MSL = male, P4 saline, P25 LPS; MES = male, P4 *E. coli*, P25 saline; MEL = male, P4 *E. coli*, P25 LPS; FSS = female, P4 saline, P25 saline; FSL = female, P4 saline, P25 LPS; FES = female, P4 *E. coli*, P25 saline; FEL = female, P4 *E. coli*, P25 LPS.

Chapter 4

DISCUSSION

4.1 Brief Summary of Findings

Neonatal infection significantly increased instances of play behaviors observed for the test rat, independent of a subsequent immune challenge later in life. This data supports and extends upon our lab's previous findings that neonatal infection increased the instances of play behaviors for the play dyad. Additionally, we observed robust relative gene expression of D1R and D2R that were modulated by neonatal infection in a brain-region and sex-specific manner. Finally results from microglia and dopamine receptor protein analysis in the amygdala and medial prefrontal cortex revealed that a neonatal infection leads to an increase in D1R density in the mPFC, while LPS immune activation on P25 significantly decreases D1R density in the amygdala. We did not observe changes in microglial density in these brain regions following neonatal and/or juvenile immune activation.

4.2 Summary of Behavioral Results

In previous experiments done by the Schwarz lab examining play behavior for both animals in the play pair, neonatal infection increased social interactions between the test and stimulus rat (Turano et, al.). The play pair consisted of a test rat infected

with e-coli on P4 who exhibited an increase in rough-and-tumble behaviors compared to their saline counterparts, independent of P25 treatment (Turano et, al.). This work directly correlates to what we found when examining the same behavioral data, but specifically examining the play behaviors in the point of view of the test animal, when did they initiate or participate in a play bout. Neonatal infection increased the instances of test-initiated behaviors; pouncing, pinning, and chasing. There were no significant differences found in test's participation in play bouts; being pounced, being pinned, and being chased.

4.2.1 Neonatal Infection Significantly Increased Social Play Behaviors in Test Juvenile Male and Female Rats

Social dysfunction as it relates to human neuropsychiatric and neurodevelopmental disorders is often stereotyped as *decreases* in social interactions. This narrow view of social dysfunction fails to encompass the bigger picture; which is that any divergence from “normal” may be seen as a deficit in social behavior. Thus social behavior deficits can range from total social withdrawal to inappropriate social approach (Porcelli et al., 2019). Thus, observing dynamic changes in social play behavior following neonatal and/or juvenile immune activation is an effective model for examining complex changes in social behavior as they may relate to human conditions. Nevertheless, it is not often that increases in social play behavior are reported in the animal literature in reference to neurodevelopmental disorders, particularly following early-life immune insults.

Neonatal infection significantly increased social play behaviors in test rats relative to controls. Specifically, play pairs that consisted of a test rat exposed to *E-coli* on P4 and a non-exposed partner showed increased instances of pouncing, pinning, and chasing behavior. These behaviors make it clear that when a test rat has been exposed to a neonatal infection they are more likely to initiate a play bout with their play partner controls. Interestingly, unlike the previous data from the Schwarz lab mentioned earlier examining the play pair, this current project did not find any significant sex differences in play behaviors. Behaviors like pouncing and pinning are classified as “rough-and-tumble” which in the previous study showed a sex difference, with males exhibiting more rough-and-tumble than females. However, within that same experiment it is important to note that females exposed to *E-coli* on P4 did not differ significantly from male rats in rough-and-tumble behaviors. The same can be said for the current project. Both sexes showed a significant increase in pouncing and pinning behaviors no matter the P25 treatment. This outcome potentially indicates that a neonatal infection increases rough-and-tumble behaviors for female rats to align with what we see in males. It is possible that exposure to *E-coli* in early life leads to the masculinization of the amygdala in females in a similar manner that testosterone does to the male amygdala through microglial activation (Argue et al., 2017). It could also be that our two-hit model creates increased sensitivity upon social isolation so that when reunited they are wanting to play more with their partner although our data doesn’t directly indicate this. Furthermore, it is possible that the increase in play behaviors can be linked to the test animal being impulsive. Impulsivity is heavily

linked in the literature to many neurodevelopmental disorders like ADHD. Although our experiment wasn't looking directly at impulsivity it is possible that our two-hit model is leading to an increase in impulsivity which is expressed by test's initiation of a play bout.

When talking about stereotypical play behaviors it's important to make the distinction between initiation and participation. Pouncing is typically described as a behavior that initiates a play encounter, being pounced on the other hand contributes to the continuation of a play bout. The same can be said for chasing, and being chased. Our results show that neonatal infection significantly increased the number of play behaviors initiated by both sexes but did not impact test's willingness to continue a play bout initiated by their stimulus partner.

As I mentioned previously, we saw a main effect of P4 treatment; however, we did not see an effect of the two-hit model of neuroinflammation on social play behavior. The current data does not support a direct connection between the two-hit immune activation and the observed behavioral outcomes. What we can see from this data is that early life immune activation, which occurs on P4, results in significant behavioral changes weeks after. The data would indicate that the second immune challenge reactivating the microglia doesn't alter behavior as we hypothesized. The fact that their first immune challenge occurs on P4 which is well within the bounds of the sensitive period of development for rats could explain why only those neonatally infected are showing alterations in play behavior. We have seen through various models of early-life immune activation that a disruption of microglia during the

sensitive period of neurodevelopment can lead to later-life vulnerabilities (Bilbo and Schwarz, 2009).

4.3 Summary of PCR Results

Relative gene expression of dopamine receptor 1 (D1R) was robust in both regions of interest (amygdala and mPFC) four hours post LPS injection, but by seventy-two hours gene expression was no longer significant. At four hours post LPS injection, D1r relative gene expression in the amygdala was more robust for female rats given a P4 injection of saline than males given saline, no matter the P25 treatment. Perhaps this indicates that the second immune challenge has little effect on expression of dopamine within the amygdala which coincides with our behavioral results. This also could mean that within the amygdala that P4 e-coli eliminates any sex differences of D1r four hours after an immune challenge. We can see that the experience of a second immune challenge later in life does not affect the expression of social play behaviors which is supported by fact that an LPS injection or a saline injection does not significantly alter dopamine receptor expression in the amygdala at 4 hours. Interestingly, at the same timepoint (4 hours post LPS) within the mPFC we see that male rats exposed to saline on P4 had more robust relative gene expression of D1r than males exposed to E. coli on P4. Again, this effect occurred no matter the P25 treatment. These results taken together show a difference in D1r responses depending on the brain region. Based on the play behavior data we would expect that animals who received e-coli that showed increased instances of play would also have robust

relative D1r expression. We would expect this because dopamine and the amygdala are greatly implicated with the reward system and play is innately rewarding to juvenile rats.

At the twenty-four-hour mark post LPS injection there was robust D1r expression within the amygdala but not within the mPFC. D1r relative gene expression was significantly decreased in the amygdala for both sexes given a P4 injection of e-coli, compared to their P4 saline counterparts. This result is in line with what we see at four hours post LPS injection since again there is no sex difference in the e-coli groups in the amygdala. By seventy-two hours (P28) post LPS injection we see that expression of D1r has normalized in the males' groups.

Relative gene expression of another dopamine receptor D2r was also significantly altered within the amygdala at seventy-two hours (P28) post LPS injection. D2r relative gene expression of the amygdala at P28 was most robust in males compared to females no matter the treatment group. There was no significant expression of D2r within the mPFC at any of the timepoints.

Based on our results of dopamine receptor relative gene expression we can see that at four and twenty-four hours post LPS injection we have robust expression of D1r in the amygdala, at seventy-two hours post LPS injection in the amygdala there is robust expression of D2r. It appears that at P25 and P26 there is a dominance of D1r expression while at P28 it is D2r that is most robust within the amygdala. Typically, adolescent rats show expression of Dr receptor's during locomotion activity, and this then transitions to be dominated by D1 receptors in adulthood (McCormick, 2016). It

could be that through immune activation during the sensitive period of development there is an inappropriate shift in dopamine receptor expression. This would begin to explain why we see the opposite, a shift from D1r to D2r within the amygdala.

4.4 Summary of IHC Results

This experiment seeks to determine how our “two-hit model of neuroinflammation” affects dopamine receptor expression across two brain regions that are particularly important for expression of social play behavior. The D1 dopamine receptor plays a crucial role in motor control and reward pathways, and in this way, is important for the expression of social play behaviors (Hasbi et al, 2011). That being said we found that animals who received a neonatal infection showed an increase in percent area of dopamine receptor density within the mPFC. This effect occurred no matter the P25 treatment and in both sexes. We also saw that rats neonatally infected had significantly higher integrated density of dopamine receptors in the mPFC. These results indicate that an early life immune activation leads to an increase in dopamine receptors specifically within the mPFC. This could mean that these animals find play more rewarding, which aligns with what came of our behavioral paradigm. Interestingly, we see a decrease in D1r relative gene expression at P25 from a neonatal infection in the mPFC but an increase in dopamine receptor density at P28. It could be that there is a level of overcompensation occurring due to the initial reduction in D1r expression. Continuing with the results of dopamine receptors we found that those that received saline on P25 had an increased density

within the amygdala. Meaning that rats who received an injection of LPS on P25 had less density of dopamine receptors. As mentioned earlier we do see an effect of LPS in PCR tissue at P28 however, we don't see such effect in the sliced tissue at P28. It could be that the second hit of LPS affects a different protein or gene than the one used in this analysis. It may be that LPS affects neural processes that we didn't measure, such as BDNF or microglia cytokines. It is also possible that we are seeing increased density of D1r because of dopamine's link to stress and the experience of injections at P4 and P25 are leading to the activation of more D1r's in the mPFC.

The results of our investigation into the density of microglia within the amygdala and mPFC yielded no significant results. We expected that with the increase in dopamine receptor density by a neonatal infection that a decrease in microglia density would also occur. Previous research found that by blocking microglial pruning of dopamine synapses during adolescence the researchers were able to show there is a robust increase in expression of D1r in the nucleus accumbens. Accompanying that increase in D1r was an increase in social play behaviors in specifically male rats (Kopeck et al, 2017). It could be that the early life immune challenge is in fact blocking the pruning of dopamine receptors without leading to the phagocytosis of said microglial cells.

4.5 Conclusions

Neonatal infection and subsequent juvenile immune activation had different impacts on social play behavior, dopamine receptor relative gene expression, and

dopamine receptor density. Neonatal infection significantly increased the instances of initiated play behaviors for both males and females. Additionally, neonatal infection led to an increase in D1r density within the mPFC for both sexes on P28, the same day play behavior was tested in a separate cohort of animals. However, we saw that neonatal infection led to a decrease in D1r relative gene expression in the amygdala on P25 and P26. Relative gene expression of D2r within the amygdala indicated that males of all treatment groups had an increase in expression relative to females. Finally, dopamine receptor density was at its greatest in the amygdala for both sexes that received saline on P25. These findings are summarized in **Table 2**. Our molecular experiments included both mRNA and protein analysis of D1r in the amygdala and mPFC. This was done in order to examine the gene expression as well as density of dopamine receptors to try and understand how our model affects the different ways dopamine is present in the brain. The results from these molecular studies don't align and it might be that the mRNA and protein analysis are looking at different aspects of dopamine receptors.

Table 2: Summary of collective results from the current study

	Play on P28	D1r expression Amygdala	D1r expression mPFC	D2r expression Amygdala	D2r expression mPFC	D1r protein density Amygdala	D1r protein density mPFC	Microglia density Amygdala	Microglia density mPFC
Effect of Neonatal Infection	↑	4 hrs: ↓ 24 hrs: ↓	4 hrs: ↓	—	—	—	↑	—	—
Effect of Juvenile Immune Activation	—	—	—	—	—	↓	—	—	—

4.6 Limitations and Future Directions

One limitation to our molecular analysis was that the tissue we used was previously collected in a previous experiment, so we did not have control over which regions of interest could be analyzed for the current experiment. As there are many regions of the brain implicated with dopamine expression collecting alternative tissue samples would allow for a more in-depth review of the effect our two-hit model has on play behavior. Additionally, the whole brain sliced we used were collected in the years prior, so the tissue was beginning to degrade. This led to our IHC analysis consisting of a small N as well as a limited number of viable slices per brain. The brains used for slicing were taken at P28, seventy-two hours post LPS injection, whole brains could be taken at similar timepoints to that of the PCR tissue to examine the immediate effects of the juvenile immune challenge. With these experiments we were able to address some limitations of the previous data collection of play behavior of the pairs. Future directions for examining play behavior could include measuring the play

behaviors of pairs comprised of the same treatment group, or of the same sex. Any of these future pursuits would provide substantial information about the impact of neonatal infection, with or without a subsequent immune activation, on the expression of juvenile social behaviors.

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Appendix

APPROVAL FOR THE USE OF ANIMAL SUBJECTS

University of Delaware
Institutional Animal Care and Use Committee
Annual Review

Title of Protocol: Sex Differences in Vulnerability to Neonatal Infection	
AUP Number: 1239-2021-2	← (4 digits only)
Principal Investigator: Jaclyn M. Schwarz	
Common Name: Rat	
Genus Species: Rattus Norvegicus	
Pain Category: <i>(please mark one)</i>	
USDA PAIN CATEGORY: <i>(Note change of categories from previous form)</i>	
Category	Description
<input type="checkbox"/> B	Breeding or holding where NO research is conducted
<input type="checkbox"/> C	Procedure involving momentary or no pain or distress
<input type="checkbox"/> D	Procedure where pain or distress is alleviated by appropriate means (analgesics tranquilizers, euthanasia etc.)
<input checked="" type="checkbox"/> E	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

Official Use Only
IACUC Approval Signature: <u>Jan T. Talker, DVM</u>
Date of Approval: <u>1.1.2021</u>

013