EFFECTS OF PLACENTAL *LISTERIA MONOCYTOGENES* INFECTION ON FETAL NEURODEVELOPMENT

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ABSTRACT

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Maternal infection can lead to adverse pregnancy outcomes. Numerous epidemiological studies have demonstrated an association between prenatal infection and neuropsychiatric disorders, including autism spectrum disorder (ASD). Different prenatal infections are associated with distinct neurological pathologies, necessitating studies of the diversity of prenatal pathogens and their consequences. *Listeria monocytogenes* (*Lm*) is a foodborne pathogen that causes listeriosis, which typically affects immunocompromised individuals, including pregnant mothers. Prenatal infection with *Lm* can cause detrimental pregnancy outcomes, such as miscarriages, stillbirths, preterm labor, and death in newborns. However, neurological outcomes of maternal listeriosis have not been characterized. Here, I sought to investigate whether placental infection with *Lm* is associated with altered neurodevelopment by using a bioluminescence strain of *Lm* and a murine model of pregnancy-associated listeriosis. I show that placental infection affects neurodevelopment during pregnancy and behavior in the offspring.

To investigate how placental infection with *Lm* dysregulates fetal brain development, I performed RNA-seq on fetal brains to quantify the enrichment of genes that were associated with the infection during gestation. The findings of RNA-seq analysis illustrated that placental infection with *Lm* altered fetal brain transcriptome and showed sexually dichotomous gene expression profiles. I further assessed the effects of different traits, including *Lm* exposure, the intensity of placental infection, and sex on the fetal transcriptome using systems biology. The genes were grouped into co-expression modules. Notably, maternal infection and its intensity measured by

bioluminescence imaging signal were significantly associated with specific modules, suggesting these traits are the main factors driving these transcriptional changes. Lastly, I showed that placental *Listeria* infection enriched ASD-associated genes. These results demonstrate that maternal listeriosis dysregulates fetal brain transcriptome during gestation.

Neurodevelopment is a complex process influenced by various environmental factors during pregnancy. To examine whether prenatal infection with *Lm* affects cortical lamination and neural activity, I performed hematoxylin and eosin staining and immunohistochemistry. Gross anatomy of the brain structure analysis showed that placental infection with *Lm* affected cortical lamination in a localized manner. Furthermore, increased neural activity was observed in *Lm*-exposed male offspring. These results illustrate that placental infection with *Lm* induces morphological changes in brain tissue during neurodevelopment.

Behavioral symptoms of neuropsychiatric disorders are an important component of the diagnosis. Animal behavioral assays and tools have been developed to examine animal behavior such as social interactions, anxiety, and repetitive behaviors. I examined behavior tests that resembled ASD to determine if mouse offspring born following placental infection displayed abnormal behavior. *Lm*-exposed offspring exhibited altered behaviors and showed sex-dependent behavioral changes. Overall, my work highlights the impact of maternal listeriosis on brain development during pregnancy and its effects on offspring's behavior and contributes to the understanding of the spectrum of fetal neurodevelopment.

This dissertation is dedicated to my family, partner, and friends.

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KEY TO ABBREVIATIONS

ASD	Autism spectrum disorder
Lm	*Listeria monocytogenes*
MIA	Maternal immune activation
CDC	The Centers for Disease Control and Prevention
SZ	Schizophrenia
TNF	Tumor necrosis factor
IL	Interleukin
IFN	Interferon
LPS	Lipopolysaccharide
Poly(I:C)	Polyinosinic-polycytidylic acid
DGE	Differential gene expression
TLR	Toll like receptor
T_H17	T helper 17
GI	Gastrointestinal
GBS	Group B *Streptococcus*
IL-1Ra	IL-1 receptor antagonist
IAV	Influenza A virus
IL-1Ra	IL-1 receptor antagonist
InlA	Internalin A
InlB	Internalin B
ActA	Actin assembly inducing protein
InlP	Internalin P
E	Embryonic day
CFU	Colony forming unit
BLI	Bioluminescence Imaging
PBS	Phosphate-buffered saline
IVIS	*In vivo* bioluminescence imaging system

H&E	Hematoxylin and eosin
ROIs	Regions of interest
NGS	Next generation sequencing
IV	Intravenous injection
DEGs	Differentially expressed genes
FDR	False discovery rate
HIF-1	Hypoxia inducible factor
GO	Gene Ontology
ER	Endoplasmic reticulum
WGCNA	Weighted Gene Co-Expression Network Analysis
BP	Biological process
SFARI	Simons Foundation Autism Research Initiative
TOM	Topological overlapping matrix
scRNA-seq	Single-cell RNA-sequencing

CHAPTER 1 – Literature review: Environmental effects on autism spectrum disorder and prenatal infection with *Listeria monocytogenes*

INTRODUCTION

Autism spectrum disorder (ASD) is a neuropsychiatric disorder that affects how people behave. There are three hallmark symptoms of ASD: impaired communication, fewer social interactions, and repetitive behavior[1]. Much progress has been made in understanding the etiology of ASD; however, the underlying mechanisms leading to this neuropsychiatric disorder remain unclear due to its complex characteristics. Although it is known to be regarded as a genetic disorder, recent studies suggest that prenatal infection is associated with an increased risk of developing ASD[2–5]. This phenomenon has been observed in maternal immune activation animal (MIA) models. Injection of an immunogen triggers pro-inflammatory cytokines during pregnancy, and MIA-induced offspring show behaviors that resemble ASD[6–9].

Listeria monocytogenes (*Lm*) is a foodborne pathogen that mainly affects immunocompromised individuals, including pregnant women[10]. Ingestion of *Lm*-contaminated food by pregnant women can lead to adverse pregnancy outcomes. *Lm* can infect the placenta by utilizing its intracellular life cycle, resulting in an array of detrimental consequences, such as stillbirth and abortion[11]. For decades, *Lm* has been used as a model of placental bacterial infection because of its well characterized physiology. Although prenatal infection with *Lm* causes many severe prenatal and newborn pathologies, the neurological outcome of offspring has not been studied. In this chapter, I will give an overview of current understanding of ASD and fetal mouse brain development. I will also discuss other animal models investigating the association between prenatal infection and ASD, including the MIA model. Lastly, an overview of prenatal infection with *Lm*, such as infection of the placenta and adverse pregnancy outcomes will be discussed.

Prevalence, characteristics, and genetic risks of autism spectrum disorder

The diagnosis of ASD is challenging since there is no known biomarker for the disorder. The most common way to diagnose ASD is to observe the child's behavior over time, and the disorder can be reliably detected as early as age 2[12]. The three main core symptoms of ASD include difficulty in social interaction, communication impairments, and restricted or repetitive behaviors[1]. Additionally, anxiety, intellectual disability, and hyper- or hypo-reactivity to sensory stimuli are seen in patients with ASD[1]. According to the Centers for Disease Control and Prevention (CDC) 2018 report, the prevalence of ASD is 1 in 44 children in the United States[13]. According to this data, biological males are more susceptible to developing ASD than biological females. One in 27 biological males compared to one in 116 biological females are diagnosed with the disorder[14]. While several hormonal factors and sex-specific genetics may contribute to this sexually dichotomous phenomenon, its causes are poorly understood.

According to genome wide association studies, hundreds of genes are dysregulated in neuropsychiatric disorders, including ASD; however, the phenotypic effects of these genes are rather small. ASD was once thought to be one of the most known heritable (up to 80%) neuropsychiatric disorders[15,16]. However, recent epidemiological twin studies estimated genetic heritability of ASD to be about 37%[17]. Furthermore, only about 25% of ASD cases are identified by genetic risk factors, such as copy number variants[18]. Over the past decade, numerous studies have shown that environmental exposures during pregnancy, such as zinc deficiency[19,20], air pollution[21,22], maternal stress[23], and prenatal infections[24,25] have been associated with an increased risk of developing neuropsychiatric disorders, including ASD. These environmental risk factors perturb fetal neurodevelopment during pregnancy, which has been proposed as a possible explanation for the etiology of ASD.

Epidemiological studies of association between prenatal infection and neuropsychiatric disorders

The association between prenatal infection and neuropsychiatric disorders was first uncovered by studying the direct effects of neurotoxicity on fetal neurodevelopment. Among 243 children who were exposed to rubella during pregnancy, 91 children (37%) were diagnosed with intellectual disability, 18 children (7.4%) had ASD and 8 children (3.3%) showed neurologic damage between the ages of 2.5 and 5[26]. Consistent with congenital rubella studies, exposure to other viral pathogens, such as measles, mumps, and influenza during gestation was associated with an increased risk of developing ASD in offspring[27]. Furthermore, recent epidemiological studies showed similar results with bacterial exposure during gestation[28,29].

Several other factors were examined, including hospitalization due to maternal infection, the timing of the exposure, ethnicity, educational level, and maternal/parental age[5,28,30]. One epidemiological study found that while no association between maternal infection and ASD was observed, prenatal infection leading to hospitalization was significantly linked to an increased risk of development of ASD[5]. In addition, they found that bacterial infections, along with multiple infections during pregnancy, were strongly associated with ASD. The timing of maternal infections has not consistently been associated with an elevated risk of ASD based on epidemiological findings. For example, according to a Swedish study, increased risk of ASD was associated with maternal infection in all trimesters[28], whereas a Danish study found that viral exposure in the first semester and bacterial or any infection in the second trimester was associated with ASD[5]. Other confounders, such as ethnicity, educational level, and parental age did not influence risk of developing ASD. Together, these data suggest maternal infection and its severity influence fetal neurodevelopment and increases risk of developing ASD.

Additionally, pathogen-specific effects of prenatal infection have been observed in neuropsychiatric disorders. Epidemiological studies show that prenatal infection with *Toxoplasma gondii*, a parasite that causes toxoplasmosis, was associated with a risk of developing schizophrenia (SZ), a neuropsychiatric disorder that affects ability to think and behave[31]. However, they did not find significant association between herpes simplex virus type 1 or -2 and SZ[32]. In addition, prenatal infection with influenza was associated with increased risk of developing SZ, but no association was observed with cytomegalovirus[33]. While general maternal bacterial infection has been associated with increased risk of ASD and SZ, the effects of bacterial pathogen specificity on fetal neurodevelopment remain unclear. Future studies may examine the association with different pathogenicity of bacteria, including the placental, urinary tract, and gut infections.

A potential mechanism for the development of neuropsychiatric disorders such as ASD, is inflammatory cytokines. Several epidemiological studies show that infections associated with fever during pregnancy can increase the risk of developing ASD[2,34,35]. Fever is induced by proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, and interferons (IFNs). Inflammatory cytokines are thought to be involved as immune modulators, but they also influence fetal neurodevelopment, including neurogenesis, neuronal differentiation/migration, vascularization, synapse function, and neuronal survival[36]. Interestingly, epidemiological studies show significant levels of cytokines, such as IFN-γ, IL-4 and IL-5 were detected in maternal sera[37], as well as elevated levels of IL-4, IL-10, TNF- α and TNF-β in amniotic fluid during gestation were associated with ASD[38,39]. In addition, pregnancy complications such as preterm birth have been suggested as possible mechanisms with an increased risk of ASD[40,41]. Together, numerous epidemiological studies suggest that prenatal infections and

their level of severity are associated with an increased risk of developing neuropsychiatric disorders, including ASD.

Behavioral animal models of ASD

Behavioral symptoms of ASD are one of the most important components of diagnosis. The three main core symptoms of ASD include deficits in social interaction, communication impairments, and restricted or repetitive behaviors. Additionally, anxiety, intellectual disability, and hyper- or hypo-reactivity to sensory stimuli are seen in patients with ASD. Many behavioral tests have been developed to examine the resemblance of clinical features of ASD in various animal models using mice, rates, and nonhuman primates[42–44]. Social interactions are crucial for bonding and reproducing for rodents. To provide social interaction settings for rodents, the three-chamber social approach assay was developed to assess sociability in rodents[45]. A schematic of the three-chamber social approach is illustrated in Figure 1.1A and the protocol is as follows. A test subject is allowed to freely interact with either an inanimate object or an unfamiliar mouse (sex, age, and treatment matched) for ten minutes. Time spent with an unfamiliar mouse and an inanimate object is recorded for social interaction preference. Furthermore, social novelty assay can be performed to test cognitive and memory function (Figure 1.1B). Self-grooming, defined as time spent washing the face or scratching with a foot, can be timed during the three-chamber social approach assay to evaluate repetitive and persistent behavior[46].

The marble burying test is another method of measuring repetitive behavior[47]. Marbles are placed on top of the bedding (usually 4 by 5 or 4 by 4) and test subjects are given a certain time (usually 10 minutes) to dig and bury marbles (Figure 1.1C). More marbles buried indicate a high

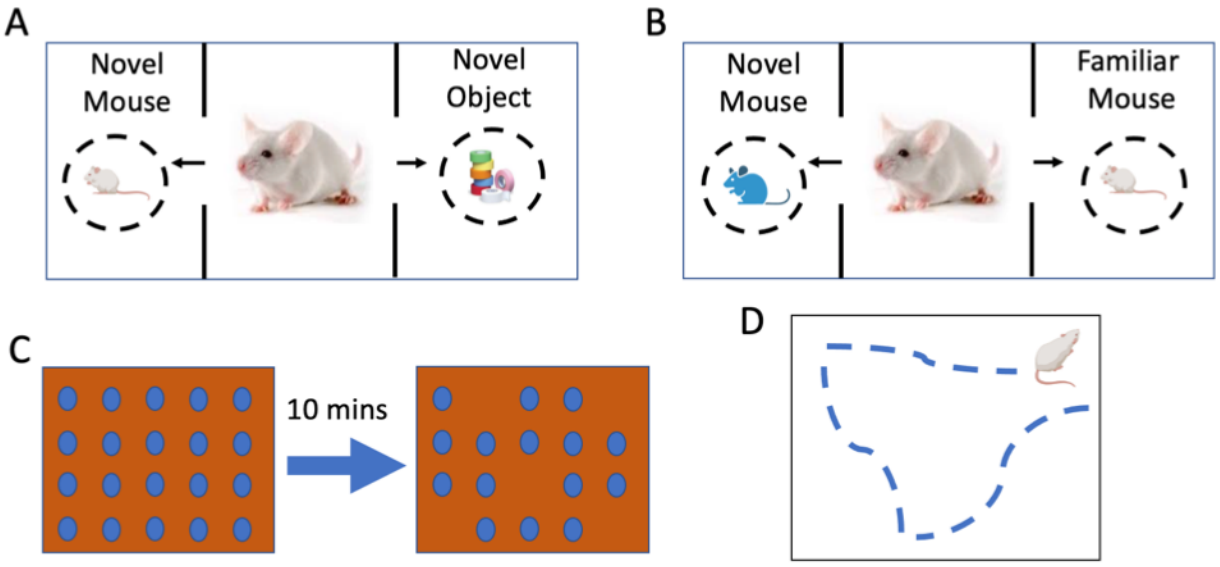

Figure 1.1. Schematic illustrations of different behavior assays. A. Three-chambered apparatus for social interaction test. Animals (same age, sex, and treatment group) will be placed with a cup containing a stimulus mouse, a center chamber, and a cup containing lab tap. **B.** Social novelty test. A lab tape will be removed and replaced with a novel mouse (same sex, age, and treatment group). A mouse from social interactions will be moved to the other side of the chamber. **C.** Schematic of the marble burying test. Each mouse will be given 10 minutes and scored at the end of the experiment. **D.** Each mouse will be given 10 minutes to explore and monitored using a camera.

level of repetitive and restricted behaviors. The level of anxiety and locomotion can be assessed using an open field exploration assay[48] (Figure 1.1D). Animals with heightened level of anxiety will spend more time exploring close to the walls rather than in the open center.

Animal models of maternal immune activation

To elucidate the association between prenatal infection and neuropsychiatric disorders, animal models have been developed. An immunogen, lipopolysaccharide (LPS) or double stranded RNA (polyinosinic-polycytidylic acid [poly(I:C)]), is injected into pregnant animals to mimic a bacterial or viral infection, respectively. These immunogens activate the immune system of pregnant animals and result in atypical behaviors in offspring that are notably relevant to ASD and SZ[7,43,49]. Because these outcomes are observed in the absence of an active infection, findings

suggest that maternal immune activation (MIA) is driving these changes in fetal brain development[42]. Subsequent analyses have demonstrated that MIA induces anatomical changes in the fetal brain, such as reduced brain volume and loss of neurons, resulting in abnormal cortical lamination[7,50]. These brain alterations suggest that synaptic function, which plays a role in the formation of memory and learning, is impaired in MIA offspring. In addition, analysis of differential gene expression (DGE) in the fetal brain has revealed that injecting different immunogens into the pregnant animals results in distinctive gene expression profiles, suggesting that MIA is not a homogenous phenomenon[51]. This may be due to the specificity of toll like receptors (TLRs) related to pathogen-associated molecular patterns. TLR4 recognizes LPS, a surface molecule presents in most Gram-negative bacteria, whereas TLR3 recognizes double stranded RNA molecules, such as poly(I:C). Fetal brain transcriptome analysis shows that many ASD-associated genes are strongly enriched in early MIA fetal brain development[52,53]. Taken together, these data suggest that injection of an immunogen induces ASD- and SZ-like behaviors and brain morphology.

Injecting pregnant mice with the cytokine IL-6 has revealed one possible mechanism for MIA[42]. This study was motivated by the association between prenatal infections and elevated levels of proinflammatory cytokines. Injection of IL-6 to pregnant mice induced alterations in pre-pulse inhibition and latent, which are behaviors relevant to SZ, in offspring. Administration of an anti-IL-6 antibody after poly(I:C) injection rescued offspring from having abnormal behaviors. In addition, an anti-IL-6 antibody normalized gene expression of brains exposed to poly(I:C) during gestation. Similarly, direct injection of IL-17a to the fetal brain induced abnormal cortical lamination and ASD-relevant behaviors in offspring[7]. T helper 17 (T_H17) cells are responsible for the production of cytokine IL-17a. T_H17 cells play an important role in modulating the immune

system by recognizing extracellular pathogens[54]; however, their altered levels of presence have been implicated in inflammatory and autoimmune disorders, including multiple sclerosis and asthma[55]. Together, these studies suggest that proinflammatory cytokines play important roles in the development of the fetal brain during pregnancy.

Numerous studies suggest composition of gut microbiota has been implicated in neuropsychiatric disorder. For example, recent animal studies demonstrated that dysbiosis of maternal gut microbiota during pregnancy is also associated with developing ASD in the offspring. Kim and colleagues demonstrated that poly(I:C) injection into pregnant mice triggered T_H17 cell differentiation by maternal gut microbiota and led to increased production of IL-17a, which ultimately induced ASD-like phenotype in the offspring[56]. Furthermore, they showed that injection of poly(I:C) increased the levels of IL-1β, IL-23 and IL-6, which triggered more production of IL-17a by stimulating T-cells. More recently, Kim and colleagues demonstrated that poly(I:C) exposure during pregnancy altered development of naive $CD4^+$ T cells in the offspring, and MIA offspring were more prone to develop inflammatory diseases[57]. Unfortunately, individuals with ASD often suffer from gastrointestinal (GI) related issues, such as diarrhea, abdominal pain and constipation[58,59]. Studies showed increased levels of proinflammatory cytokines and intestinal pathologies such as gastritis[59,60], were observed in ASD. One hypothesis regarding GI issues in ASD is dysbiosis or altered composition of gut microbiota. For example, several studies demonstrate that ASD individuals showed higher levels of *Clostridium* species, which are often associated with colitis by producing toxins, in their stool samples[61,62]. Furthermore, higher relative abundance of *Sutterella* and *Lactobacillus* species was observed in ASD individuals, suggesting altered gut microbiota composition[63–65]. The importance of gut microbiota was also demonstrated in MIA-induced animal models. Hsiao and colleagues illustrated that oral treatment with

Bacteroides fragilis, a commensal probiotic reside in the gut, rescued ASD-like behavioral symptoms in poly(I:C)-induced offspring by correcting gut permeability[66]. Collectively, these observations demonstrate the importance of gut microbiota and highlight the therapeutic potential of targeting the maternal gut bacteria when they are exposed to infections during pregnancy.

Several studies used infectious pathogens to elucidate the association between prenatal infection and neuropsychiatric disorders. Studies by Allard and colleague studied the effects of prenatal infection with group B *Streptococcus* (GBS; *Streptococcus agalactiae*) on fetal neurodevelopment[67,68]. GBS is an opportunistic pathogen that can cause chorioamnionitis, resulting in preterm delivery or stillbirth, which is a major health concern during pregnancy[69]. GBS-exposed rat offspring showed ASD relevant behaviors, such as impaired social interaction and communication, and altered sensory processing. Furthermore, abnormal gross anatomy of the brain structure was observed, including altered thickness of white matter lesions. Offspring exposed to influenza A virus (IAV) during pregnancy also showed deficit in social interaction, exploratory behavior, and pre-pulse inhibition (sensorimotor gating)[70]. Furthermore, IAV-exposed offspring were more susceptible to early life infections (either bacterial or viral) than poly(I:C)-exposed offspring due to impaired pathogen clearance by alveolar macrophages[71]. Overall, these findings suggest that prenatal infections with infectious pathogens can lead to abnormal brain development during pregnancy and cause altered behaviors relevant to ASD and SZ. Further studies should address different effects between actual infections with pathogens and artificial immunogens on neurodevelopment during pregnancy.

Sexual dimorphism of neuropsychiatric disorders and MIA

In neuropsychiatric disorders, sexual dimorphism is well-recognized. However, it is unclear why these differences persist. Similar to humans, in murine models of MIA, male offspring display more severe ASD-like abnormal behaviors than female offspring. MIA-exposed male offspring showed impaired socialization and communication and repetitive behaviors, whereas female MIA offspring show heightened levels of anxiety[72,73]. One possible explanation for these observations could be sex specific vulnerabilities and responses to MIA or infections during gestation. Recent studies demonstrate that male fetal mice experience more prominent placental damage and brain hypoxia, whereas female mice exhibit acute inflammation in the fetal brain and postnatal growth delay following LPS exposure during pregnancy[72]. Studies of prenatal hypoxia animal models show altered brain morphology, such as disrupted cortical layers[74], altered neurogenesis[75], and dysregulated cell proliferation/differentiation[76]. It also has been linked to neuropsychiatric disorders in humans.

Recent studies by Kalish and colleagues observed sexually dichotomous responses induced by poly(I:C), as well. According to their findings, poly(I:C) only induces the integrated stress response in males, resulting in an overall reduction in protein synthesis[77]. Dysregulated translation has been observed in ASD[78]. The increase in repetitive and abnormal social behaviors were rescued by blocking the activation of the integrated stress response induced by MIA. It is important to note that they observed perturbed fetal brain transcriptome using single-cell RNA-seq analysis in both sexes. Their findings suggest that females are more resilient to intrauterine inflammation during gestation, and future studies should therefore examine the mechanisms.

Maternal intraperitoneal inoculation with GBS also induced sex-biased responses in rat offspring. GBS-exposed male offspring showed ASD-like behaviors, including impaired social

interaction and communication, compared to female offspring[67]. There were also more polymorphonuclear cells in male placentas, a marker for chorioamnionitis, in the labyrinth layer (fetal compartment) than in female placentas. In addition, elevated levels of cytokines and chemokines, such as IL-1β and cytokine-induced neutrophil chemoattractant-1, were observed in male placentas. Interestingly, the production of proinflammatory cytokines, such as IL-6 and TNF-α, was not significantly different between the sexes. Cytokine IL-1β plays an important role in response to GBS infection. However, IL-1 has been shown to cross the blood barrier, causing neuronal cell death and inducing abnormal myelination. One possible mechanism of sexual dimorphic susceptibility in neuropsychiatric disorders may be due to different basal levels of IL-1 receptor antagonist (IL-1Ra) between the sexes. Several clinical studies indicate that higher levels of IL-1Ra have been detected in the amniotic fluid of females than males during pregnancy. One of the functions of IL-1Ra is to inhibit the effect of IL-1β, possibly suggesting that females are more "protected" against IL-1β inflammation. These results highlight the therapeutic potential for sex-biased responses of prenatal environmental risk factors. Together, these studies suggest that males and females show sexually dimorphic responses to environmental risk factors during gestation.

Pathogenesis of *Listeria monocytogenes*

Listeria monocytogenes (*Lm*) is a pathogen of health concern during pregnancy that can cause placental dysfunction. *Lm* is a ubiquitous Gram-positive facultative anaerobic bacterium that causes human listeriosis, an infection caused by consuming contaminated food[79]. Symptoms of listeriosis include fever, confusion, muscle aches, and death in severe cases[11]. Due to its ability to grow at low temperatures and pH, various types of food such as meat, dairy products, and soft

cheeses are susceptible to contamination with *Lm*[80,81]. An estimated up to 2,500 cases of listeriosis occur in the U.S. annually. Among those at high risk of contracting listeriosis are immunocompromised individuals, such as elders and pregnant women[81]. Clinical studies show that pregnant women are up to 18 times more likely to contract *Lm* than non-pregnant individuals[82].

Lm has a unique intracellular lifecycle which allows it to cross various host barriers. After consuming food contaminated with *Lm*, the bacterium can cross the intestinal barrier, spread into the bloodstream through the lymph nodes and disperse to different organs, such as the liver and spleen[79]. This intracellular lifecycle has been well studied. First, *Lm* can be internalized by either phagocytosis or receptor mediated endocytosis using internalin proteins[79]. These surface proteins, internalin A and B (InlA and InlB) bind to host surface proteins and induce *Lm* uptake. These virulent factors interact with epithelial cadherin[83] (expressed at the surface of epithelial cells) and the hepatocyte growth factor receptor c-Met[84] (expressed at the cell surface of hepatocytes), respectively. Once *Lm* is internalized inside the vacuole, it expresses the cholesterol dependent cytolysin listeriolysin O[85] and two phospholipases (phospholipase A and B) to escape the vacuole and enter the host cytosol. *Lm* then expresses actin assembly inducing protein (ActA), a surface anchored virulence factor that mediates actin polymerization[86,87]. ActA allows *Lm* to spread from one cell to another once sufficient force is generated via actin rockets. Once *Lm* enters neighboring cells, it restarts the intracellular lifecycle.

Pregnancy associated infections with *Lm* and immune responses

One pathogenic feature of *Lm* in the context of prenatal infection is its ability to breach the placenta, a transient organ that plays an essential role in providing nutrients, protection, and oxygen between the mother and the fetus during pregnancy. When pregnant women consume *Lm*-

contaminated food, the bacterium infects intestinal epithelial cells and ultimately spread to the placenta. Prenatal infections with *Lm* typically occur during the second or third trimester of pregnancy by infecting the subtype of trophoblasts, specifically syncytiotrophoblasts and extravillious trophoblast cells in the placenta. The syncytiotrophoblasts are the protective outer epithelial cells of the placenta that provide nutrients, waste, and gas exchanges between the pregnant woman and the fetus during pregnancy. In human placental explants, *Lm* has been shown to invade the placental barrier utilizing the InlA and InlB virulence factors. Interestingly, internalin P (InlP), a virulence factor specific to the placenta, has recently been identified. InlP is thought to play a role in colonization of the placenta by interacting with afadin (a protein associated with cell-to-cell junction).

Immune responses are induced when *Lm* infects trophoblasts. Recent studies by Johnson and colleagues demonstrated that human placental trophoblasts infected with *Lm* triggered production of an array of cytokines and chemokines. According to their transcriptome analysis of *Lm*-infected trophoblasts, genes involved in pattern recognition receptors (TLR2 and cytosolic receptors), nucleotide-binding oligomerization domain-like receptors, proinflammatory cytokines, and activation of leukocyte related pathways were upregulated, whereas transcription regulation and inflammation and immune function related pathways were downregulated. Furthermore, infected trophoblasts secreted significant levels of proinflammatory cytokines (IL-1β, IL-6, IL-1α, and TNF-α) and chemokines involved in recruitment of neutrophils, monocyte, and natural killer cells. These immune responses have been associated with poor pregnancy outcomes.

Perturbations of the maternal-fetal interface by placental infection with *Lm* can result in deleterious effects in conjunction with maternal inflammation. Adverse outcomes, such as spontaneous abortions, preterm birth, and fetal death, can occur with maternal listeriosis, while

pregnant mothers are often asymptomatic[88] or exhibit flu-like symptoms, including fever, headache, or diarrhea[89,90]. Recent cohort studies show that more than 80% of prenatal infection with *Lm* led to adverse pregnancy outcomes, including pregnancy loss (26/107) and preterm birth (48/107), whereas only 5 pregnant women out of 107 had normal delivery[91]. Among 82 infants born alive, only 10 showed normal physical attributes[91]. While symptoms of prenatal infection by *Lm*, including low birth weight, respiratory distress, cyanosis, and apnea, have been observed in neonates[92], little is known about its postnatal effects, and this should be the focus of further investigation.

Consequences associated with placental infection using *Lm* in animal models

Lm has been used to study placental and fetal infection for many years due to its well characterized pathogenicity. Hardy and colleagues demonstrated that placental infection with *Lm* caused various adverse pregnancy consequences[93]. Pregnant mice were infected on embryonic day 14 (E14) with 5 x 10^5 colony forming unit (CFU) of bioluminescent *Lm* which resulted in abortion and stillbirth, representing subclinical infection in humans. They also illustrated that bioluminescence imaging (BLI) signal, which measures the intensity of infection, was correlated with poor pregnancy outcomes. The fetuses with a high BLI signal showed slower rate of heartbeat, whereas the fetuses with a low BLI signal showed normal rate of heartbeat within the same infected pregnant mouse. The results indicate a localized rather than systemic effect of placental infection. Interestingly, pregnancy outcomes were correlated with timing of the infection. When pregnant mice were infected on E8, fetal resorption occurred. These findings illustrate the importance of timing and severity of infection, which have been associated with poor pregnancy outcomes in several epidemiological studies. One possible mechanism of preterm labor by *Lm* placental

infection has been demonstrated in our lab. Conner and colleagues showed increased levels of eicosanoids, which are fatty acids known to induce labor and cervical ripening in pregnant women, due to placental infection by *Lm*. These eicosanoids highlight potential therapeutic targets for *Lm* placental infection; however, this warrants further study.

Concluding Remarks

Prenatal environmental risk exposures, such as maternal infection, during pregnancy may lead to adverse pregnancy outcomes, including an increased risk of developing neuropsychiatric disorders. Although *Lm* causes detrimental pregnancy outcomes, its effect on neurological outcomes has not been investigated. In Chapter 2, I examine the sex-specific fetal brain transcriptome and brain morphological changes induced by *Lm* infection during pregnancy. I also investigate the behavioral effects of prenatal infection by *Lm*. In Chapter 3, I investigate the fetal brain transcriptional changes at the systems level and examine the enrichment of ASD associated genes induced by *Lm* placental infection. Furthermore, I also explore how the severity of placental infection changes the fetal brain transcriptome. In conclusion, these findings show that placental infection with *Lm* affects neurodevelopment during pregnancy and results in ASD-like behaviors.

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CHAPTER 2: Placental *Listeria monocytogenes* Infection Induces Sex-Specific Responses in the Fetal Brain

Work presented in this chapter has been submitted to *Pediatric Research* as Kun Ho Lee[1,2], Matti Kiupel[3], Thomas Woods[3], Prachee Pingle[2] and Jonathan Hardy[1,2]

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ABSTRACT

Epidemiological data indicate that prenatal infection is associated with an increased risk of several neurodevelopmental disorders in the progeny. These disorders display sex differences in presentation. The role of the placenta, which is a target of prenatal infection, in the sex-specificity of neurodevelopmental abnormalities is unknown. We used an imaging-based animal model of the bacterial pathogen *Listeria monocytogenes* to identify the sex-specific effects of placental infection on the neurodevelopment of the fetus. Pregnant CD1 mice were infected with a bioluminescent strain of *Listeria* on embryonic day 14.5 (E14.5). Excised fetuses were imaged on E18.5 to identify the infected placentas. The associated fetal brains were analyzed for gene expression and altered brain structure due to infection. The behavior of adult offspring affected by prenatal *Listeria* infection was analyzed. Placental infection induced sex-specific alteration of gene expression patterns in the fetal brain and resulted in abnormal cortical development correlated with placental infection levels. Furthermore, male offspring exhibited abnormal social interaction, whereas females exhibited elevated anxiety. Placental infection by *Listeria* induced sex-specific abnormalities in neurodevelopment of the fetus. Prenatal infection also affected the behavior of the offspring in a sex-specific manner.

INTRODUCTION

The molecular and cellular mechanisms leading to most neuropsychiatric disorders, such as autism spectrum disorder (ASD), remain unclear due to their complex polygenic etiology. Epidemiological data indicate that prenatal infection with bacterial, viral, or parasitic pathogens during pregnancy is associated with an increased risk of neuropsychiatric disorders in the progeny, including ASD[1] and schizophrenia[2,3]. Injection of bacterial endotoxin lipopolysaccharide (LPS) or polyinosinic-polycytidylic acid [poly(I:C)], which mimics viral infections, activates the immune system of pregnant rodents and results in altered brain gene expression[4] and atypical behavior in offspring[5]. These behavioral abnormalities are notably relevant to ASD core symptoms, such as repetitive behaviors and deficits in social interaction. Furthermore, animal studies show sex biased behaviors and responses in offspring after exposure to LPS and poly(I:C) during pregnancy, which resembles sex differences in neuropsychiatric disorders, including ASD[6,7]. Maternal immune activation (MIA) induced by LPS or poly(I:C) causes the changes in fetal brain development. Although injection of immunogens in pregnant animals results in consistent altered behavior and brain abnormalities in the progeny, they do not elicit the complex immune responses induced by actual infection. Prenatal pathogens exhibit tissue and cell-specificity as well as directed immune modulation, such that the different pathogens may regulate MIA differently. For example, infection of rats with Group B *Streptococcus* elicits distinct MIA patterns including neutrophil infiltrates that differ from immune stimulants such as LPS and poly(I:C)[8]. In addition, prenatal influenza is a risk factor for schizophrenia[2], whereas no such association was found with prenatal infection with either maternal type 1 herpes simplex virus[9,10] or cytomegalovirus[11]. Thus, the induction of MIA is complex and cannot be completely replicated by any single approach. It is

therefore critically important to examine different prenatal infection models and their specific effects on fetal brain development and behavior.

Listeria monocytogenes (*Lm*) provides an excellent animal model for prenatal infection[12,13]. This Gram-positive bacterium is a foodborne pathogen and is a significant health concern during pregnancy because pregnant mothers are up to 10 times more likely to be infected with *Lm*[14]. An important hallmark of prenatal listeriosis is the infection of the placenta[15–17]. Placental infection by *Lm* can lead to many overt fetal and newborn pathologies, including spontaneous abortions, stillbirth, and other neonatal illnesses, even while pregnant mothers can be largely asymptomatic[12,18–20]. Previously, we reported that bradycardia was only observed in fetuses with infected placentas within the same infected pregnant mouse as those with normal heart rates[21]. These studies demonstrated that bradycardia induced by placental infection was not systemic but localized.

Infection with the appropriate dose of intravenous *Lm* on embryonic day 14.5 results in abortion, stillbirth, and fetal bradycardia in the absence of overt maternal disease symptoms. Although placental infection by *Lm* causes adverse outcomes in newborns, neurodevelopmental consequences of this infection have not been characterized. In addition, sex-specific consequences of placental infection have not been defined for any living pathogen. The aims of this study were to understand how bacterial infection of the placenta affects fetal neurodevelopment, to determine if sex-specific responses occur, and to assess effects on the behavior of the offspring.

METHODS

Animal care and use

All animal procedures were approved by the Institutional Animal Care and Use Committee and the Biosafety of Michigan State University under protocol number 201800030. Michigan State University (MSU) has approved Animal Welfare Assurance (A3955-01) from the NIH Office of Laboratory Animal Welfare (OLAW). In addition, all components of the University are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC Unit #1047). Standard BSL-2 containment and handling procedures were used for all animals including the offspring. These procedures were also approved according to the specific MSU Biosafety Protocol 0000058, and all laboratories, procedure rooms and facilities are inspected by MSU Environmental Health and Safety. Timed CD1 pregnant mice purchased from Charles River Laboratories were used for all studies and housed in temperature controlled, 12:12 hour light and dark cycle rooms. Euthanasia was performed by cervical dislocation under isoflurane anesthesia by trained personnel according to NIH and MSU approved protocols.

In vivo bioluminescence imaging (BLI) and tissue processing

The bioluminescent strain of *L. monocytogenes* used in this study (Perkin Elmer Xen32) was generated in a 10403S strain background[22]. Cultures were incubated overnight at 37°C in brain heart infusion (BHI) broth. The overnight culture was sub-cultured in fresh BHI broth to an optical density (OD_{600}) of 0.5. Timed embryonic day 11 (E11) pregnant CD1 mice were house at the Michigan State University Clinical Center animal facility under BSL-2 containment. Pregnant mice were administered a tail vein injection of 2 x 10^5 colony-forming units (CFU) of Xen32, diluted in 200 uL phosphate-buffered saline (PBS), or an equivalent volume of PBS vehicle on E14.5. On E18.5, pregnant mice were anesthetized using isoflurane and imaged using the *In vivo*

bioluminescence imaging system (IVIS; Perkin Elmer Inc.), and then humanely sacrificed by cervical dislocation while under isoflurane anesthesia according to the approved animal protocol. Uterine horns were excised immediately and imaged again using the IVIS. Individual fetuses could be imaged separately for high-resolution BLI. Signal levels from the placenta at this dose and timing vary over orders of magnitude within one pregnant mouse, permitting the analysis of systemic versus localized effects and allowing for comparisons of fetal brains with and without high placental BLI signal from the same pregnant animal. Fetal brains were collected and transferred into sterile Eppendorf tubes, flash-frozen, and stored at -80°C until analyzed.

Histology

For histology of the fetal brain, excised fetuses and placentas were imaged with ex vivo BLI to determine signal levels of the associated placentas. The heads were removed and fixed overnight in 4% paraformaldehyde for sectioning. Following sectioning, the brains were routinely processed and embedded in paraffin and matched coronal sections were stained with hematoxylin and eosin (H&E). Matched coronal sections were also obtained from PBS-injected pregnant mice and from fetuses with and without detectable BLI signals from the placenta from infected pregnant mice. BLI signals from the placenta were measured with identical regions of interest (ROIs). For immunohistochemistry of brains of the adult offspring, the animals were euthanized with CO_2 according to the approved animal protocol. The brains were removed, fixed in 4% paraformaldehyde and matched coronal sections were obtained as described above. Several sections from each brain were stained with H&E following the same routine methods or immunohistochemically labeled with a rabbit monoclonal anti-c-Fos antibody (dilution 1:1,000, EPR21930-238, Abcam, Boston, MA). Immunohistochemistry was performed on the Dako link 48 Automated Staining System (Agilent Technologies, Santa Clara, CA) using a high pH antigen

retrieval and peroxidase-conjugated EnVision Polymer Detection System (Aligent Technologies) with 3,3'-diaminobenzidine (DAB) as the chromogen and hematoxylin counterstaining.

RNA-seq

Total RNA was isolated using the phenol/guanidine based QIAzol lysis reagent (Qiagen, Valencia, CA), according to the manufacturer's recommendations. The concentration and quality of RNA samples were measured using Qubit (ThermoFisher) and BioAnalyzer (Agilent), respectively. Samples with RNA integrity number values of 9 or above were selected for sequencing. Fetal brains (positive BLI signal n = 19; control n =6) were collected and total RNA was submitted for next generation sequencing (NGS) library preparation and sequencing to Research Technology Support Facility at Michigan State University. Libraries were prepared using the Illumina TruSeq Standard mRNA Library Preparation Kit with IDT for Illumina Unique Dual Index adapters following manufacturer's recommendations. Completed libraries were quality checked and quantified using a combination of Qubit dsDNA High Sensitivity and Agilent 420 TapeStation HS DNA1000 assays. Libraries were pooled in equimolar proportions for multiplexed sequencing. The pool was quantified using the Kapa Biosystems Illumina Library Quantification qPCR kit. This pool was loaded onto two lanes of an Illumina HiSeq 4000 flow cell (two technical replicates) and sequencing was performed in a 1 x 50 single read format using HiSeq 4000 SBS reagents. Base calling was done by Illumina Real Time Analysis v2.7.7 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.1. The raw single-end (SE) reads were processed to trim sequencing adapter and low-quality bases. The clean SE RAN-seq reads were mapped to the mouse reference genome (GCRm38.p6/mm10) using STAR (Spliced Transcriptions Alignment to a Reference) v2.3.2[23]. Mapped reads were assigned to genes with FeatureCounts in the subread package[24].

Differential gene expression analysis and functional enrichment analysis

Differential gene expression analysis was performed using DESeq2 v1.32.0[25] in R v4.1.1. Genes with minimum 5 raw reads in at least 20 samples were filtered out, resulting a total of 19,180 of genes. Differentially expressed genes with p-adj < 0.05 were used to perform functional enrichment analysis using the g:Profiler system (https://biit.cs.ut.ee/gprofiler/gost)[26]. Biological pathways with p-adj < 0.05 were considered significant. To examine the sex dependent effects of placental infection, a female specific gene, *Xist*, was used to identify the sex of fetal brains from RNA-seq samples. Differential gene expression and functional enrichment analyses were performed using the same parameters as placental infection. Volcano plots were generated using EnhancedVolcano package in R[27].

Social interactions and repetitive behaviors

The three-chamber social approach assay has been widely used to test for assaying sociability in mice[28]. This assay measures interaction between animals that are provided choices between unfamiliar animals and inanimate objects (social interaction). We used a custom three-chamber apparatus (63 cm x 30 cm x 31 cm) with an empty middle chamber and accessible side chambers on either end that contain cylindrical open barred cages in which mice or objects are placed. An inanimate object is placed in one of the barred cages in one side chamber, and an unfamiliar mouse is placed in the barred cage in the other side chamber. A test subject mouse is placed in the central chamber and allowed to freely interact with the mice or objects in the side chambers. The social interaction test we employed had three phases. First, the test subject (prenatal *Lm*-exposed male = 10 and female = 8; control male = 5 and female = 5; 8 – 12 weeks of age) was habituated in the center of chamber for 10 minutes and two doorways in the chambers were closed. Second, the test subject was habituated to all three chambers for 10 minutes. Third, the subject was

confined to the middle chamber, a novel object (lab tape) was placed in the barred cage in one side chamber, and a novel mouse (a treatment, sex, and age matched unfamiliar mouse) was placed in the other side chamber.

The social interaction in each test was recorded for 10 minutes. Sniffing time for each subject was recorded. Self-grooming, which is defined as time spent rubbing the face, scratching with a foot, or licking paws, was examined to measure repetitive and persistent behavior[28]. During the three-chamber social approach assay, self-grooming was measured by using a stopwatch.

Open field exploration

Open field exploration tests measure anxiety, exploration and locomotion[29]. Mice (prenatal *Lm*-exposed male = 8 and female = 10; control male = 4 and female = 3, 8 – 12 weeks of age) were acclimated for 30 minutes before the assay. Mice were place in the middle of the testing area (63 cm x 60 cm x 31 cm) and underwent a 10-minute exploration period. Sessions were video recorded and analyzed using the ANY-maze Video Tracking System software.

RESULTS

BLI and postnatal effects of placental infection

All infections were performed by intravenous (IV) injection into pregnant CD1 mice with 5 x 10^5 colony forming units (CFU) of the bioluminescent *Lm* strain 2C (Perkin Elmer Xen36) on E14.5. The dose and timing were selected based on our prior studies[21]. The IV route of *Lm*-infection in pregnant mice is employed rather than oral infection for several reasons; the foremost being that oral infection requires over 10^{11} CFU in CD1 mice and is not reproducible between laboratories. In contrast, IV infection is highly reproducible and bypasses the intestine, seeding the placenta directly in a dose-dependent manner. The selected dose results in stillbirth, abortion, and developmental abnormality, resembling listeriosis in pregnant mothers. An IVIS image of *Lm*-infected pregnant CD1 mouse is shown in Figure 1.1a, with BLI signals indicating different infection sites, including gallbladder, placentas, and fetuses. BLI of excised uterine horns shows that there is a range of infection severity indicated by the intensity of the BLI signals from the placentas (Figure 2.1b). In addition, BLI demonstrated that *Lm*-infection was much greater in the placenta than the fetus (Figure 2.1c), as most often at this dose the signal was only detectable in the placenta. This result was consistent with other studies that showed that fetal infection only occurs at high doses[12,30].

When the *Lm*-infected pregnant CD1 mice gave birth, the pups showed a range of postnatal effects of placental infection. Some pups showed extreme low birth weight and altered body morphology (4 weeks old; Figure 2.1d). These effects were correlated with signal levels in the live pregnant dam. Higher signal levels of >10^5 photons/sec produced more severe effects such as stillbirth and extremely low birth weight, whereas signals <4x10^4 photons/sec yielded litters of

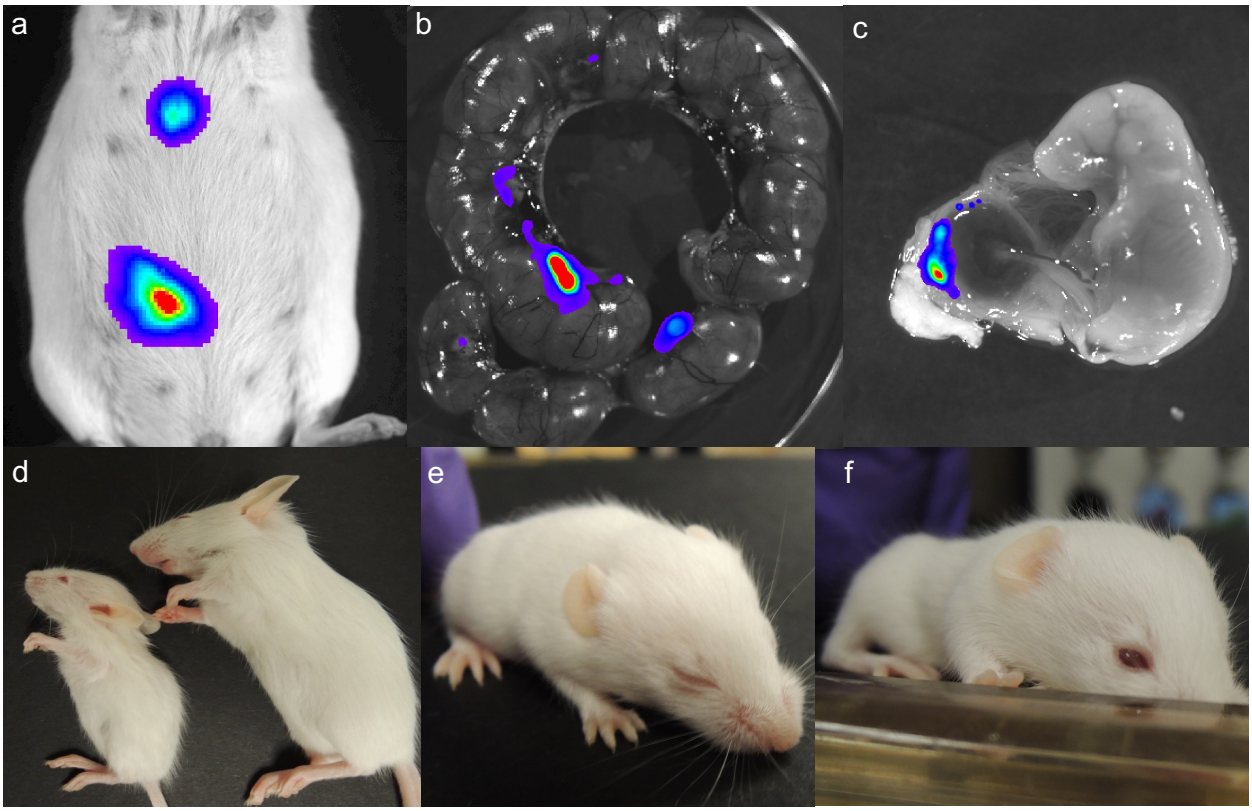

Figure 2.1. Postnatal effects of placental infection. (**a-c**) *In vivo* bioluminescence imaging (BLI) of prenatal *L. monocytogenes* (*Lm*) infection. **(a)** Live pregnant CD1 mouse on embryonic day 18.5 (E.18.5). (**b**) Excised uterine horns and **(c)** placenta. (**d**) Low birth weight due to *Lm* placental infection in a 4-week-old mouse (left) compared to a littermate (right). **(e)** *Lm*-exposed offspring exhibiting delayed eye opening compared to controls **(f)** on postnatal day 13.

normal-sized pups. In addition, pups from infected pregnant dams that showed $<4x10^4$ photons/sec and were indistinguishable from controls exhibited delayed eye opening (postnatal day 13; Figure 2.1e). These findings show that placental infection with *Lm* affects fetal and postnatal development. The range of effects was correlated with overall signal intensities from the live pregnant animal. At the dose we employed, none of the pregnant dams exhibited overt symptoms and they were outwardly indistinguishable from PBS-injected controls. Although some pregnant dams showed BLI signals from the area of the liver and/or gallbladder, all of them survived to give birth if they were allowed to do so.

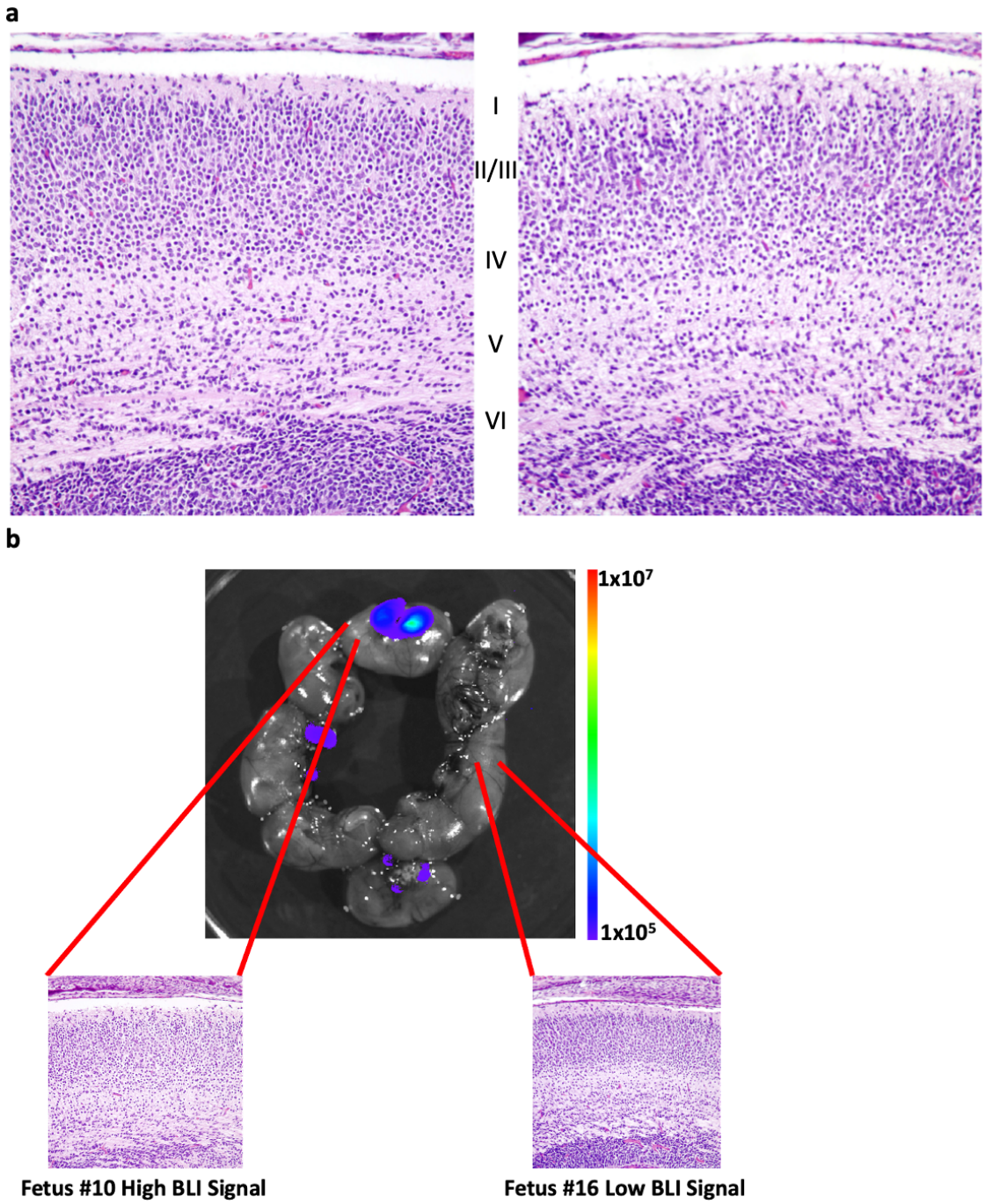

Figure 2.2. Placental infection promotes abnormal cortical lamination. (a) Coronal sections of fetal brain with normal layering in cortex from PBS-injected pregnant mice (left) and abnormal layering cortex from *Lm*-infected pregnant mice (right). **(b)** Abnormal layering in brains from mice with high placental BLI signal versus low placental BLI signal from the same pregnant animal. BLI signal is indicated in photons/s/cm^2/str. Layers: I: molecular, II: external granular, III: external pyramidal, IV: internal granular, V: internal pyramidal, VI: multiform.

Effect of placental infection on fetal cortical development

To determine whether placental infection promotes morphological changes in the fetal cortex, we performed hematoxylin/eosin (H&E) staining of the cortical sections of fetal brains. Pregnant CD1 mice were infected as described above and imaged to ascertain infection levels. We compared fetuses from infected and PBS-injected controls, but also fetuses within one pregnant dam that exhibited high and low signals from the placenta. The latter observation allowed us to distinguish effects due to systemic MIA from localized effects of the placenta. In the sections, layering was abnormal in the fetal brains from mice that originated from infected dams compared to controls (Figure 2.2a), and fetal brains from mice where the placentas exhibiting BLI signals above background showed layering alterations compared to fetuses from the same dam where the placenta had background BLI signals from the same dam (Figure 2.2b).

Infection of the placenta alters gene expression in the fetal mouse brain

We next investigated the effect of placental infection on transcriptomic alterations in fetal brain. For these investigations, we used fetal brains from mice in which no BLI signal over background was detectable in the placenta. A total of 25 whole fetal brains (6 control and 19 *Lm*-exposed samples) were harvested on E18.5 to generate RNA-seq datasets and performed differential expression analysis using a DESeq2 package in R. The analysis revealed that IV injection of bioluminescent *Lm* into pregnant CD1 mice at E14.5 altered gene expression in the fetal mouse brain. Overall, *Lm*-exposed fetal brains had a total of 268 upregulated and 139 downregulated differentially expressed genes (DEGs) with a false discovery rate (FDR) <0.05 threshold, and 1697 upregulated and 1247 downregulated with a p <0.05 threshold (Figure 1.3a). Among DEGs, most significant genes include upregulated *Lyrm7*, *Flt1*, *Vegfa* and *Kdm3a*, and downegulated *Zfp125*, *Mfsd5*, *slc38a5*, *Mblac1,* and *Chd15* (Figure 2.3b). The Gene Ontology

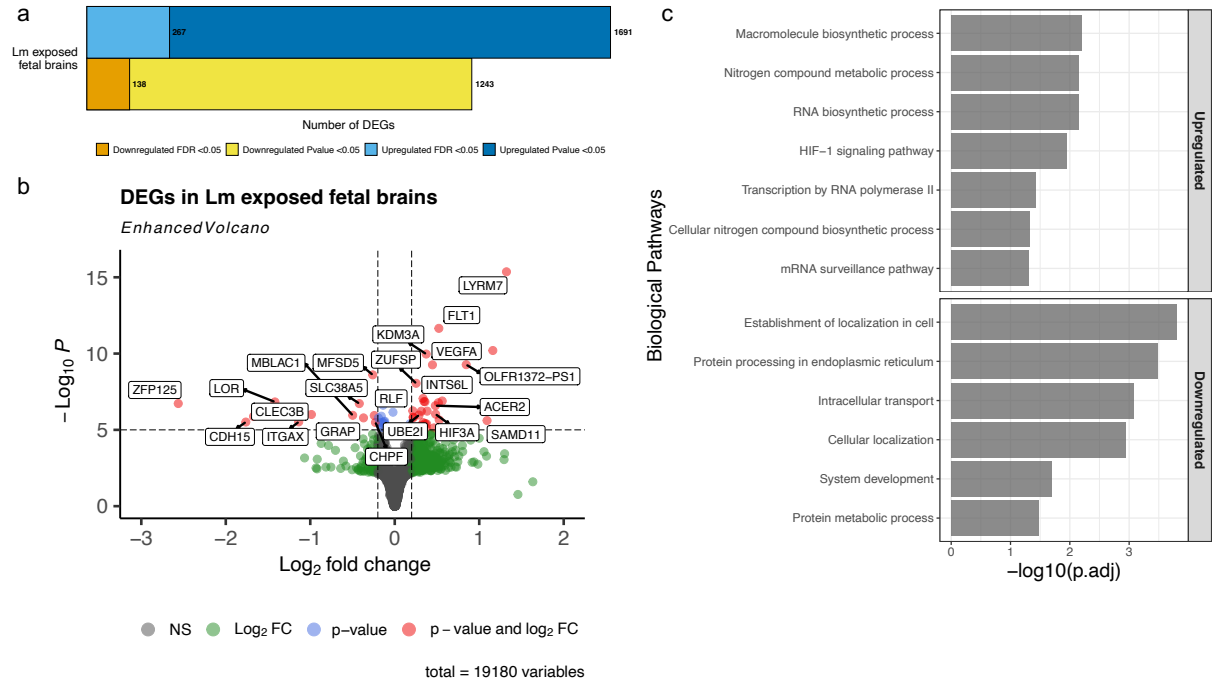

Figure 2.3. Gene expression changes in the fetal brain due to placental infection with *Lm*. (**a**) Total number of differentially expressed genes (DEGs) of fetal brains in response to placental infection. (**b**) Volcano plot of DEGs in *Lm*-exposed fetal brains of *Lm*-exposed mice at E18.5. Red dots indicate statistical significance (p-value < 10^6) and $\log_2$(fold change) greater or less than 0.2. Total variables indicate the total number of genes that were used to generate a volcano plot. (**c**) Gene Ontology analysis of DEGs (p-adj < 0.05) in fetal brains of *Lm*-exposed mice at E18.5. Biological pathways of downregulated and upregulated fetal brains of *Lm*-exposed mice were identified using g:ProfileR.

(GO) enrichment and KEGG analysis of upregulated DEGs revealed pathways, such as macromolecule biosynthetic and nitrogen compound metabolic processes, and hypoxia inducible factor-1 (HIF-1) signaling pathway (Figure 2.3c). Furthermore, pathways such as establishment of localization in cell and protein processing in endoplasmic reticulum were identified among significantly downregulated DEGs (Figure 2.3c). Many of these genes are associated with brain development or neurological function[31–34]. Together, differential expression analysis demonstrated that placental infection by *Lm* causes disruption of neurodevelopment during pregnancy.

Male and female fetal brains exhibit distinct gene expression profiles in response to placental infection

To examine sex-specific gene expression patterns, we used *Xist*, a female specific gene, to identify sex of each fetal brain RNA-seq sample (males: 9 *Lm*-exposed and 3 controls; females: 10 *Lm*-exposed and 3 controls). We used DESeq2 in R to identify DEGs and investigated overlapping genes between both *Lm*-exposed sexes. A total of 44 and 42 downregulated DEGs were identified for males and females, respectively, with one gene overlapping between the sexes (Figure 2.4a). Interestingly, females had 171 upregulated DEGs while males had 50 upregulated DEGs with 7 DEGs overlapping between the sexes (Figure 2.4b). GO enrichment and KEGG analysis of upregulated DEGs of *Lm*-exposed male fetal brains identified pathways, such as VEGF receptor 2 binding, HIF-1 signaling, and microtubule organizing center (Figure 2.4c). In addition, ribosome, mitochondrial translation elongation and termination, and oligosaccharyltransferase complex pathways were identified in downregulated DEGs of *Lm*-exposed male fetal brains (Figure 2.4d). Analysis of the GO enrichment and KEGG analyses of upregulated DEGs in *Lm*-exposed female fetal brains demonstrated organelle related and nuclear speck pathways, whereas catenin complex and postsynaptic actin cytoskeleton pathways were identified in downregulated DEGs. (Figure 2.4c and d) These findings demonstrated that placental infection had different effects on male versus female brains during neurodevelopment.

Placental infection induces sex-specific behavioral alterations in adult offspring

We sought to determine if altered behaviors were induced in the progeny of dams infected with *Lm*. Bacterial chorioamnionitis, which is not placental, leads to autism-like alterations in the behavioral of progeny in animals[35], so we selected behavioral tests that are used as correlates for human ASD. For this purpose, we screened the pregnant dams with BLI to identify those with

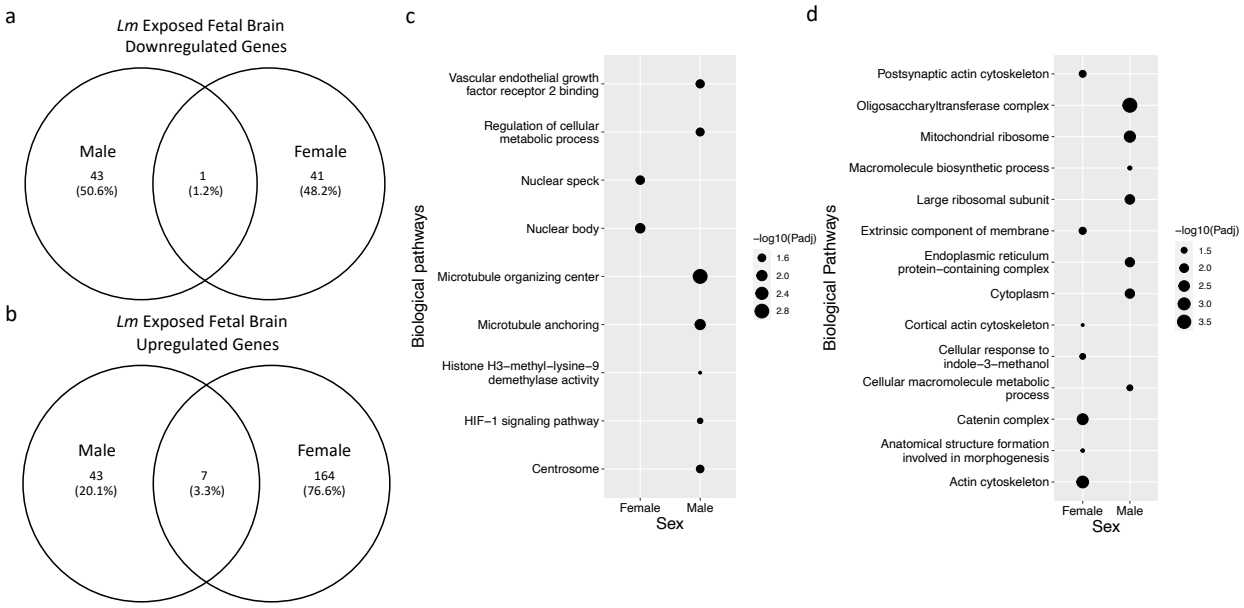

Figure 2.4. Sexually dichotomous gene expression profiles induced by placental infection. (a, b) Venn Diagrams representing the number of overlapping downregulated and upregulated genes in **(a)** and **(b)**, respectively. **(c, d)** Enrichment analysis of DEGs (p-adj < 0.05 of fetal brains from female and male *Lm*-exposed mice. Upregulated and downregulated biological pathways are shown in **(c)** and **(d)**, respectively.

signals less than $4x10^4$ photons/sec. These mice give birth to normal-sized pups, which cannot be grossly distinguished from controls from PBS-injected dams. When the pups were 8 to 12 weeks of age, we performed behavioral assays to determine if the adult mouse offspring exhibit abnormal behavioral due to placental infection by *Lm*. We separated mice tested with behavior assays by sex to determine if placental infection results in a sex bias of these effects. First, we analyzed social interaction by using the three-chamber social approach assay to assess social impairment. Social interactions are important for forming bonds for rodents, and autism-relevant behavior mouse models have demonstrated reduction in reciprocal social interactions. *Lm*-exposed male adult offspring presented with significant reduction in social interaction time with an unfamiliar mouse,

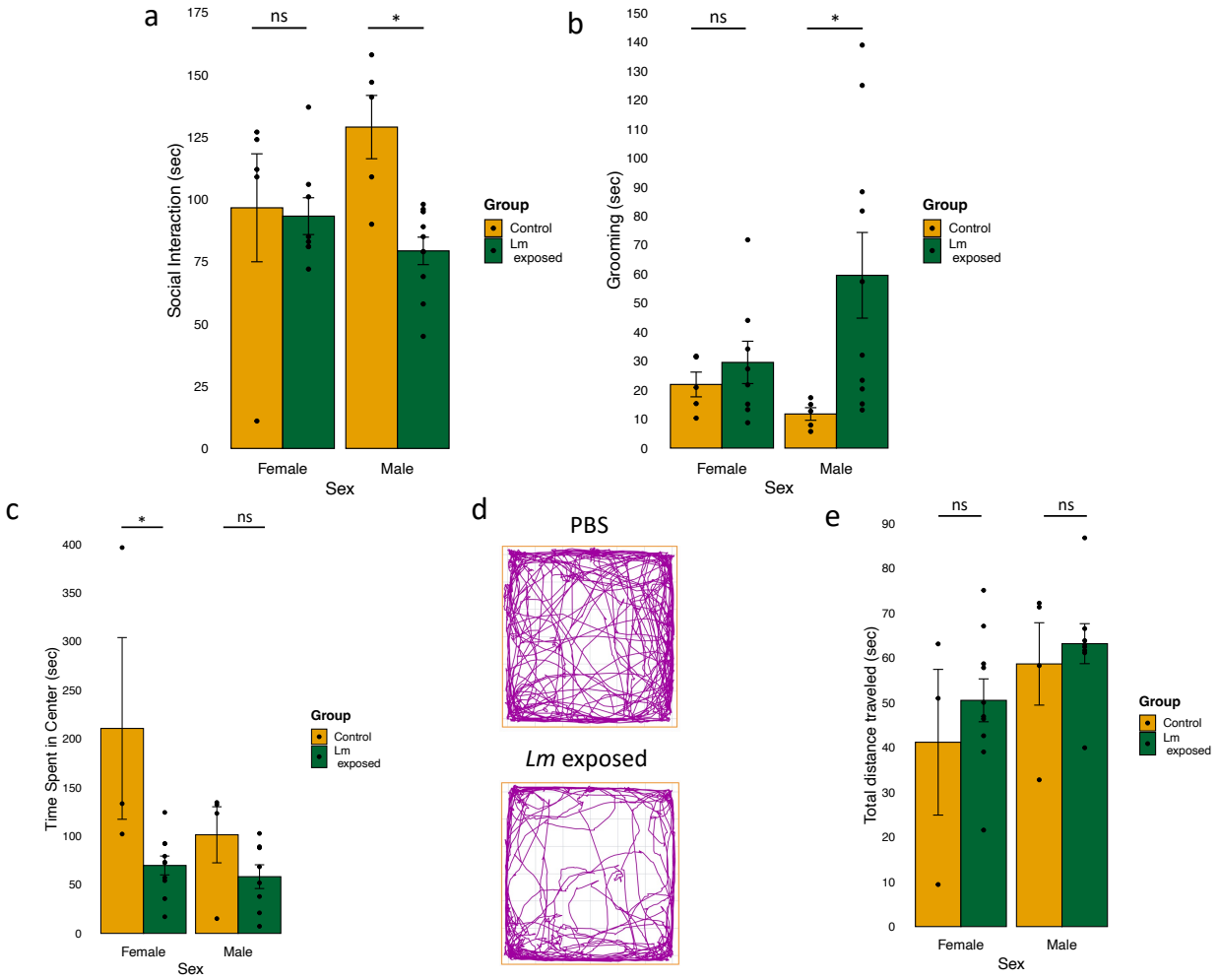

Figure 2.5. Sex-specific abnormal behaviors in the offspring of *Lm*-infected pregnant mice. **(a)** *Lm*-exposed adult male mouse offspring display deficits in social interaction (sniffing of unfamiliar mice versus inanimate objects) whereas *Lm*-exposed adult female mouse offspring show no altered behavior. **(b)** *Lm*-exposed adult male mouse offspring exhibit high levels of grooming (resembles repetitive behavior). Number (*n*) of offspring: control male ($n = 5$), *Lm*-exposed male ($n = 10$), control female ($n = 5$), and *Lm*-exposed female ($n = 8$) (a, b). **(c)** Heightened level of anxiety observed only in *Lm*-exposed adult female mouse offspring. **(d)** Differences in tracked movement during the open field exploration assay in *Lm*-exposed adult mouse offspring versus PBS controls. **(e)** No significant change in total distance traveled during open field exploration. Control male ($n = 3$), *Lm*-exposed male ($n = 8$), control female ($n = 3$), and *Lm*-exposed female ($n = 10$) used in open field exploration. Data are shown as the mean ± SEM. The behavioral assay data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test. $*P < 0.05$.

whereas *Lm*-exposed female adult offspring did not exhibit impairment in socialization (Figure 2.5a; two-way ANOVA, $p = 0.016$ by Tukey's HSD test).

To assess repetitive behavior with restricted interests, the duration of self-grooming behavior was examined during the three-chamber social approach assay. *Lm*-exposed male adult offspring spent significantly more time self-grooming compared to the PBS treated male mice (Figure 2.5b; two-way ANOVA, $p = 0.044$ by Tukey's HSD test). However, self-grooming behavior of female adult offspring was not affected by placental infection.

Next, we examined the level of anxiety and locomotion using an open field exploration test. Rodents are hesitant to enter an unfamiliar brightly lit open field, but they gradually explore the area. Higher level of thigmotaxis, a subject remaining close to walls, is usually indicative of heightened anxiety[36]. Compared with PBS treated controls, *Lm*-exposed female adult offspring spent less time in the center of the field (two-way ANOVA, $p = 0.011$ by *post hoc* test). However, *Lm*-exposed male adult offspring did not show difference in total time spent in the center compared to the PBS treated male group (Figure 2.5c). In addition, both *Lm*-exposed male and female groups showed no difference in total travel distance, which indicates locomotion activity was not affected (Figure 2.5e). Together, placental infection causes abnormal behaviors in offspring that are relevant to human neuropsychiatric disorder symptoms, including elevated anxiety, increased repetitive behavior, and impaired social interaction.

Differential expression of the activation marker c-Fos

To begin to identify changes in the adult brain due to placental infection, we immunohistochemically labeled brain sections of mice from different test groups with anti-c-Fos (Figure 2.6), which has been used to characterize neuronal activity differences in MIA models[37,38]. In these preliminary experiments, we labeled coronal sections of four male and female *Lm*-exposed mice brains and two controls mice with anti-c-Fos, a marker of brain cell activation. The results are shown in Figure 2.6. Sections from male mice exposed to prenatal *Lm* infection had increased

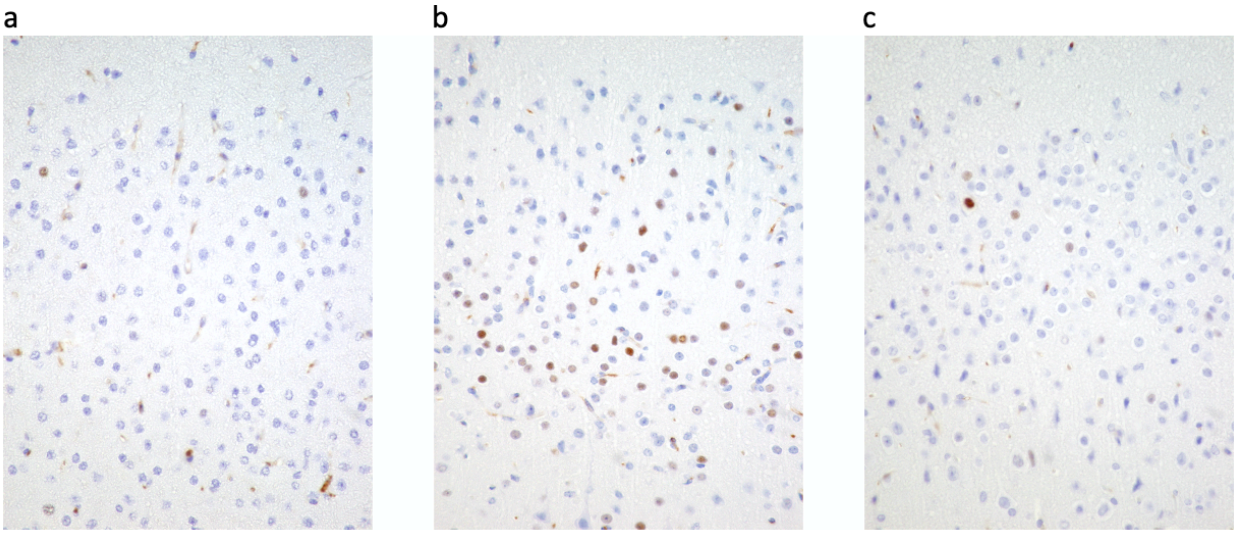

Figure 2.6. Increased nuclear c-Fos labeling in adult mouse brain due to prenatal *Lm* infection. Brains of mice that were analyzed for behavior in Figure 5 (2 infected males, 2 infected females, 2 uninfected females and one uninfected male) were immunohistochemically labeled for c-Fos. Representative sections of (a) control male; (b) *Lm*-exposed male; (c) *Lm*-exposed female. DAB chromogen (brown), hematoxylin counterstain.

c-Fos labeling compared to exposed brains from female mice and control mic. While these results are based limited in numbers of tested animals, they suggest increased neuronal activation in male mice exposed to *Lm* as a possible corollary of the sex-specific alterations of behavior

DISCUSSION

Prenatal infection is highly diverse and leads to a wide variety of outcomes both for the pregnant mother and the developing fetus. Animal models continue to reveal important mechanisms of fetal abnormality due to infection, including effects on fetal brain development that lead to abnormal behavior. Although injecting pregnant mice with immunogens, such as LPS or poly(I:C), consistently results in altered behavior and brain abnormalities in the progeny, the results of these studies are quite heterogenous[39]. In addition, these chemicals cannot be used to determine the effects of localized bacterial infections. The placental infection model using *Lm* reflects a typical subclinical infection in humans and our methods of infection allows for the analysis of abnormalities that are not due to symptomatic disease of the pregnant subject. In addition, *Lm* is well characterized and has been used for decades in placental infection models. Although listeriosis may cause serious and even fatal consequences for pregnant mothers and their offspring, its effect on neurodevelopment of the adult offspring has not been characterized. Here, we used bioluminescent *Lm* and the IVIS imaging system to determine if placental infection affects fetal neurodevelopment and the behavior of offspring in mice.

The identification of biological pathways in exposed whole brain transcriptome data suggests that placental infection with *Lm* dysregulates transcriptional levels of several different processes during neurodevelopment. First, the HIF-1 signaling pathway was upregulated, suggesting placental infection induces hypoxic conditions in the fetal brain during neurodevelopment. Numerous studies indicate that prenatal hypoxia results in various postnatal deficits, including reduced body and brain weight, delayed development, and impaired synaptic plasticity. Notably, *Vegfa* (vascular endothelial growth factor A), which promotes cortical interneuron proliferation, migration, and vasculature in the forebrain[40,41], and *Flt1* (Fms related

receptor tyrosine kinase), which plays an important role in regulation of angiogenesis and development of embryonic vasculature[42,43], are among the main genes that are upregulated in the HIF-1 signaling pathway. Dysregulation of these genes has been identified in neuropsychiatric disorders[33,44]. In addition, recent MIA studies demonstrated induction of hypoxia in the brain[4,6]. Identifying elements of conservation between MIA and bacterial infection models should be examined. Interestingly, previous rodent studies have shown that prenatal hypoxia is associated with alterations in biochemical pathways during brain development, including nucleic acids process and metabolic process pathways[45,46]. Among these pathways, *Kdm3A* (lysine demethylase 3A), which plays an important role in regulating mitochondrial biogenesis by sensing oxygen availability[47], and *Vegfa* genes were upregulated. Lastly, protein processing in the endoplasmic reticulum (ER) pathway was downregulated due to placental infection by *Lm*. Dysregulation of protein synthesis has previously been suggested as one of the cellular responses to a hypoxic condition[48] and implicated in various neuropsychiatric disorders[49]. Furthermore, a recent study found that poly(I:C) induced MIA triggers ER stress as a cellular response to inflammation and results in reduced protein synthesis[38]. Future work should examine the effect of placental infection on different types of cells in the fetal brain during neurodevelopment using single-cell RNA-seq.

Sexual dimorphism in neuropsychiatric disorders is well recognized. However, the basis of this dichotomy is unknown. One hypothesis proposes sex-specific vulnerability and response to environmental insults during pregnancy as one cause of sex dimorphism in these disorders. Recent MIA studies demonstrate that inflammation during pregnancy caused sex-biased placental and fetal pro-inflammatory responses[6]. Although we did not observe significant differences in BLI signals of *Lm* from placentas between male and female mice, sexually dichotomous responses are consistent with our transcriptional analysis. Interestingly, we observed more upregulated number

of DEGs in brain from *Lm*-exposed female mice compared to brains of *Lm*-exposed male mice (Figure 2.4b), but we did not find many biologically meaningful pathways in females. Consistent with previous MIA study, upregulation of HIF-1 signaling pathway was only enriched in *Lm*-exposed males, suggesting males are more susceptible to hypoxia during pregnancy. Future work should examine at the protein level by performing proteomic analysis to better understand how the male and female brain development is impacted by *Lm* infection during pregnancy.

Our behavioral results highlight possible pathogen specificity among rodent MIA-associated models. Injection of immune stimulants such as LPS or poly(I:C), into pregnant animals results in behavioral abnormalities in offspring that are notably relevant to ASD. Similar to these MIA-associated studies, *Lm*-exposed male offspring, but not female offspring, showed a significant reduction in social interaction and more frequent repetitive behaviors (Figure 1.5a and b). These behavioral changes, and male-biased sex ratio, are observed in human ASD patients. Interestingly, we only observed significantly increased anxiety levels in *Lm*-exposed female offspring (Figure 2.5d), whereas MIA-associated male offspring exhibited heightened anxiety levels during open field exploration. It is important to note that numerous MIA studies have investigated behavioral changes only using male offspring[50–52] because the prevalence of developing ASD is higher in males than in females. This difference remains to be further investigated; however, women are more likely to be diagnosed with human anxiety disorders. Another behavioral discrepancy was observed in locomotor activity. In our studies, placental infection did not alter locomotor activity in both sexes. Interestingly, Allard et al. demonstrated that prenatal infection with live Group B *Streptococcus*, a major health concern during pregnancy implicated in preterm birth and stillbirth, led to hyper-locomotor and elevated anxiety behaviors in male rat offspring, but not in female rat offspring[35]. Our contrasting results highlight the need

to examine diverse prenatal pathogens, as it is becoming clear that different infections result in distinct neurological abnormalities. Studies have shown that if the locomotor activity is altered due to a treatment effect, it has a confounding effect on the movement of the animal subject during open field exploration[29]. In preliminary results, we have shown increased c-Fos labeling in brain of male but not female mice when they were exposed to *Lm* in utero (Figure 2.6). This result suggests that hyperactivity of cortical neurons may be one underlying mechanism of the sexual dimorphism. Large scale and more in-depth studies will be needed to confirm this hypothesis.

One of the limitations of our studies is that individual placental BLI signals cannot be correlated with the behavior of individual offspring. Although the BLI signal of the pregnant dam can be measured using an IVIS, severity of each placental infection cannot be quantified except by sacrificing the animal. In our model, individual placentas are differentially infected by *Lm* (Figure 2.1b), and our previous findings show that fetal pathologies, such as bradycardia and fetal resorption, are correlated with BLI signals from pregnant dams. Since high BLI signals in pregnant mice may result in severe postnatal consequences for the offspring, we used the animals that showed relatively low BLI signals for the behavioral analysis. We wished to compare healthy, normal-sized offspring and did not perform behavioral studies of stunted animals such as shown in Figure 2.1d. Another limitation of this study is the limited dose and timing of *Lm* infection we selected. According to epidemiological studies, developing psychiatric disorders is highly associated with severity and timing of the infection[53]. Furthermore, MIA-associated brain transcriptomic data from LPS and poly(I:C) have demonstrated different profiles of DEGs were observed in fetal brains that were collected at various time points[4]. Different doses and timing of *Lm* infection are likely to yield different outcomes in our behavioral and transcriptomic outcomes studies and should be performed. We have not studied the consequences of direct infection of the

fetal brain, which occurs in mice with higher BLI signals, nor the effect of infection of maternal organs such as the liver or spleen, which would induce MIA. Finally, we are very interested in ascertaining the role of maternal antigen-specific immunity. These studies could be performed by vaccinating the dams before pregnancy.

In summary, we have established that placental infection by *Listeria* affects the trajectory of fetal neurodevelopment during pregnancy. We showed sex-specific dysregulation of the fetal brain transcriptome due to *Lm* infection during pregnancy. We also demonstrated that prenatal infection causes sex-specific behavioral abnormalities in offspring that resemble human ASD and anxiety-related disorders, which are known to have sexually dimorphic effects. Altogether, we have identified neurodevelopmental effects of placental infection by bacteria and expanded models of prenatal infection-associated sexual dimorphism of behavior, thus improving our understanding of the development of neuropsychiatric disorders.

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CHAPTER 3: Placental Listeria Infection Induces Autism-Related Gene Expression Patterns in the Fetal Mouse Brain

Work presented here is in preparation for submission, BMC Bioinformatics.

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ABSTRACT

Background: Prenatal infection during pregnancy has been associated with an increased risk of developing neuropsychiatric disorders, including autism spectrum disorder (ASD). *Listeria monocytogenes* (*Lm*) is a bacterial pathogen can cause adverse pregnancy outcomes, including preterm birth and miscarriage by infecting the placenta. Although maternal *Lm* infection can cause an array of consequences, the effects of placental infection on fetal brain transcriptome are poorly understood.

Results: We identified modules that were significantly associated with *Lm* placental infection and its severity using the weighted gene co-expression network analysis. We found an enrichment of genes that may affect fetal brain development during pregnancy and as well as ASD-associated genes. Lastly, we identified overlapping genes between immunogen-induced and placental infection animal models

Conclusions: Our findings show that placental infection with *Lm* affects fetal brain transcriptome during pregnancy.

BACKGROUND

Development of the fetal brain can be affected by many factors during pregnancy, which may result in altered behavior in adulthood. Autism spectrum disorder (ASD) is a neurodevelopmental disorder that affects social, communication, and repetitive behaviors[1]. The underlying mechanisms of ASD have been challenging to elucidate due to its complex genetic heterogeneity. Although ASD is known to have strongly associated with genetic factors, recent studies show that up to 55% of variance in ASD susceptibility is determined by environmental insults[2]. For example, concordance rates of developing ASD in monozygotic twins yielded 50-77% and even lower for dizygotic twins (31-36%)[2]. Epidemiological studies indicate that environmental insults, such as hospitalization with prenatal infection, may increase the risk of developing ASD[3,4]. One hypothesis is that prenatal infection disrupts fetal-maternal homeostasis and alters the trajectory of fetal brain development. The associations between prenatal infection and neuropsychiatric disorders from epidemiological studies have been extensively studied by inducing maternal immune activation (MIA) in animal models[5–8]. During pregnancy, an immunogenic substance such as lipopolysaccharide (LPS) or polyinosinic:polycytidylic acid [poly(I:C)] is injected to elicit MIA. LPS and poly(I:C) mimic bacterial and viral infections, respectively. MIA studies demonstrated that injecting these immunogens into pregnant animals results in dysregulation of the fetal brain transcriptome[9–11] and altered behaviors that resemble clinical features of ASD in humans[8,12,13]. Although these experiments have revealed much about MIA and altered brain development, much less is known about the consequences actual prenatal infection.

Listeria monocytogenes (*Lm*) is a pathogenic bacterium that mainly affects immunocompromised individuals, such as pregnant women, causing fever, diarrhea, and

meningitis in severe cases[14,15]. Listeriosis can be 20% fatal even when the patient is hospitalized and treated with antibiotics[16]. Processed meats, vegetables and dairy products are susceptible to contamination with *Lm*[17]. After ingesting contaminated food with *Lm*, the bacterium can disseminate through the body by utilizing its intracellular lifecycle, largely in monocytes and macrophages, which are mobile cells that spread the infection[18]. In pregnant women, *Lm* infects the placenta, leading to many overt fetal and newborn pathologies, including spontaneous abortions, stillbirth, and neonatal meningitis[19]. Although pregnant women are highly susceptible to listeriosis, these infections can be subclinical and barely noticeable, while still affecting the fetus or newborn[20–22]. Little is known about the neurological outcomes of offspring exposed to *Lm* during pregnancy, or the effects of placental infection on fetal brain development.

In this study, we analyze fetal brain RNA-seq data that was previously generated using a pregnant CD1 mouse model of *Lm* to investigate how the transcriptome is altered by placental infection. Utilizing bioinformatics approaches and the Weighted Gene Co-Expression Network Analysis (WGCNA) R package[23], we demonstrate that placental infection with *Lm* is significantly associated with co-expression modules of specific genes in the fetal brain and identified enriched pathways that may affect neurodevelopment. The altered expression of these pathways in the fetal brain correlates with the degree of placental infection. We also identify conservation between ASD-associated genes and co-expression modules. We then compare our findings with an existing MIA RNA-seq dataset to identify elements of conservation between the effects of placental infection and immune activation on neurodevelopment during pregnancy. Together, we demonstrate that placental infection with *Lm* induces ASD-associated gene expression patterns in the fetal brain during pregnancy at the systems level.

RESULTS

Identifying modules associated with sex, placental infection, and infection severity traits

RNA-seq data was generated from fetal brains excised from pregnant mice infected with bioluminescent *Lm*. Infection of the individual placentas was quantified with bioluminescence signals (Figure 3.1A and B), and the associated fetal brains were excised for RNA-seq. In this manner, changes in the fetal brain transcriptome could be correlated with the degree of placental infection. In our study, we used WGCNA to identify co-expressed modules that are associated with traits known to contribute to having significant pregnancy outcomes and their key hub genes. To minimize background noise, we filtered out lowly expressed genes (fewer than 10 raw reads and expressed in at least 20 fetal brain samples), working with 17,106 genes. The value of soft-threshold power (adjacency matrix weighting) was set to 12, which was when the square value of the correlation coefficient reaches 0.9 as developers' recommendation. A total of 26 co-expression modules, which are arbitrarily named after colors, were identified after merging modules whose expression profiles are similar to each other using the consensus network using a 0.25 threshold (Figure 3.2B). We then examined the association between different traits, placental infection with *Listeria monocytogenes* (control vs. infected, regardless of bioluminescence signal), severity of placental infection (based on bioluminescence signal from the placenta), and sex (males and females regardless of bioluminescence signal) with identified modules. Modules with p-value < 0.05 were considered significant. A total of nine and six modules were significantly correlated with infection and severity, respectively. None of the modules were significantly correlated with sex independent of infection or bioluminescence signal (Figure 3.2C). These results suggest that prenatal infection and its severity dysregulate at the systems level; however, sex by itself does not have an impact on the gene networks.

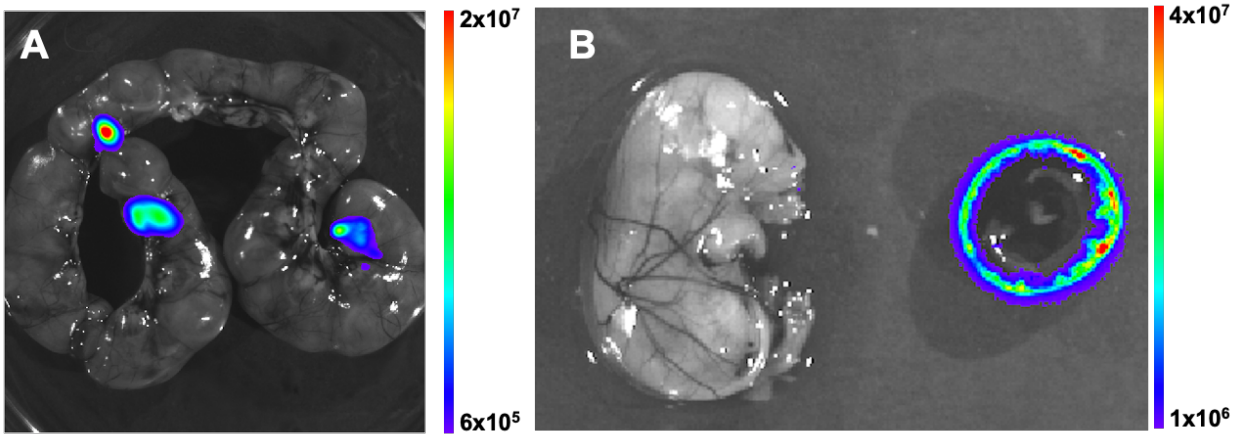

Figure 3.1. Bioluminescence imaging of placental infection by *Lm* (A) Bioluminescence image of excised uterine horns from infected pregnant mouse. **(B)** Image of fetus (left) and the infected placenta (right). BLI signal is indicated in photons/s/cm^2/str.

Pathways of infection- and severity-associated modules are associated with abnormal neurodevelopment in the fetal brain

Having identified co-expressed modules, we then performed gene ontology biological process (GO BP) analysis to identify functional pathways of these gene clusters. Several metabolic and biological pathways were enriched in genes within placental infection-associated modules, including vesicle-mediated transport, protein ubiquitination, mitochondrial gene expression, and neurogenesis (Figure 3.3A). We also observed enriched pathways related to hypoxia, fetal development, and gene regulation in modules associated with the severity of placental infection (Figure 3.3B). Interestingly, we found biological pathways that are enriched in modules that are associated with placental infection and also correlated with the severity of that infection as measured by bioluminescence signal in the placenta. Enriched pathways, such as cilium organization, response to stress, and DNA repair/recombination were correlated in both infection vs control and infection level datasets (Figure 3.3C). Hub genes, the most correlated gene in each module, were identified and listed in Table 3.1. Our pathway enrichment results suggest that placental infection with *Lm* and its severity may affect neurodevelopment during pregnancy by

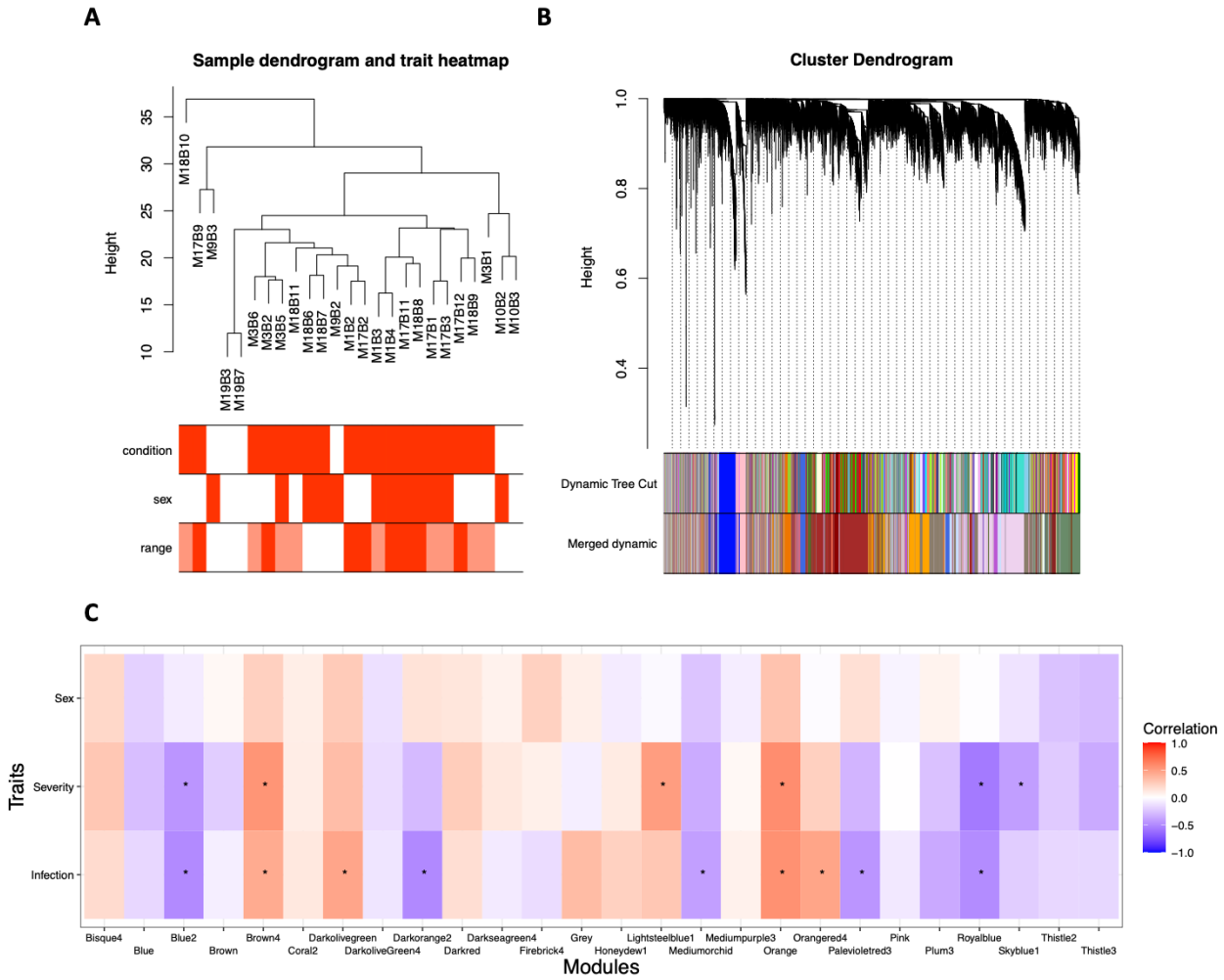

Figure 3.2. Alterations in gene networks in the fetal brain due to placental infection with *Lm* identified by using Weighted gene co-expression analysis (WGCNA). (A) Hierarchical clustering of sample dendogram and trait heatmap. Red indicates brains from infected mice in condition trait; red indicates brains from male mice in sex trait; white color indicates low, salmon color indicates medium, and red indicates high BLIs. **(B)** Cluster of dendogram of all genes were assigned into modules based on topological overlap. A total of 26 co-expression modules were identified after merging modules that were similar in gene expression which are arbitrarily named after colors. **(C)** Heatmap of the association between module eigengenes and different traits; * indicates a significant association between the module and trait ($p < 0.05$). Red indicates high and purple indicates low correlation. Correlation strength is represented by the intensity of color.

indirectly activating and repressing specific genes in the fetal brain.

Dysregulation of eigengene expression due to prenatal infection with *Lm*

We then investigated the levels of dysregulation of gene expression induced by prenatal

Table 3.1. List of hub genes from modules that are significantly associated with different traits.

Module	Ensembl.ID	Gene.symbol	Function
Blue2	ENSMUSG00000033508	Asprv1	Aspartic peptidase
Brown4	ENSMUSG00000032580	Rbm5	Regulates alternative splicing of a number of mRNAs
Darkolivegreen	ENSMUSG00000056476	Med12l	RNA polymerase II transcription
Darkorange2	ENSMUSG00000030374	Strn4	May function as scaffolding or signaling protein
Lightsteelblue1	ENSMUSG00000023852	Chd1	Transcription regulatory histone acetylation
Mediumorchid	ENSMUSG00000058833	Rex1bd	Required for excision 1-b domain containing
Orange	ENSMUSG00000022822	Abcc5	A multispecific organic anion pump
Orangered4	ENSMUSG00000050930	Map10	Regulate cell division and promote microtubule stability
Palevioletred3	ENSMUSG00000018411	Mapt	Promotes microtubule assembly and stability
Royalblue	ENSMUSG00000018865	Sult4a1	May have a role in the metabolism of neurotransmitters
Skyblue1	ENSMUSG00000032289	Thsd4	Promotes FBN1 matrix assembly

infection with *Lm* and its severity. The modules that were positively correlated (upregulated) showed a higher average gene expression profile in the *Lm*-exposed fetal brain during pregnancy, whereas negatively correlated (downregulated) modules showed a lower average gene expression profile in the *Lm*-exposed fetal brains, except the Darkorange2 module (Figure 3.4A). Interestingly, the gene expression profile was correlated with the severity of placental infection by *Lm*. The placental infection associated with a high bioluminescence signal, which indicates higher infection levels of *Lm* in the placenta, showed the highest gene expression profile compared to medium and low placental infection severity in the upregulated modules. Conversely, a low bioluminescence signal from the placenta showed the highest gene expression profile in the downregulated modules (Figure 3.4B). Together, these data demonstrate that placental infection with *Lm* alters gene expression profile in the fetal brain that is correlated with placental bioluminescence signal. Thus, the degree of placental infection correlates with the gene expression changes in the fetal brain.

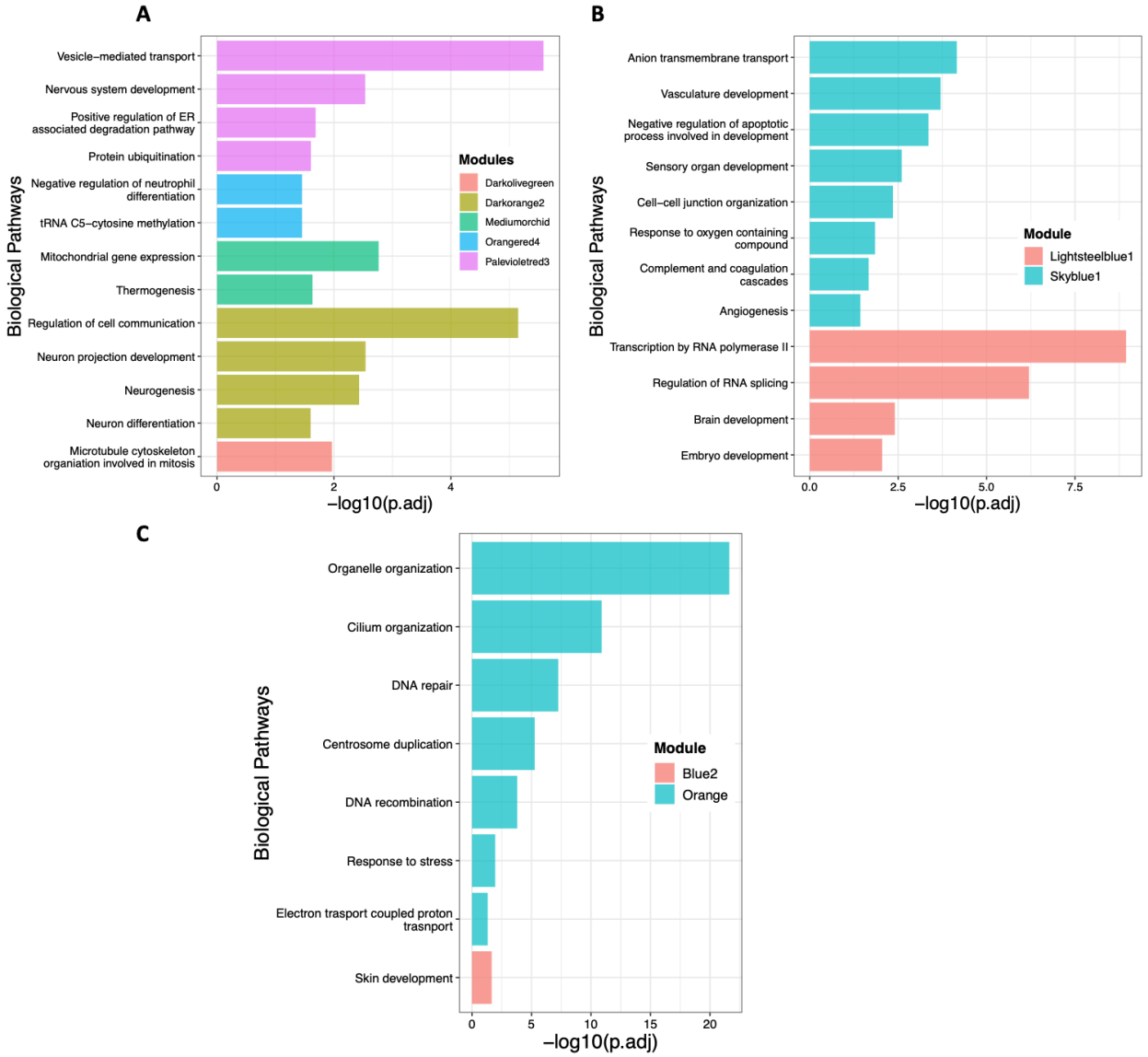

Figure 3.3. Gene ontology (GO) pathway enrichment analysis of all the genes in the co-expression modules. **(A)** GO biological process (BP) analysis of modules that are significantly associated with prenatal infection with *Lm* (padj < 0.05), **(B)** GO BP analysis of severity of placental infection, and **(C)** GO BP analysis of both traits, infection, and severity.

Association of Lm-enriched pathways with ASD

Because prenatal infection with other bacteria has been associated with ASD, we next examined the enrichment of ASD-associated genes within modules that were significantly correlated with the traits we previously defined in our data. To do this, we downloaded the mouse

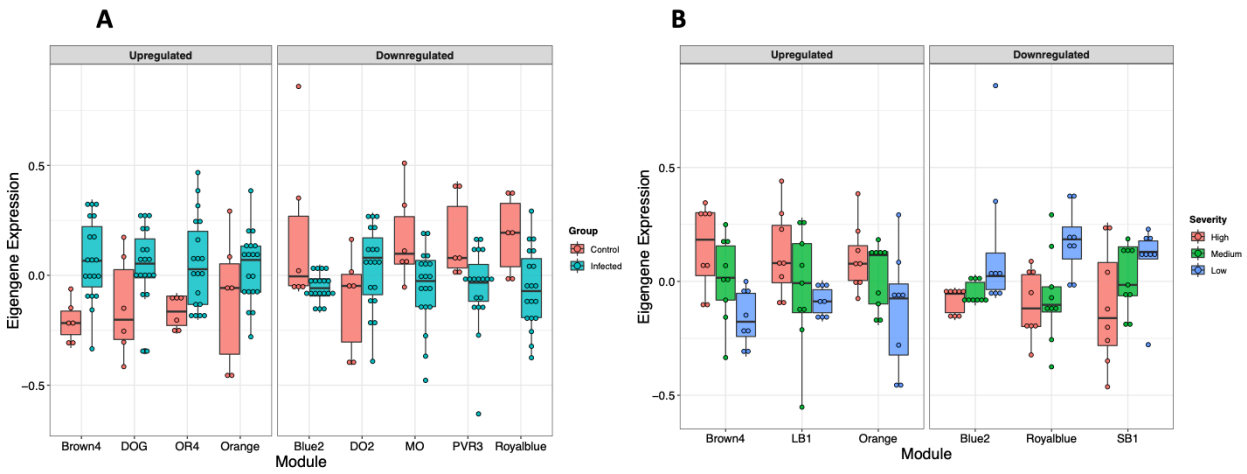

Figure 3.4. Prenatal infection with *Lm* induces dysregulation of eigengene expression profile **(A)** Eigengene expression (Y-axis) of modules that are associated with prenatal infection with *Lm* and control. DOG: Darolivegreen, OR4: Orangered4, DO2: Darkorange2, MO: Mediumorchid, PVR3: Palevioletred3. **(B)** Module eigengene expression of severity of placental infection with *Lm*. LB1: Lightsteelblue1, SB1: Skyblue1.

models of ASD associated gene-set from the Simons Foundation Autism Research Initiative (SFARI) Gene database and compared our modules to this dataset[24]. In the upregulated modules, a total of 29 ASD associated genes overlapped with infection and severity traits (Figure 3.5). Interestingly, modules that are associated with prenatal infection with *Lm* showed higher number of overlapping genes compared to genes enriched within modules that are associated with severity. Similarly, a higher number of ASD associated genes were detected within the downregulated modules associated with infection. These results suggest that placental infection with *Lm* alters the neurodevelopment of the fetus at systems level and further support the evidence that dysregulation of fetal brain transcriptome is correlated with bioluminescence signal from the placenta.

Elements of conservation with another prenatal infection model

We sought to identify conserved elements between distinct systems by comparing gene expression datasets to those observed in another animal model that stimulated prenatal infection. We selected an RNA-seq dataset resulting from the induction of MIA by injection of poly(I:C), an

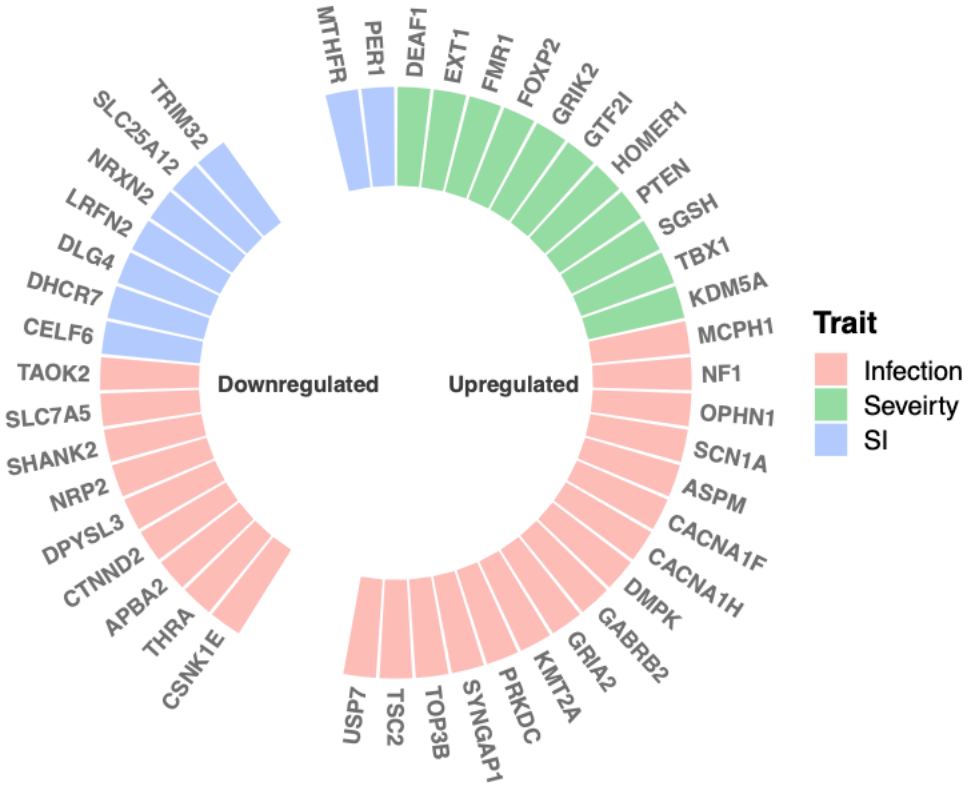

Figure 3.5. Autism spectrum disorder associated genes are enriched in the co-expression modules associated with infection and severity. Circular bar plot of autism spectrum disorder associated genes enriched in modules that are significantly associated with different traits. Infection: control vs infected; severity: bioluminescence signal from the placenta; SI: severity and infection. Upregulated and downregulated indicate positive and negative correlation in modules, respectively. Upregulated and downregulated indicate positive and negative correlation in modules, respectively.

immunogen that mimics a viral infection. We used the RNA-seq dataset generated on embryonic day 17.5 (E17.5) using the developing cortex and pallium of the fetal brain based on similar harvesting days. For the poly(I:C) dataset, we performed differential gene expression analysis to identify up- and downregulated genes, and we compared overlapping genes within modules. A total of 350 upregulated genes were identified, whereas 220 downregulated genes were observed between systems (Figure 3.6A and B).

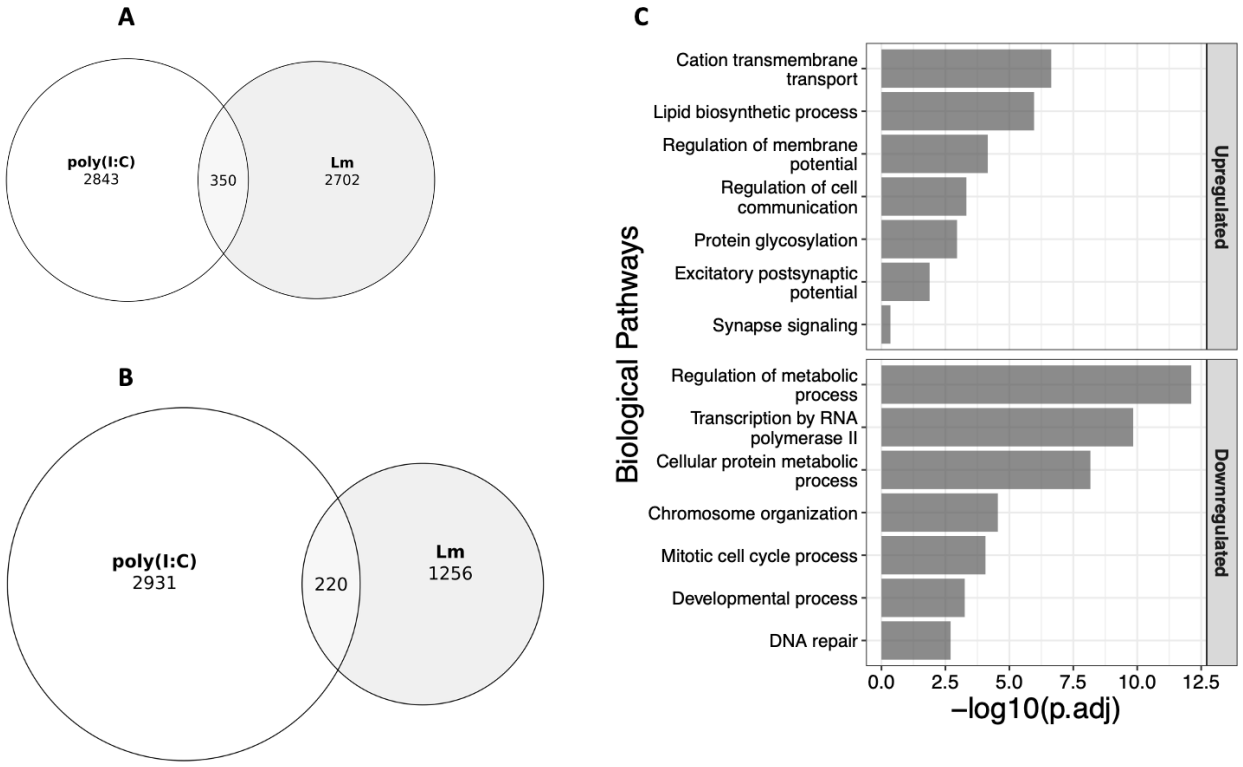

Figure 3.6. Elements of conservation between poly(I:C) and *L. monocytogenes* infection animal models. (A, B) Venn Diagram representing the number of overlapping upregulated and downregulated genes between poly(I:C) and *Lm* models in **(A)** and **(B)**, respectively. **(C)** GO BP enrichment analysis of overlapping upregulated and downregulated genes between maternal immune activation animal model with poly(I:C) and prenatal infection with *L. monocytogenes*. Y-axis represents GO BP terms and x-axis represents -log10(padj) value.

We then performed pathway analysis to determine which biological processes were enriched in the overlapping gene sets. *Lm*-exposed and poly(I:C) upregulated genes displayed some similar enriched functions, including cation transmembrane transport, regulation of membrane potential, and excitatory postsynaptic potential. Genes downregulated by *Lm* and poly(I:C) exposure showed pathways, such as transcription by RNA polymerase II, chromosome organization, and developmental processes. The overlapping enriched pathway analysis revealed the similarity of the two different animal models, which both induce abnormal brain development. However, there were many genes that were regulated differently, which highlights the need to understand different prenatal infections.

DISCUSSION

Fetal brain development is a subtle process that can be perturbed in many ways, leading to a variety of neurodevelopmental and psychiatric disorders. Multiple epidemiological studies demonstrated that prenatal infections may increase the risk of developing neuropsychiatric disorders, such as ASD[4,25–28]. The hypothesis behind this phenomenon is that maternal infections during pregnancy can alter the trajectory of early-stage development of fetal brain, which results in long term consequences for neurodevelopment. However, the molecular mechanisms leading to ASD remain unclear. *Lm* is a foodborne pathogen that can cause detrimental outcomes to pregnant women by invading the placenta. Even though placental infection with *Lm* causes an array of severe consequences in both the mother and the fetus, its effects on neurological outcomes are poorly understood. In this study, we used bioluminescent *Lm* to investigate fetal brain transcriptome changes caused by placental infection at the systems level by using the WGNCA analysis.

We detected co-expression modules that were only associated with prenatal infection by *Lm* and severity of placental infection measured by bioluminescence signals, but not sex itself in and of itself, independent of all other factors. Sex is a well-known risk factor in numerous neuropsychiatric disorders, and sexually dichotomous responses to environmental insults have been observed in numerous MIA studies[10,12,29,30]. Consistent with these reports, our data indicate that sex alone is not associated with any dysregulation of fetal brain transcriptome[9]. Prenatal infection with *Lm* and severity of the placental infection were the main drivers of altered gene expression of the fetal brain. GO analyses suggest that co-expression modules associated prenatal infection by *Lm* may induce abnormal fetal neural development. We found dysregulation of cell communication, neuron projection, differentiation, and neurogenesis (Figure 3.3a). Neurogenesis

is a tightly regulated process that plays a critical role in generating neurons and can lead to abnormal brain development if it is disrupted[31]. As consequences, studies have shown altered neuron projection[32], abnormal brain structure[33], and dysregulation of neural migration[34] and maturation[35] in patients with ASD. It will be important to identify the placental factors responsible for altered fetal brain development in the context of *Lm* infection. The severity of the placental infection was also correlated with the disruption of fetal brain gene networks. This result indicates that the effects are localized to the placenta rather than systemic throughout the entire pregnant animal, because placentas with high and low bioluminescence signals occur in the same animal. Previous studies show positive correlation between the risk of developing ASD and the degree of maternal fever during pregnancy[27,28,36]. One possible mechanism is that overall protein expression levels are related to the severity of infection. In our studies, we found GO terms, such as transcription by RNA polymerase II and regulation of RNA splicing, that may affect overall gene expression levels and result in dysregulated protein synthesis (Figure 3.3b). Previous MIA studies show an injection of poly(I:C) during pregnancy induced dysregulation of protein synthesis[10]. Future studies should examine infection dose-dependent protein synthesis levels in the fetal brain during pregnancy.

Prenatal infection with *Lm* and its severity enrich ASD associated genes. Notably, both positively and negatively correlated genes within the co-expression modules are enriched in synaptic signaling. These genes are involved in trans-synapse signaling (EXT1, GRIK2, HOMER1, PTEN, NF1, OPHN1, DMPK, GABRB2, KMT2A, and SYNGAP1) and post-synapse organization (CTNND2, NRP2, SHANK2, DLG4, LRFN2, and NRXN2). Synaptic signaling is important for communication between neurons, and alterations in synapses have been observed in both animal models and patients with ASD[37–39]. It will be important to examine the effects of

prenatal infection with *Lm* on the balance between excitatory and inhibitory neurons in the fetal brain. MIA-induced fetal brain transcriptome studies also revealed enrichment of dysregulated synapse development[9,40]. Although it is not clear whether the same genes that are involved in synapse signaling, both MIA and prenatal infection models suggest that the ASD associated genes are affected in brain development.

We also examined an existing MIA-induced transcriptome dataset using poly(I:C) to identify elements of conservation between systems. Numerous studies have shown that injection of poly(I:C), which mimics a viral infection and induces proinflammatory cytokines, results in altered morphology of cortex[5], dysregulated fetal brain transcriptome[9,10,40–42], and abnormal behaviors that resemble ASD[5,13,43]. Interestingly, overlapping downregulated genes were significantly enriched in biological pathways such as transcription by RNA polymerase II and mitotic cell cycle. In contrast, biological pathways such as regulation of membrane potential and synapse signaling were observed in upregulated genes. These pathways further suggest that environmental insults such as prenatal infection or inflammation influence protein synthesis and result in abnormal brain development, including imbalance of excitatory and inhibitory neurons.

CONCLUSION

Collectively, our data associate placental infection by *Lm* with dysregulation of gene networks in the fetal brain. Co-expression modules correlate with the severity of infection as measured by the bioluminescence signal of the placenta, suggesting localized rather than systemic effects. GO terms relevant to neuronal development and gene expression were enriched in the modules that were associated with placental infection by *Lm*. In the co-expression modules associated with the severity of placental infection with *Lm*, GO terms related to neurogenesis and gene expression that may affect neurodevelopment were identified. We observed that gene expression levels of co-expression modules correlated with the severity of placental infection. Overall, our studies identified specific ASD-associated pathways induced in the fetal brain by infection of the placenta, suggesting that placental infection may cause autism.

METHODS

Animals

All animal experiments were approved under Michigan State IACUC protocol 201800030, and Biosafety Protocol 0000058. BSL-2 animal procedures were followed according to these protocols. The infection of pregnant CD1 mice with bioluminescent *Listeria* has been described. Briefly, the bacteria were grown to logarithmic growth phase and diluted in phosphate buffered saline (PBS) for injection via tail vein. $2x10^5$ colony forming units (CFU) of bioluminescent *Listeria* strain 2C (Xen32) were injected into pregnant mice on gestational day 14.5. the mice were imaged using an IVIS system (Perkin Elmer Inc.) on gestational day 18.5. Uterine horns were excised and imaged to determine infection levels and associated fetal brains were removed for RNA purification. Processing of tissues and RNA preparation have been described. Briefly, total RNA was extracted using Qiagen QIAzol reagent, quantified on a Bionalyzer and Illumina TrueSeq mRNA libraries were prepared and sequenced by the Michigan State University Genomics Core. For a complete description of these methods see Lee *et al*., BioRxiv, 2022.

Weighted gene co-expression network analysis

The WGNCA package (v1.70-3) in R was used to identify highly correlated gene networks[23]. Before WGCNA, lowly expressed genes (raw counts fewer than 10 in 20 samples) were filtered out to minimize background noise. After filtering, raw counts were normalized using the vst() function from the DESeq2 package. We set the soft-threshold power value to 12, which was the lowest power for the scale-free topology fit index that reached 0.9, as developers' recommendation. Co-expression networks were constructed using the blockwiseModules function (minModuleSize = 30 and maxBlockSize = 25,000). Topological overlapping matrix (TOM) and topological dissimilarity matrix (1 – TOM) were created by transforming the adjacency matrix. We then

identified modules using the cutreeDynamic function and merged them whose expression profiles were similar using the threshold of 0.25. Student asymptotic p-values were used to calculate correlation between sample traits and modules. Modules were summarized by the module eigengenes, which named after colors. Modules with less than p-value of 0.05 were considered significant and were used for further analysis. Hub genes from each module were identified using the chooseTopHubInEachModule() function.

Autism spectrum disorder associated genes overlapping analysis

The animal models of autism spectrum disorder (ASD) associated gene-set (1/11/22 released) from SFARI (https://gene.sfari.org/) was downloaded on 4/28/22. A total of 218 ASD associated genes were included in the gene-set. Overlap of genes in the co-expressed modules (positive and negative correlation) that are significantly associated with traits (infection and severity) with animal models of ASD associated genes were analyzed.

MIA developing cortex transcriptome dataset and differential gene expression analysis

A gene count matrix was downloaded from Gene Expression Omnibus (ID: GSE166376) published by Canales *et al*. Briefly, maternal immune activation of pregnant C57BL/6N mice was induced by injecting poly(I:C), which mimics a viral infection, on embryonic day 12.5 (E 12.5). Parts of fetal brains (telencephalon/pallium or developing cortex) were harvested at E12.5 + 6 hour, E14.5, E17.5, and postnatal day 0 and proceeded to bulk RNA-seq. Based on similar brain harvesting days, we used the E17.5 RNA-seq dataset to observe elements of conservation between two animal models. Differentially expressed genes (DEGs) of MIA dataset were then identified using DESeq2 in R. Lowly expressed genes (raw counts < 5 across in at least 10 samples) were filtered out prior to DEG analysis, resulted in 14,995 genes. DEGs with padj < 0.05 were considered significant and were used for further analysis.

Functional enrichment analysis

Gene Ontology biological pathway (GO BP) and KEGG enrichment analysis were performed using the g:Profiler system (https://biit.cs.ut.ee/gprofiler/gost). Biological pathways with padj < 0.05 were considered significant.

DECLARATIONS

Ethics approved and consent to participate

All mice procedures were approved by Institutional Animal Care and Use Committee and the Biosafety Committee of Michigan State University (MSU) under animal protocol number 201800030.

Consent for publication

Not applicable.

Availability of data and materials

RNA-seq fastq and raw counts of the dataset can be access through the NCBI Gene Expression Omnibus.

Competing interests

The authors declare no competing interests.

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Author's contribution

K.H.L. designed the experiment, performed bioinformatics/systems biology analyses, wrote the draft and edited the manuscript A.K. supervised the team with feedback and edited the manuscript. J.H. conceptualized the project, supervised the team with feedback and evaluation of the project, and edited the manuscript. All authors read, edited, and approved the final manuscript.

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CHAPTER 4 – Concluding Chapter

INTRODUCTION

Clear causes of many neuropsychiatric disorders have yet to be identified. Recent large-scale genetic studies have revealed a limited genetic association with autism spectrum disorder (ASD)[1]. A possible etiology of ASD has been suggested as disturbed brain development during pregnancy. Environmental insults, including prenatal infection, can cause detrimental outcomes to the pregnant mother and the fetus during pregnancy. Numerous epidemiological studies have demonstrated the association between prenatal infection and neuropsychiatric disorders, including ASD[2–6]. It is important to understand the specific consequences of infection by diverse prenatal pathogens because they are associated with different neurodevelopmental disorders. The foodborne pathogen *Lm* is a health concern that can cause placental dysfunction during pregnancy. Listeriosis during pregnancy can result in stillbirth, abortion, and neonatal meningitis[7,8]. Despite pregnant mothers being highly susceptible to listeriosis, the neurological outcomes in offspring subject to placental *Lm* infection have not been characterized. The studies in my dissertation focused on the consequences of prenatal infection with *Lm* on fetal brain development during pregnancy and its effect on behavior in the offspring. The main objectives of my dissertation were to (1) determine how placental *Listeria* infection alters fetal brain gene expression pertaining to ASD and evaluate sexually dichotomous gene expression response to maternal listeriosis (2) determine its effects on the gross anatomy of the brain structure, and (3) evaluate the behavior of mouse offspring exposed to maternal listeriosis.

Summary and future studies for Chapter 2

In chapter 2, I investigated the neurological outcomes of placental infection with *Lm*. Particularly, I studied the effects of prenatal infection with *Lm* on fetal brain gene expression, brain

morphology, and behaviors in the offspring. The *Lm*-exposed fetal brain showed dysregulated gene expression profiles. According to differential gene expression (DGE) analysis, females and males responded differently to placental infection. Genes that are involved in hypoxia, vascular endothelial growth factor, and histone activity were enriched in males, whereas females showed nuclear body/speck pathways. The morphology of the fetal brain is also altered due to placental infection with *Lm*. The H&E staining showed that placental infection disrupted cortical lamination during gestation. We observed a more prominent alteration of cortex development with a high BLI signal, whereas normal cortical lamination was observed with a low or absence of BLI signal within the same infected pregnant mouse. Our findings suggest an effect of localized placental infection rather than a systemic effect. Furthermore, we investigated neural activity using IHC. Although it was preliminary, my results indicated that males showed an increased number of nuclear c-Fos labeling in the mouse brain, suggesting an imbalance between inhibitory and excitatory neurons. The altered ratio of neurons has been observed in individuals with ASD. We also observed postnatal effects of *Lm* placental infection. *Lm*-exposed offspring displayed stunted growth (low body weight) and delayed eye-opening. Lastly, offspring exposed to *Lm* during pregnancy exhibited phenotypes resembling ASD. Female offspring exhibited normal social behavior, while male offspring showed deficits in social interaction and repetitive behavior. Furthermore, females showed a heightened level of anxiety during open-field exploration. Lastly, locomotion behavior was not altered in either sex, indicating that the behavior assays were not affected by their ability to move. Taken together, these results demonstrated that placental infection by *Lm* alters fetal brain development during pregnancy and affects the offspring behavior in a sex-specific manner.

Although cortical layers showed prominent disruption due to *Lm* placental infection, we have not identified which layers were affected. The cerebral cortex is a complex laminated structure with six different layers (I–VI) containing various types of cells. To study the integrity of cortical layers, immunofluorescence staining using neuronal layer-specific markers can be used. Biomarkers such as special AT-rich sequence-binding protein (layer II-IV)[9], T-box brain 1 (layer VI)[10], and COUP-transcription factor-interaction protein 2 (layer V and subset of VI)[11] can be used to evaluate the cortex development. These biomarkers also represent excitatory neurons. One can target parvalbumin interneurons to quantify inhibitory neurons and observe the ratio between inhibitory and excitatory neurons[12]. If we see an imbalance between inhibitory and excitatory neurons, this would confirm that placental infection with *Lm* causes abnormal neural activity. Analysis of morphological brain differences between females and males should be investigated.

Future studies should perform more behavior tests. In Chapter 2, we only investigated social interaction, repetitive and anxiety-related behavior assays. Different tests can be utilized to assess offspring's behaviors in other neuropsychiatric disorders. For example, the novel object recognition and fear conditioning tasks can be performed to test the subject's cognitive function and memory[13,14]. Furthermore, impaired communication is one of the hallmark behaviors of ASD. The ultrasonic vocalization test can be performed to examine subject's communication impairment[15]. Lastly, the olfactory and the wire hang tests can be used to measure the ability to smell and evaluate the motor function, respectively[16,17].

To strengthen the analysis of fetal brain transcriptome, single-cell RNA-seq (scRNA-seq) should be performed to investigate how different cells are affected in the brain due to placental infection with *Lm* during gestation. We can classify major cell subtypes in the brain by using the clustering method (the Seurat package in R)[18] and perform DGE analysis to investigate the effects

of placental infection during pregnancy using scRNA-seq. Sex differences should be investigated, as well.

Summary and future directions of Chapter 3

In chapter 3, I investigated the effects of placental infection with *Lm* on fetal brain transcriptome at the systems level using WGCNA. Several modules (clusters of genes that are highly correlated with each other based on gene expression patterns) were identified and then tested for association with different traits. Notably, modules were correlated with prenatal infection and the severity of the infection, which was measured by BLI signal. I found that sex, independent of prenatal infection, was not significantly correlated with any of the modules. These findings suggest that prenatal infection and its severity are the main traits that dysregulate the fetal brain gene expression profiles. The enrichment analysis identified biological pathways that may affect neurodevelopment during pregnancy. The gene expression profiles were correlated with the severity of placental infection, indicating that severe prenatal infections are associated with poor pregnancy outcomes. I identified ASD-associated genes that were enriched in both traits. There were more ASD-associated genes enriched in co-expression modules associated with infection than the severity, indicating the detrimental consequences of prenatal infection with *Lm* during pregnancy. The enrichment analysis of ASD-associated genes showed that several genes were involved in synaptic transmission and behavior-related pathways. Alterations in synaptic transmission have been observed in ASD patients, as well as MIA-induced fetal brain transcriptome. Lastly, I investigated the elements of conservation between poly(I:C) exposure and *Lm* infection in animal models. Biological pathways involved in cell communication and synapse signaling were upregulated, whereas cell cycle and protein synthesis-related pathways were downregulated. These results illustrated the similarity between MIA and *Lm* placental infection

animal models. Importantly, many genes were distinctively expressed in *Lm*-exposed fetal brain gene expression, which warrants further investigation. Overall, Chapter 3 identifies ASD-associated genes induced in the fetal brain by maternal listeriosis and highlights the effects of placental *Listeria* infection and its severity on brain development during pregnancy.

While Chapter 3 mainly focused on fetal brains that were harvested on E18.5, additional time points should be performed to better define the impact of maternal listeriosis on fetal brain development. To study how placental *Listeria* infection alters the trajectory of brain development during pregnancy, 3 different time points should be investigated. We infected pregnant mice with *Lm* on E14.5 because pregnant mice do not display disease symptoms, which reflects typical subclinical infection in humans. Fetal brains should be harvested on E15.5, 16.5, and 17.5 to perform RNA-seq analysis. The correlation between time points and modules can be analyzed using WGCNA. Previous MIA-induced time point study showed a strong correlation with age (time points) of the brains. Lastly, brains should be collected after completing behavioral tests (12 weeks old) for RNA-seq analysis. It would be interesting to see whether gene expression profiles are correlated with the severity of abnormal behavior of the *Lm*-exposed offspring. Furthermore, different regions of the brain should be dissected to study the effects of placental *Lm* infection during pregnancy. My study used the whole brain to perform gene expression analysis. Braun and colleagues demonstrated that the cortex showed more prominent hypoxia than other brain regions after injection of LPS during pregnancy, suggesting different susceptibilities to inflammation during brain development.

In Chapter 3, I used ASD genes from the SFARI Gene database (https://gene.sfari.org/) to study the association between placental *Listeria* infection and neuropsychiatric disorders[19]. I chose ASD because the genetic component of the animal model is well characterized. As of today (May

2022), a total of 220 ASD-associated genes have been identified. To strengthen the association between maternal infection and neuropsychiatric disorders, other illnesses should be investigated, including SZ. It is a complex neuropsychiatric disorder that affects the individual's behavior and cognitive ability. Genetic and environmental factors, including maternal infections and asphyxia, are thought to play roles in the etiology of SZ; however, its exact mechanism remains unclear[20–23]. To investigate whether SZ-associated genes are enriched within the placental *Listeria* infection gene network, the schizophrenia exome meta-analysis consortium database (SCHEMA) (https://schema.broadinstitute.org/) can be used for the analysis[24]. This database was generated using sequencing data of SZ patients. In addition, studying the elements of conservation with other animal models is needed. In Chapter 3, a poly(I:C)-induced bulk RNA-seq dataset was utilized to study the conservation. Poly(I:C), a synthetic double stranded RNA, is used to induce immune responses via Toll-like receptor binding. This molecule is used in many prenatal infection models and induces specific responses in the fetal brain. Comparing the dataset in this study with other RNA-seq datasets that were generated using environmental factors, such as LPS, would be interesting to see whether placental *Listeria* infection has its own unique pathogenicity.

Concluding remarks

Prenatal infection can cause detrimental outcomes for the pregnant mother and the fetus, as well as long-term postnatal effects on the offspring. In this study, I have demonstrated perturbation of the fetal brain transcriptome due to placental infection with *Lm*. The main drivers of these changes are placental infection and its severity. Sex-biased gene expression profiles were observed in *Lm*-exposed fetal brains. We also identified genes that were associated with ASD in our gene networks. Placental infection also induced alterations in behaviors that resembled ASD

symptoms in the offspring. In addition, placental infection altered cortical lamination was correlated with BLI signal, which illustrated the localized effect of placental infection. In conclusion, this work illustrated the neurological outcomes of placental infection by *Lm*.

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