

MICRORNA AS A POTENTIAL MEDIATOR IN MATERNAL/FETAL  
INTERACTION IN NEURODEVELOPMENTAL DISORDERS

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NEURODEVELOPMENTAL DISORDERS

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## DEDICATION

My rock(s), Atira, Sokka, Suki, Sabrina

My family, close and far, inherited and chosen

My physical health, Datesh, Marlene, Susan, Shanaz, Thomas, Jennie, Courtney, Elena

My mental health, Erica, Zane, Kimberly

My village, thank you

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*“Perfect is the enemy of good.”*

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## LIST OF ABBREVIATIONS

5-HT – Serotonin

ACE – Adverse Childhood Events

ADDM – Autism and Developmental Disorders Monitoring Network

ADHD – Attention-Deficit Hyperactivity Disorder

AFP/*afp* – Alpha-fetoprotein

*AGO2* – Argonaute 2

ANOVA – Analysis of Variance

ASD – Autism Spectrum Disorder

BDNF – Brain-Derived Neurotrophic Factor

BMI – Body Mass Index

BTBR – BTBR T+ Itpr3tf/J inbred mouse strain

C19MC – Chromosome 19 microRNA cluster

CD – Communication Disorders

CES-D – Center for Epidemiologic Studies Depression Scale

CI – Confidence Interval

CNV – Copy Number Variants

*CRFR2* – Corticotropin-Releasing Factor Receptor 2

CVS – Chronic Variable Stress

*CYP1A1* – Cytochrome P450 family 1 subfamily A member 1

DD – Developmental Delay

*DGCR8* – DiGeorge syndrome critical region 8

DMR – Differentially Methylated Regions

DOHaD – Developmental Origins of Health and Disease hypothesis

DSM-5 – Diagnostic and Statistical Manual of Mental Disorders, 5th Edition

E0/6/14 – Embryonic Day 0/6/14

EPM – Elevated Plus Maze

EV – Extracellular Vesicle

FMRP – Fragile X Mental Retardation Protein

HPA – Hypothalamic-Pituitary-Adrenal axis

ID – Intellectual Disability

IL-1 $\alpha$ /1 $\beta$ /4/6 – Interleukin 1 $\alpha$ /1 $\beta$ /4/6/8, IL-1 $\alpha$ , IL-1 $\beta$ , and IL-6

I.P. – Intraperitoneal

I.V. – Intravenous

iPSCs – induced Pluripotent Stem Cells

LPS – Lipopolysaccharide

MADRES – Maternal and Developmental Risks from Environmental and Social Stressors

MD – Motor Disorders

MetS – Metabolic Syndrome

MIA – Maternal Immune Activation

miRNA – microRNA

MREs – miRNA Response Elements

mRNA – messenger RNA

MVs – Microvesicles

NCD – Non-Communicable Disease

NDDs – Neurodevelopmental Disorders

NHPs – Non-Human Primates

*NR3C1* – Nuclear receptor subfamily 3 group C member 1

OA – Open Arm of EPM

OCD – Obsessive-Compulsive Disorder

OR – Odds Ratio

PACAP – Pituitary Adenylate Cyclase-Activating Polypeptide

PD 35/60 – Postnatal Day 35/60

PDQ – Pregnancy Discrimination Questionnaire

pre-miRNA – precursor-miRNA

pri-miRNA – primary miRNA

PSD-95 – Postsynaptic density protein 95

PSS – Perceived Stress Scale

RISC – RNA-Induced Silencing Complex

RRB – Restrictive and Repetitive Behaviors

SEM – Standard Error of the Mean

*SLC6A4*/ SERT/ 5-HTT – Solute Carrier Family 6 Member 4/ Serotonin Transporter

SLD – Specific Learning Disorders

SNI – Social Novelty Index

SPI – Social Preference Index

SRRS – Social Readjustment Rating Scale

STAT3 – Signal Transducer and Activator of Transcription 3

TNF- $\alpha$  – Tumor necrosis factor alpha

UTR – Untranslated Region

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## ABSTRACT

The Developmental Origins of Health and Disease hypothesis (DoHaD) proposes that environmental stress exposure to the intrauterine and maternal systems at key developmental periods may lead to long-term impacts on the health of the offspring. Our previous mouse model study demonstrated that the combination of maternal serotonin receptor transporter (SERT) heterozygosity and prenatal stress exposure resulted in reduced social preference and increased repetitive behaviors in male offspring, compared to wild-type controls.

Additionally, we identified three differentially expressed maternal microRNAs (mmu-miR-7684-3p, mmu-miR-5622-3p, mmu-miR-6900-3p) in the SERT-het/stress group, demonstrating the epigenetic changes in the miRNA profile at the time of pregnancy. To investigate the mechanistic role of the prenatal stress-associated miRNAs, they were replicated as oligonucleotide miRNA mimics and combined into a miRNA mix diluted to 50 ug/100 uL. C57Bl/6J pregnant mice were then injected with 100 uL on gestational day 6 (G6) and again on G14.

Starting from post-natal day 60, offspring from injected and injection naive mice were behaviorally tested for social communication, repetitive behaviors, and other anxiety measures. Offspring displayed no differences in social interactions or repetitive grooming behaviors but, during open field testing, 18/23 (80%) of tested offspring from injected dams displayed spontaneous, and repetitive jumping directly into the wall of the chamber. They exhibited decreased duration within the center of the open field and this was corroborated by higher thigmotaxis. However, prenatally miRNA exposed offspring also displayed increased distance travelled during the elevated plus maze as well as longer duration spent on open arms than non-injected controls, suggesting differing

anxiety-like phenotypes. These findings suggest maternal intravenous exposure to the three prenatal stress-related miRNA recapitulates specific behavioral patterns of hyperlocomotion and wall-directed repetitive behaviors independent of social ability and self-grooming behaviors.

# 1. Introduction & Literature Review

## 1.1 Developmental Origins of Health and Disease Hypothesis

Once described as the “Barker’s Hypothesis”, the Developmental Origins of Health and Disease (DOHaD) proposes the importance of the fetal environment during pregnancy and the temporal relevance of stressors in poor health outcomes (Wadhwa et al., 2009; Gage et al., 2016). The DOHaD framework provides mechanistic explanations for how environmental factors may interact with genetic susceptibility to influence non-communicable disease (NCD), neuropsychiatric, and neurodevelopmental risk.

### 1.1.1 Historical Perspective

In a series of observational studies, David Barker investigated the geographic, temporal, and diagnostic parallels between fetal mortality and ischemic heart disease. Barker and his colleagues would subsequently publish a series of articles detailing the relationships between infant mortality, low fetal weights, and the development of heart disease later in life, particularly in specific areas of England and Wales. In 1986, they published findings after reviewing infant mortality in 1921-1925 and the standardized mortality ratios of adults from 1968-1978, identifying 5 out of 24 most common causes of death as highly correlated (D. Barker, 1986). Barker anticipated diseases historically linked to poor living conditions, such as chronic bronchitis, stomach cancer and rheumatic heart disease, followed the same geographic pattern as infant mortality. Ischemic heart disease, however, a disease that had previously been related to increased prosperity, was also highly correlated with geographic area and infant mortality. This led to the hypothesis, “*adverse influences in childhood*,

*associated with poor living standards, increase susceptibility to other influences, associated with affluence, encountered in later life”* (D. Barker, 1986, p.1080).

In subsequent works, Barker and colleagues would continue to investigate this relationship with the “thrifty phenotype hypothesis”. Barker and Hales further expound on outcome differences in Holland and Leningrad, which experienced drastically shorter and longer bouts of famine and quicker or slower recoveries, respectively. Low birthweight, while related to the later development of type 2 diabetes, was not the best or only predictor of adult health outcomes. The children exposed to the Holland famine during mid to late gestation exhibited altered insulin-glucose metabolism, and this adaptation was incongruent to the post-war abundance they were born into. In contrast, children exposed to intrauterine malnutrition from the siege of Leningrad were not found to be associated with glucose intolerance compared to those exposed as infants, but prenatal exposure to the siege was related to obesity and blood pressure. They suggested this was not solely due to the lack of nutritional availability *in utero*, observed in both crises, as the elevated glucose concentration after 2 hours was not explainable by reduced birthweight alone. However, children exposed to the famine in Leningrad did not see the same elevated insulin load and increased fasting proinsulin as seen in Holland (Stanner et al., 1997, Ravelli et al., 1998). Due to the fetus’s ability to adapt under stressful conditions, the authors suggested metabolic alterations, consistent with insulin resistance, during development preceded the sacrifice of normal growth. The development of type 2 diabetes was not contingent on malnutrition, but rather if the altered glucose-insulin metabolism was appropriate for the nutritional availability after birth. Developmental

plasticity, as Barker put it, describes the ability of developing organisms to remain malleable and adaptive during the myriad of events in early development, but they are critically vulnerable to external stimuli. The authors attributed this adaptation to maternal malnutrition and thus fetal malnutrition to “fetal programming”, adapting for the womb’s environmental pressures under the assumption that the same would be imposed upon them outside of the womb. Instead, their adaptation was ill-suited for an environment of relative nutritional “abundance” compared to the starved uterine environment they grew within (Hales & Barker, 2001). So, while on average, fetal development can follow a predictable pattern, developmental plasticity helps explain how a “single genotype” is capable of producing more than one behavioral or physiological profile (D. Barker, 2004; D. J. P. Barker, 2007).

#### *1.1.2 Critical Periods of Development and Maternal Stressors*

Barker’s ideas of “fetal programming” and “developmental plasticity” have expanded into the DOHaD framework. The field continued to broaden past fetal undernutrition to encompass a wider array of environmental influences such as infections, exposure to chemical agents, and metabolic and hormonal perturbations during critical developmental stages. Maternal psychological stressors, as described further below, result in various short- and long-term adverse outcomes in the offspring. Metabolic syndromes (MetS) and neuropsychiatric effects alike reflect mid to late critical windows during which the developing fetus is especially vulnerable to the maternal system.

Studies over the past two decades have continued to support the DOHaD hypothesis as observational human studies and experimental animal models alike

have described various MetS and neuropsychiatric diagnoses related to environmental insults. Glover and colleagues extensively reviewed prenatal stress and its role in programming the Hypothalamic-Pituitary-Adrenal (HPA) axis, describing incredible variability in human and animal studies of prenatal stress. These outcomes may depend on the timing of exposure in gestation, nature and intensity of the stressor, and the sex and genetic vulnerabilities of the individual offspring. Maternal anxiety, daily life events, and extreme disasters all have the potential to impact neurodevelopment, but described numerous confounds unstudied previously such as delivery method and methodological oversights in stress measuring paradigms (Glover et al., 2010).

Earlier work by Wadhwa and colleagues identified pregnant populations and surveyed them twice during their pregnancies, using surveys measuring acute and chronic stress as well as levels of trait anxiety and pregnancy-related anxiety. Independent of pre-existing biological conditions, prenatal life stress events decreased birthweight and pregnancy-related anxiety was negatively associated with gestational age. However, it should be noted that biomedical risk accounted for 20% of the variance in these findings (Wadhwa et al., 1993). Mueller and Bale replicated this in part in a mouse model deficient of corticotropin-releasing factor receptor 2 (*CRFR2*), exposing stress-susceptible (*CRFR2-KO*) dams to a variable stress paradigm, during early (days 1-7), middle (days 8-14), or late gestation (days 15-21). The dams exhibited persistent elevated corticosterone levels, but no differences in maternal body weight or gestational age of the offspring. They also revealed *increased* body weight of prenatally stressed *KO* males that remained elevated through postnatal week 16. Wild-type mice exposed to stress late in gestation showed similar increases

at birth, but these differences diminished by postnatal measurements (B. Mueller & Bale, 2006).

A recent review of 45 studies identified maternal stress and resulting glucocorticoid exposure as a risk factor in several cardio-metabolic disorders, including diabetes mellitus, hypertension, and hyperglycemia, among others, as well as fetal growth restriction and low fetal birthweight. Maternal stress and distress during early gestation were related to child body mass index (BMI) at age 2-4, adiposity at age 2.5, and rat adulthood blood pressure (Eberle et al., 2021). However, another review of maternal psychological distress during pregnancy on neurological structural and functional connectivity of the offspring found converging evidence of amygdala and medial prefrontal cortices dysregulation in children exposed to maternal depression and maternal trait anxiety, and implicated the second and third trimesters (Y. Wu et al., 2024).

Maternal overnutrition can alter placental structure, blood flow, and nutrient transfer to the fetus. High maternal glucose levels can increase fetal insulin production, exposing the developing fetal brain to a hyperinsulinemia environment, which can alter the development of neural circuitry and prime for future insulin resistance in the offspring brain. Maternal leptin can cross the placental barrier and act on the developing fetal brain, with changes to leptin signaling during early life altering neural circuitry development. Maternal obesity is a state of low-grade inflammation, causing placental inflammation and disrupting neurodevelopment, potentially altering the development and function of astrocytes and microglia. Low vitamin D status is linked to adverse neurological outcomes such as autism spectrum

disorder (ASD), schizophrenia, depression, multiple sclerosis, and dementia. Specifically, gestational VDD (maternal 25(OH)D <25nmol/L) was associated with an increase in autism-related traits in 6-year-old offspring, and increasing maternal 25(OH)D concentrations were linked to a lower risk of ADHD-like symptoms in children. Furthermore, maternal vitamin D deficiencies can determine offspring susceptibility to obesity and related metabolic complications later in life, with lower pregnancy 25(OH)D concentrations linked to higher fat mass and insulin resistance in offspring.

### *1.1.3 Epigenetic Mechanism of Action*

Epigenetics, from the Greek *epi-* meaning “on top of”, is the study of heritable genetic expression that does not involve the manipulation of one’s DNA sequence (Waterland & Michels, 2007). Epigenetic mechanisms, encompassing histone modifications, DNA methylation, and microRNA (miRNA) expression, are fundamental directors of neuronal proliferation, differentiation, and integration throughout various life stages (Park et al., 2022; Tompkins, 2022; Yao et al., 2019). These mechanisms serve as a crucial interface between external stimuli and the genome, demonstrating responsiveness to environmental cues on a molecular level. Canonical epigenetic processes, including DNA methylation, histone modification, and chromatin remodeling, are essential for neurodevelopment and help explain how cells consisting of identical DNA sequences differentiate into diverse cell types with specialized functions (Jakovcevski & Akbarian, 2012).

Exposure to different environmental insults has been strongly linked to adverse outcomes, including in maternal cigarette or alcohol consumption. Maternal exposure

to nicotine during pregnancy can result in extensive DNA methylation patterns in the placenta and fetal environment. Nicotine readily crosses the placental barrier, resulting in increased epigenetic alterations in fetal samples. Extensive placental methylation patterns in epigenome-wide analyses of placenta and umbilical cord blood samples, including in the *CYP1A1* gene, critical for handling carcinogenic compounds (such as from tobacco smoke) and the mitigation of cellular damage (Suter et al., 2010, 2011).

Maternal stressors such as maternal depression and trait anxiety have also been related to epigenetic regulation. For example, increased neonatal methylation of *NR3C1*, a glucocorticoid receptor gene, and subsequent HPA reactivity of the offspring at three months of age was related to depressed maternal mood during the third trimester. DNA methylation was observed at the NGFI-A consensus binding site in the promoter of *NR3C1* in neonatal blood samples, and increased methylation at the 5' CpG3 was the only segment of *NR3C1* correlated to increased HPA reactivity to visual novel stimuli. Decreased methylation at this site also accounted for 11% of the variance associated with decreased salivary cortisol response at three months. However, maternal depression scores were also associated with methylation at CpG2 during the second trimester (Oberlander et al., 2008). The methylation of *NR3C1* was also related to high fundamental frequencies in healthy infant crying, a measure of potential stress reactivity, and possible neurobehavioral implications (Sheinkopf et al., 2016).

## 1.2 Neurodevelopmental Disorders

Neurodevelopmental disorders (NDDs) constitute a heterogeneous group of conditions with onset during the developmental period. The Diagnostic and Statistical Manual of Mental Disorders 5<sup>th</sup> edition (DSM-5) currently recognizes 6 distinct categories, including Attention Deficit Hyperactivity Disorder (ADHD), Autism Spectrum Disorders (ASD), Intellectual Disability (ID), Communication Disorders (CD), Motor Disorders (MD), and Specific Learning Disorders (SLD) (*Diagnostic and Statistical Manual of Mental Disorders*, 2013). The term autism, originally “Autismus” in German, was first attributed to Eugen Bleuler in his text *Dementia Praecox or The Group of Schizophrenias* (Bleuler, 1912). In 1912, Bleuler used the term to define patients he considered “the most severe schizophrenics” due to the “predominance” of their inner worlds and “detachment from external reality”. It was not until 1943 when Dr. Leo Kanner wrote in his *Autistic Disturbances in Affective Contact*, the cases of 11 children with “infantile autism”, differentiating the diagnosis from schizophrenia-related disorders, which appear from birth rather than developing over time (Kanner, 1943).

### 1.2.1 Characterization & Symptomology

NDDs can be broadly defined by development-related impairments, specifically within the central nervous system, resulting in abnormal brain function (Morris-Rosendahl & Crocq, 2020). As a result, numerous challenges arise across personal, social, academic, and occupational domains across the lifespan for those with ASD and ADHD (Hillier et al., 2007; Danckaerts et al., 2010; Mason et al., 2018); the same can be said of ID, CD, MD, and SLDs but the literature here is severely lacking (Mahjoob et al., 2024). Frequently, NDDs appear comorbid,

particularly among ASD (Maenner, 2023; Shaw et al., 2025), ADHD (Salari et al., 2023), and Intellectual Disability (Yang et al., 2022), suggesting overlapping pathways to their development (Parenti et al., 2020; Berg et al., 2023; Khachadourian et al., 2023). NDDs frequently present in a sex-specific manner, as seen in higher diagnostic rates of males with ASD and ADHD, but sexually dimorphic mechanisms of action have yet to be conclusively elucidated. A common theory for the predominance of male diagnoses is the “female protective effect” (FPE), which includes factors such as etiological “load”, sex chromosome heterogeneity, and hormonal exposure differences (Skuse, 2000; Baron-Cohen et al., 2005; Robinson et al., 2013). However, psychosociological factors may also contribute due to male-centric diagnostic criteria or professional diagnostic biases, which may result in inflated sex ratios found in some prevalence studies (Lai & Baron-Cohen, 2015; Loomes et al., 2017). Still, the gap in these sex differences is narrowing with improvements in public awareness and increased access to diagnoses (Lyll et al., 2017; Francés et al., 2022; Gallin et al., 2024).

Autism spectrum disorder is a neurodevelopmental disorder commonly characterized by social communication deficits and restricted and repetitive behavior (American Psychiatric Association, 2013). ASD diagnoses continue to rise, according to the Autism and Developmental Disorders Monitoring (ADDM) network 8-year-old children were diagnosed at a rate of 1 in 150 in 2000 but has since risen to 1 in 31 in 2022 (Shaw et al., 2025). Prevalence has risen particularly in response to broadening diagnostic criteria, greater access to mental health services, and expanded school and work documentation access (Grosvenor et al., 2024; Napolitano et al., 2022; Salari et

al., 2022; Zeidan et al., 2022). Not only has overall prevalence shifted, but so have the demographics of diagnoses. Across 16 sites in the year 2022, prevalence continues to rise among historically minority populations. From data collected in 2020, for the first time, Black, Hispanic, and Asian and Pacific Islander groups surpassed rates of ASD in white populations, possibly indicating growing accessibility and awareness amongst these specific populations (Maenner, 2023; Shaw et al., 2025).

### *1.2.2 Prenatal Stress Related Etiology*

Human epidemiological studies have identified several environmental insults capable of resulting in various NDDs, such as maternal immune activation (MIA), natural disasters, and maternal psychosocial stressors. Some of these environmental contributors, such as air pollution or pesticides in the case of ASD, have significant evidence of causal contribution (Lyall et al., 2014; Nevison, 2014; Rossignol et al., 2014; Kundakovic & Jaric, 2017; Bekkar et al., 2020; Rahman et al., 2022); which may can be mediated by genetic susceptibility as seen between air pollution and the functional promoter variant of the MET receptor tyrosine kinase (*MET*) gene (Volk et al., 2014; Beversdorf et al., 2018). However, some exogenous contributors, such as vaccinations containing thimerosal, have been vigorously studied and provide no evidence of etiological contribution to ADHD or ASD (C. S. Price et al., 2010; Yoshimasu et al., 2014).

Continuing work has implicated infectious insults on the maternal-fetal environment in neurodevelopmental disorders (like ASD and ADHD) as well as bipolar disorder and schizophrenia risk (Osman et al., 2024). In response to

immunological triggers, the maternal immune system instigates a cytokine cascade. Interleukin-6 (IL-6) and tumor-necrosis-factor alpha (TNF), for example, are involved in oligodendrite survival and synaptogenesis during development, but are also both proinflammatory cytokines found in higher concentrations in the fetal brain after maternal treatment of Poly (I:C) or lipopolysaccharide (LPS) in rodent models (Deverman & Patterson, 2009). Interestingly, maternal serum cytokine and chemokine profiles collected during 15-19 weeks of gestation were distinct from mothers of children with ASD and intellectual disability (ASD+ID) compared to developmental delay (DD), ASD without ID (ASD+noID), and controls. Collected from participants of California's Department of Public Health's Project Baby's Breath, mothers of children with ASD+ID exhibited elevated inflammatory cytokines TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , and IL-6, and the chemokines IL-8, MCP-1, and MIP-1 $\alpha$ . Higher levels of both the Th1 inflammatory cytokine IFN- $\gamma$  and the Th2 cytokine IL-4 were also associated with an increased risk of ASD+ID relative to ASD+noID. (Jones et al., 2017). Authors also noted mothers and fathers of autistic children were older on average than DD or GP parents; advanced paternal and maternal age has also been a noted risk factor for ASD (D'Onofrio et al., 2014; Sandin et al., 2016).

Natural disasters are an extreme environmental stressor humankind continues to contend with and can impact development in behavioral and biological manners. For example, Nomura and colleagues found that children whose mothers were pregnant during Superstorm Sandy had an elevated risk of psychopathology, including ADHD and anxiety, adding to evidence that prenatal stressors instigate stress responses transmitted to the fetus, imparting enduring neuropsychiatric risk (Nomura et al.,

2022). The tragedy of Hurricane Maria, which struck Puerto Rico in 2017, provided evidence of altered DNA methylation profiles in blood samples of two-year-old children that had been exposed *in utero* to the natural disaster. Differentially methylated regions (DMR) were associated with exposure during the second half of pregnancy (>20 weeks), maternal reported perceived stress, and the presence of PTSD symptomology (Kello et al., 2023). Similarly, research on maternal stress experienced during the Quebec Ice Storm of January 1998 revealed several cognitive and language development changes of children exposed *in utero*. During a series of power outages in the dead of winter from a series of ice storms, researchers of *Project Ice Storm* followed found significantly lower scores in Bayley Scales of Infant development at 2 years of age, with similar deficits seen in functional and stereotypical play in higher objective maternal stress, which included domains of scope, loss, change and threat (STORM32), during the first and second trimesters. However, language ability differences were only stress, and not trimester, dependent (King & Laplante, 2005). Follow-up studies found a significant correlation with objective storm-related hardship experienced, pregnant during or conceived within three months of the storm, and altered DNA methylation in the children at thirteen years of age evident in their T cells and peripheral blood mononuclear cells as well as saliva samples collected from the same children at age eight (Cao-Lei et al., 2014). and identified early exposure, rather than mid to late exposure, was predictive of ASD traits in 6 ½ year old children (Walder et al., 2014).

A meta-analysis by Manzari et al. (2019) indicated that prenatal stress was significantly associated with an increased risk of both ASD and ADHD. For ASD, the

synthesis of 15 eligible articles revealed a pooled odds ratio (OR) of 1.64, with a 95% confidence interval (CI) of 1.15 to 2.34. This indicates that, on average, children whose mothers experienced significant stress during pregnancy were 64% more likely to receive an ASD diagnosis compared to children of non-stressed mothers. A similarly strong association was found for ADHD. The analysis of 12 articles yielded a pooled OR of 1.72 (95% CI 1.27–2.34), suggesting a 72% increased likelihood of an ADHD diagnosis. While the authors did advise cautious interpretation of these results due to heterogeneity of stress surveys, they noted a 48% increased risk of ASD when maternal bereavement occurred during the third trimester (Manzari et al., 2019). A more recent meta-analysis of 55 longitudinal studies focusing specifically on externalizing behaviors, which encompass the hyperactivity and impulsivity core to ADHD, found a significant correlation between prenatal psychological distress, particularly in the second and third trimesters, and offspring externalizing behaviors, and this effect was unchanged after controlling for postnatal distress (Tung et al., 2024). Earlier work from Beversdorf and colleagues provided evidence of mothers reported significantly more prenatal stressors (32.4 per 100 surveys) than control (18.9) or Down syndrome (21.7) groups using the Social Readjustment Rating Scale (SRRS). Tabulation for stressors by 4 week intervals revealed elevated reports of stressors in the autism group, specifically between 21-32 weeks gestation, with a peak at 25-28 weeks, even after adjusting for stress severity. Stress exposure during these weeks was also associated with a higher likelihood of language delays in autistic children. Additionally, mothers of autistic and Down syndrome children reported more prenatal medical illnesses. Collectively, these studies provide ample support for

a variety of maternal stressors, particularly in mid to late gestation, are capable of resulting in increased rates of specific neurodevelopmental pathologies, especially ASD.

### *1.2.3 Animal Models of Neurodevelopmental Disorders*

Animal models have enabled our understanding of basic mechanisms through pre-clinical research, and continue to enable researchers to investigate various etiological pathways of NDDs otherwise difficult to parse from the constant confounds of human life. They offer significant advantages over human tissues, such as rapid growth and reproductive cycles, necessary sample size, and the control of the experimental environment. Maternal immune activation (MIA) has been identified as a significant possible mechanism in the investigation of adverse offspring outcomes (R. M. Woods et al., 2023), including neurodevelopmental disorders (Hall et al., 2023), but is remarkably difficult to study in humans due to differences in viral load, genetic differences in reaction, and the clear ethical implications. Precise manipulation of animal models, such as in experimental studies of prenatal stressors and genetically susceptible models, are necessary to investigate the causal mechanisms underpinning observed neuropsychological alterations and provide potential therapeutic purposes for complex heterogenous disorders such as ASD.

Today, a wide variety of experimental species are precisely reproduced, controlling and manipulating relevant genes, and enabling researchers to investigate the plethora of human NDDs through species-specific lenses. Zebrafish (*Danio rerio*) and fruit flies (*Drosophila melanogaster*) are valuable for screening multiple candidate genes rapidly due to their short life cycles and high genomic similarity with humans (e.g., *Drosophila*

has ~87% of genes involved in neurological function (Gatto & Broadie, 2011), zebrafish ~70% homology with human genes (Howe et al., 2013; de Abreu et al., 2020)). Mouse models are widely used given their relatively fast reproduction time, multiple offspring, and over 95% similarity with the human genome, allowing for accurate genomic manipulation of candidate genes (Monaco et al., 2015; Breschi et al., 2017). Advanced genetic manipulation techniques, such as in utero electroporation and CRISPR/Cas9, have made it economical to generate genetically manipulated non-human primates (NHPs), which serve as significant research tools for studying genetic aspects of NDDs with translational potential (Zuo et al., 2017; Liu et al., 2020).

Rodent models, and other placental mammals in particular, are foundational to prenatal stress research due to their physiological, genetic, and developmental similarities to humans, which allow researchers to investigate how early-life environments influence neurodevelopmental outcomes (Breschi et al., 2017; Christmas et al., 2023). Non-human primates, though more limited in use due to ethical and logistical constraints, offer the highest fidelity to human neurodevelopment, and recent advances in genetic engineering have expanded their utility in modeling specific mechanisms underlying neurodevelopmental disorders (NDDs) (Nelson & Winslow, 2009; Watson & Platt, 2012). The capacity to manipulate both genetic and environmental variables across these species enables researchers to identify molecular and epigenetic pathways by which prenatal stress can program lasting, often sex-specific, neurobehavioral outcomes. While species-specific differences in placental structure and stress physiology present challenges, and ethical considerations must be weighed carefully, the translational relevance and experimental precision of animal models—particularly placental

mammals—make them indispensable for elucidating the biological foundations of prenatal stress and informing clinical interventions for human neurodevelopmental health (Bale, 2016; Weinstock, 2016; Mukherjee et al., 2022) Despite these breakthroughs, animal models present several limitations. Zebrafish, fruit flies, and chicken embryos are evolutionarily distant from humans. Mouse models, while widely used, lack several human-specific features, such as the gyrification (folding) of the cortex (Boroviak et al., 2018), and their behavioral tests cannot precisely recapitulate higher brain functions observed in the human brain (van der Staay et al., 2009). For example, in conditions like Rett Syndrome, *Mecp*-mutant mice exhibit neurological symptoms that are less severe and do not fully recapitulate the human phenotype (Guy et al., 2001; Stearns et al., 2007).

Given these advantages and drawbacks of using animal models, prenatal stress paradigms necessitate their use for researchers to investigate G x E interaction effects on biological systems at a molecular level. Rodent models, particularly mice and rats, are commonly used, and ethologically validated stressors have been extensively studied to produce physiological and behavioral effects in the pregnant mouse as well as the offspring. For example, the chronic variable stress (CVS) paradigm calls for novel stressors daily for a week, and is capable of inducing depression-like phenotypes in male offspring, sex-specific HPA axis reactivity, and placental-related immune activation characterized by the upregulation of IL-1b and IL-6 in male placentas and corresponding hyper locomotor behaviors (B. R. Mueller & Bale, 2008; Goel & Bale, 2010; Bronson & Bale, 2014). Similarly, transgenic GAD67GFP mice exposed to acute restraint-stress (45 minutes three times daily starting at E12) exhibited GABAergic progenitor disruption as early as E13 (Stevens et al., 2013). The same paradigm produced decreased social

approach behaviors and increased anxiety in male mice (Lussier & Stevens, 2016). Specific manipulations provide stress-susceptible animal models with targeted genes like the serotonin transporter (5-HTT/Slc6a4/SERT), which, when knocked out and bred to produce heterozygous SERT dams and combined with prenatal stress, has been shown to produce offspring with behaviors consistent with autism-like characteristics (Jones, et al., 2010; Matsui et al., 2018). Other genetic factors explored include the DISC1 gene, fibroblast growth factor 2, GABAergic enzymes (GAD67), transcription factors like Npas4, and synaptic function regulators like Snap-25 (Abbott et al., 2018).

## 1.3 MicroRNA

### 1.3.1 Biogenesis and Mechanisms

MicroRNA (miRNA) are small non-coding RNA approximately 18-22 nucleotides in length responsible for post-transcriptional regulation of messenger RNA (mRNA). First identified by researchers Viktor Ambros and Gary Ruvkin, *lin-4* in the model organism *Caenorhabditis elegans* (*C. elegans*), the two described a small RNA that repressed translation of the *lin-14* mRNA by binding to its 3' untranslated region (UTR), thus controlling the timing of larval developmental progression (Ruvkun & Giusto, 1989; Lee et al., 1993). After the discovery of *let-7* (also in *C. elegans*), this class of small endogenous RNAs (miRNAs) was found to be highly conserved across species, with homologs identified in *Drosophila*, humans, and other vertebrates (Friedman et al., 2009).

The “canonical” pathway for microRNA is as follows: first transcribed in the nucleus by RNA polymerase II, primary miRNA (pri-miRNA) is formed in a hairpin-like structure that is then identified by RNase III enzyme (Drosha) and DiGeorge syndrome critical region 8 (*DGCR8*), which together form the “Microprocessor complex” that then

cleaves the base of the pri-miRNA, resulting in precursor-miRNA (pre-miRNA). Exportin 5 provides safe passage of pre-miRNA out of the nucleus into the cytoplasm where the endoribonuclease Dicer and the TAR RNA-binding protein (TRBP) awaits to cleave the stem loop and enabling Argonaute (Ago) to load the “guide” strand of the miRNA, now mature, into the RNA induced silencing complex (RISC) while the “passenger” strand is degraded. The miRNA-RISC complex then represses gene expression, inhibiting translation or promoting mRNA degradation based on partial or complete nucleotide pairings in the seed region (O’Brien et al., 2018; Saliminejad et al., 2019).

### *1.3.2 Neurodevelopmental Relevance*

Key actors in the miRNA biogenesis pathway as well as miRNAs themselves can produce aberrant effects in the embryo and its neural development. Post-transcriptional gene regulation orchestrates neural developmental progression, from differentiation to synaptogenesis, via temporally dynamic miRNA profiles (Davis et al., 2015). Phenotypic alterations cannot always be easily detected, as some miRNAs appear functionally redundant, but the ablation of specific miRNAs or their requisite machinery, particularly during development, results in much larger consequences (Bushati & Cohen, 2007; Cherone et al., 2019; Cho et al., 2019). Human research using brain tissue, saliva and blood samples have provided evidence of unique miRNA signatures found in patients with NDDs, but animal studies allow for the direct manipulation of miRNA during embryonic development; subsequently providing key insight into life outcomes through biological and behavioral lenses (Xu et al., 2010; Wiegand et al., 2017; Godoy et al., 2018; DeVeale et al., 2021).

The dysfunction of miRNA biogenesis machinery, which can be regulated by miRNAs themselves, can also result in developmental and physiological abnormalities. *DGCR8*, for example, required for the processing of pri-miRNAs to pre-miRNAs (Landthaler et al., 2004; Gregory et al., 2004), and its deficit can result in the promotion of apoptosis in conjunction with reductions in cell proliferation of vascular smooth muscle cells. Similarly, *Dicer*, critical for miRNA maturation, along with *DGCR8*, mutant mice can result in malformations of the nervous and cardiovascular system, often resulting in embryonic or perinatal death (J.-F. Chen et al., 2008; Huang et al., 2010; Z. Chen et al., 2012).

MiRNAs, in contrast, have critical roles in the timing and progression of neurogenesis, neuronal maturation, and proliferation. During gastrulation, the ectoderm, endoderm, and mesoderm are formed. In a study of human embryonic stem cells and *Xenopus laevis*, evolutionarily conserved miRNAs *miR-430*, *miR-427*, and *miR-302* were shown to play a crucial role in germ layer specification, particularly in early vertebrate development, and exhibit species-specific target selection. The neuroectoderm, differentiated from the ectoderm around 7.5 days after conception in mice (Behringer, 2014), is the precursor to the neural tube and the CNS, but induction of *miR-302* can interrupt this cellular lineage in favor of mesendodermal cells (Rosa et al., 2009). Loss or suppression of specific miRNAs, such as those in the *miR-17-92* cluster, and central-nervous-system (CNS)-specific, like *miR-9* and *miR-124*, can result in the death of entire litters, aberrant neuronal migration, and halt neurogenesis (Mendell, 2008; Bian et al., 2013).

Another example would be microRNAs *miR-9* and *miR-124a*. These non-coding RNAs are highly expressed in neurons and astrocytes, modulating STAT3 phosphorylation, an intracellular signaling molecule that mediates the inhibition of neuronal terminal differentiation. The overexpression of the two, and their coregulation, would result in STAT3 phosphorylation at Tyr-705, mediating DNA binding activity, thereby inhibiting astrocyte neurogenesis (Boeuf et al., 2001; Krichevsky et al., 2006; Ivey & Srivastava, 2015). *MiR-124a*, along with *miR-9*, is also among the highest-expressed miRNAs in the nervous system, including the cerebral cortex, midbrain, and cerebellum (Lagos-Quintana et al., 2002). In a double knock-out model (*miR-124a-1* & *miR-124a-2*) in transgenic *Drosophila*, perinatal mortality and reduction in brain development were observed, and ectopic expression of pre-*miR-124a* resulted in the reduction of dendritic branching in dopaminergic neurons compared to wildtypes (Chaya et al., 2022)

*MiR-125b* has been observed to play a role in regulating cellular processes critical for brain development (Cui et al., 2012). In induced pluripotent stem cells (iPSCs) derived from Down Syndrome patients, overexpression of miR-125b has been linked to an influence on *CDKN2A* expression and, consequently, glial proliferation (Pogue et al., 2010). Furthermore, its upregulation leads to the suppression of *EPHA4*, a gene crucially involved in guiding murine interneurons during cellular migration (Edbauer et al., 2010). While this suggests a potential role in cortical organization, its direct control over cortical neuron migration during embryogenesis requires further elucidation. More broadly, miR-125 has been found to regulate synaptic plasticity in cortical neurons through the translational inhibition of the post-synaptic protein PSD-95, partly mediated by the

phosphorylated form of FMRP attaching to the 3'UTR, the protein associated with Fragile X syndrome, but is insufficient alone to regulate PSD-95 (Muddashetty et al., 2011).

### *1.3.3 Maternal-Fetal Communication*

Placental transfer of nutrients and other forms of molecular transit have been extensively studied (Rossant & Croy, 1985; Adamson et al., 2002; L. Woods et al., 2018), but miRNA placental transfer remained an open question until surprising evidence arose from plant miRNA was found in human biological tissue (L. Zhang et al., 2012; Li et al., 2015). The placenta is not merely a passive barrier or a simple organ for nutrient exchange; it actively integrates maternal stressors and can amplify dysregulation in the fetal compartment (H. Zhang et al., 2010; St-Pierre et al., 2016). Moreover, the placenta is a significant source of neurotrophic signals, such as serotonin (5-HT) (Bonnin et al., 2011; Bonnin & Levitt, 2011) and brain-derived neurotrophic factor (BDNF) (Kodomari et al., 2009), to the developing fetal brain. Maternal inflammation or stress can disrupt these signals, impacting fetal neurodevelopment. The placenta also exhibits sexual dimorphism, responding differently to maternal stressors based on the sex of the fetus, which may underlie sexually divergent brain development and behavioral outcomes in offspring (B. R. Mueller & Bale, 2008; Bronson & Bale, 2014, 2016; Bale, 2016). This highlights the placenta as a crucial, yet often underappreciated, target for understanding and potentially intervening in DOHaD-related outcomes.

Experimental studies using mouse models have recently elucidated the dynamics of miRNA transfer. The detection of circulating miRNAs, along with other microparticles and exosomes, primarily within microvesicles (MVs) suggested intercellular

communication (X. Chen et al., 2012), and other work by the same group provided evidence that intravenously injected MVs are secreted into circulation, selectively packaged, and delivered to recipient cells. THP-1 derived MVs containing miR-150 were delivered into human HMEC-1 cells, resulting in direct suppression of a known target gene, *c-Myb* (Y. Zhang et al., 2010). They subsequently identified small non-coding RNA, MIR168a found commonly in rice, in the circulatory system of participants and C57Bl/6J mice, providing evidence of miRNA transfer between tissues, and luciferase reporter assay demonstrating LDLRAP1 binding and thus miRNA's potential for cross-kingdom regulation (L. Zhang et al., 2012; Zhou et al., 2015).

MV-mediated pathways are also involved in the maternal-fetal communication interface, as discovered with another plant miRNA, MIR2911, found in honeysuckle. By gavage feeding pregnant mice, researchers detected elevated concentrations of MIR2911 in maternal plasma, placenta, and the fetal liver. Subsequent gavage feeding of synthetic siRNAs targeting Alpha-fetoprotein (AFP), expressed in fetal liver during development, resulted in the decreased expression of *afp* mRNA in fetal tissue. Another group created transgenic mice expressing the primate-specific chromosome 19 miRNA cluster (C19MC), a group of miRNAs primarily expressed in the placenta (Bortolin-Cavaillé et al., 2009). Using a lentivirus-based placenta-specific expression and embryo transfer experiments, they demonstrated that maternal miRNAs were also shown to traffic predictably through the placenta using a time course (11.5, 14.5, 17.5 gestational days) of miR-517a expression in the transgenic decidua, spongium, and labyrinth. Transgenic dams also displayed an elevation in miR-517a in other maternal organs, but placental

expression was the highest. The fetal compartment also saw significant increases, particularly in the brain, liver, and kidneys of the embryo (Chang et al., 2017).

These findings highlight a vital communication network mediated by the placenta and integral to the development and maintenance of both the maternal and fetal compartments. The dynamic profiles of extracellular vesicle-associated miRNAs throughout pregnancy are thought to characterize the maternal-fetal interaction and some indicators of gestation. For example, a series of studies involving the Maternal and Developmental Risks from Environmental and Social Stressors (MADRES) cohort in Los Angeles, CA has investigated extracellular vesicle (EV)-associated microRNA (miRNA) profiles with a variety of pregnancy-related outcomes. 64 miRNAs significantly increased and 26 decreased with gestational age, suggesting their involvement in healthy pregnancy progression and maternal-fetal communication, with predicted gene targets related to energetic and metabolic processes (Foley et al., 2021). Building on this, subsequent work revealed that maternal stress (PSS & PDQ), depression (CES-D), and childhood trauma (ACE) were all associated with specific circulating EV-miRNA alterations, indicating their potential as biomarkers for mental health during pregnancy, with 50 unique miRNA signatures associated with at least one of PSS, PDQ or CES-D (Foley et al., 2023). Most recently, environmental air pollution exposures, especially particulate matter, were described to significantly impact EV-miRNA expression, primarily in late pregnancy, suggesting these miRNAs mediate biological responses related to inflammation, lung conditions, and metabolic pathways, and could serve as valuable biomarkers for environmental exposures and related pregnancy complications (Foley et al., 2024). Moreover, alterations in circulating EV-associated miRNA profiles

have been linked to pregnancy complications like preeclampsia, gestational diabetes, and preterm birth, suggesting their utility as non-invasive biomarkers for assessing fetal health (Kotlabova et al., 2011; Pinson et al., 2023). Beyond normal development, this transplacental miRNA transfer also indicates that maternal factors, including genotype and environmental stressors, can influence the epigenetic and transcriptomic profiles, including miRNA expression, in developing embryo brains, and aid in the understanding of underlying mechanisms for environmental insults such as prenatal alcohol exposure (Tseng et al., 2019; Strawn et al., 2021). This understanding also opens new avenues for predictive models and therapeutic interventions.

Furthermore, the intergenerational nature of DOHaD extends beyond direct offspring. Stress affecting a pregnant woman can directly impact three generations, and can possibly be programmed via miRNAs through breastmilk postnatally (Bozack et al., 2021). Paternal prediabetic status in male rat are also able to produce glucose metabolic alterations for F1, F2, and F3 through sperm miRNAs (Dupont et al., 2019). This extends the DOHaD framework from a focus on individual life courses to a multi-generational health trajectory, both maternally and paternally, reinforcing the importance of early life interventions for long-term population health and for breaking cycles of disadvantage across generations (Hollins & Cairns, 2016).

## 1.4 Neurodevelopmental Disorder Model: $G \times E$ Model of ASD

### 1.4.1 Serotonin Transporter Gene (*SERT*)

The serotonin transporter gene (*SERT*), *Slc6a4*, is necessary for the regulation of 5-HT and its removal from the synaptic cleft (Murphy et al., 2004). An extensively studied genetic variation of this gene is the functional polymorphism (5-HTTLPR), consisting of

a 44-base pair insertion or deletion within the promoter region. Humans thus carry either a long (L) allele or a short (S) allele (Heils et al., 1996). Those with at least one short allele (approximately 64% of the general population) have reduced SERT transcription and expression, reducing reuptake of serotonin from the extracellular space by as much as half, resulting in possible dysregulation of the serotonergic system (Mueller et al., 2011; Houwing et al., 2017). Those with at least one copy of the S allele of the SERT gene are particularly susceptible to stress; for example, in humans they display higher rates of suicidal ideation and attempts (Bondy et al., 2006; Clayden et al., 2012), depressive symptoms (Goodyer et al., 2009; J. S. Price et al., 2013; Miozzo et al., 2020), as well as higher rates of substance use disorder (particularly with alcohol and marijuana) (Vaske et al., 2012; Otten & Engels, 2013; Thompson & Kenna, 2016). Interestingly, this may be related to dysregulation of the hypothalamic-pituitary-adrenal axis and sex specific neuroanatomical alterations including in the cerebral cortex, amygdala, and thalamus (Wassink et al., 2007; Murphy et al., 2008; Ellegood et al., 2018; Beversdorf et al., 2018). Most importantly, despite lack of a SERT orthologue, we can produce rodent variants with one or two copies of the SERT gene that adequately replicate human allelic variants (Bengel et al., 1998; Homberg et al., 2007; Houwing et al., 2017). Heterozygous serotonin transporter (SERT) knockout rodents (SERT<sup>+/-</sup>) are employed as a translational model for human S-allele carriers due to their similar reduced SERT expression and function. In these models, SERT<sup>+/-</sup> animals exhibit reduced SERT expression and function, with lowered brain serotonin (5-HT) turnover, though basal 5-HT levels often remain unchanged. Neuroendocrine findings reveal alterations in the hypothalamic-pituitary-adrenal (HPA) axis response, suggesting an increased sensitivity to stressors.

The most significant effects are observed when SERT<sup>+/-</sup> rodents are exposed to early life stress (ELS), which can lead to enhanced anxiety-like and depressive-like behaviors. Neuroanatomically, Slc6a4 knockout (KO) mice show smaller total brain volume, particularly in females, driven by decreases in the cortex and cerebellum, with regional reductions in areas such as the piriform, auditory, temporal association, and entorhinal cortices (Houwing et al., 2017; Ellegood et al., 2018; Beversdorf et al., 2021).

#### *1.4.2 Gene and Environmental Contributions in ASD*

Early research of ASD pointed to a largely genetic etiology, as twin studies such as that conducted by Folstein & Rutter found approximately 82% concordance among monozygotic twins in contrast to the 10% found in dizygotic twin pairs-pointing to a heavy genetic influence (Folstein & Rutter, 1977). However, these estimates range as other studies have identified populations approximating up to 95% heritability between monozygotic twins and up to 20% in dizygotic twins, with the most recent estimate being 83% (Sandin et al., 2017). Proband analyses also revealed the extent of neurodevelopmental disorders within a family, as siblings and close relatives have been found to also be diagnosed with other mental health concerns at a higher rate than the general population (Jokiranta-Olkonemi et al., 2016). This held, particularly in the context of subthreshold traits of various neurodevelopmental disorders. While these individuals do not meet the full criteria, they do provide evidence of familial variation.

Numerous studies have identified relationships among rare single-gene and de novo mutations and copy number variants (CNV) related to ASD, but few can be recognized as lone or the dominant contributor in the development of ASD. Nor do ASD diagnoses usually fall into a singular etiological basket; most cases result from common genetic

variation (~49%), while commonly studied *de novo* mutations may only account for up to 3% of the variance and additive and non-additive genetic providing only ~7% more (Gaugler et al., 2014). Gaugler and colleagues therefore leave 41% unaccounted for their genetic architecture model and attributable to environmental factors. Researchers must therefore employ a multifaceted exploratory approach to common and stochastic environmental perturbations that may be moderated by genetic susceptibility (J. Chen et al., 2014; Abbott et al., 2018).

Current research paints a heterogeneous mosaic of etiologies, and predominant research in the field supports both genetic and environmental (G x E) interactions to be capable of changes at the cellular and molecular levels, facilitating ASD development (Ornoy et al., 2015; Beversdorf, 2016; Abbott et al., 2018; Beversdorf et al., 2018). While not all prenatally stressed pregnancies result in ASD diagnoses, ones that do follow a temporal pattern. Work done previously by the Beversdorf research group used retrospective surveys of mothers of ASD children reported increased prenatal stressors during the child's gestation, but this rate was particularly bound to a critical time period in development as those reporting prenatal stressors were significantly higher during the 21-32 weeks compared to mothers of down syndrome and typically developing children (Beversdorf et al., 2005). Furthermore, stress surveys were also collected depicting the gestational period of non-affected siblings, suggesting the recall of stress events was not a mediating factor.

The critical developmental period is supported in studies of *in utero* natural disaster exposure, such as from studies of hurricanes, tropical storms and floods in Louisiana from 1980-1995 and in the 1998 Quebec Ice Storm. The prevalence of ASD diagnoses

quadrupled between storm exposure at gestational ages 3-4 months (4.77 per 10,000 births) to 5-6 months (17.74 per 10,000 births), and was also found to be higher for those of gestational age 9-10 months (10.78 per 10,000)(Kinney et al., 2008). Interestingly, when comparing these more “sensitive” gestational periods, prevalence also rose with the severity of the storm, with children exposed to the most severe storms during these months reporting the highest prevalence amongst these groups (26.59 per 10,000). Such retrospective cohort studies reveal an ability to identify subsets of prenatal stress-related ASD development, but these subgroups may be linked by a common genetic factor.

#### *1.4.3 Previous Study*

Based on previously discussed work in the Beversdorf lab, we sought to evaluate the G x E interaction in a controlled experimental setting. Our lab developed a maternal serotonin transporter heterozygous knockout (SERT-het) mouse model exposed to chronic variable prenatal stress (38 hour constant light exposure, 1 hour exposure to fox odor in the dark cycle, overnight exposure to novel objects, 10 minutes of restraint in the light phase, dark cycle novel static noise exposure, repeated cage changes, and wet bedding) beginning at E6 to investigate the causal mechanisms underlying gene-environment interactions (SERT-het/stress model) in the development of ASD. This model aimed to replicate human genetic susceptibility to stress by reducing serotonin transporter function in the maternal lineage. Initial characterization of the SERT-het/stress model revealed that prenatally stressed offspring of 5-HTT<sup>+/-</sup> dams exhibited significant social deficits. This supported our hypothesis that the combined effect of maternal 5-HTT dysfunction and prenatal stress produces autistic-like behaviors. Additionally, these offspring reduced ultrasonic vocalizations (20-40 kHz range), indicating a disruption in early social communication. Our subsequent studies have

consistently replicated these findings, demonstrating reduced sociability and increased repetitive behaviors (e.g., grooming frequency) in mainly male SERT-het/stress offspring (Jones, Smith, et al., 2010a; Matsui et al., 2018).

To address and replicate genetic susceptibility, our subsequent research focused on gene-environment interactions within the vulnerable developmental period as previously discussed (Beverdors et al., 2005). In human studies, we demonstrated that mothers of children with ASD who carried the stress-susceptible short (S) allele variant of 5-HTTLPR reported a significantly greater number of stressors and higher severity of stressors during pregnancy compared to mothers carrying the long (L) allele variant. The stratification of the S-allele and prenatal stress history within the critical gestational period (weeks 21-32) was a consistent finding across two independent cohorts. Critically, this association was found to be specific to pregnancies resulting in a child with ASD; when the same mothers were queried about pregnancies of unaffected siblings, there was no reported increase in prenatal stress exposure regardless of maternal genotype. This suggests that the S-allele does not simply increase the recall of stress but serves as a genuine genetic risk factor for increased maternal stress response that interacts with ASD development (Hecht et al., 2016).

We then investigated epigenetic changes, including DNA methylation and microRNA (miRNA) expression, in both SERT-het/stressed offspring brains and maternal blood samples. A key finding in the mouse model was a striking *attenuation* of the typical embryonic response to prenatal stress in offspring from SERT-het mothers. While embryos from stressed wild-type (WS) dams exhibited significant alterations in methylation profiles and differential expression of numerous miRNAs and genes—a

possible coping mechanism—embryos from stressed SERT-het (HS) dams showed a significantly reduced transcriptomic and miRNA response, along with genome hypermethylation unlike stressed wildtypes, suggesting a stunted stress “coping” response. All mouse embryos in these studies were genotypically wild-type for *Slc6a4* (+/+), confirming that the observed changes were maternal effects on embryonic development independent of fetal genotype (Sjaarda et al., 2017). Translating these findings to humans, we examined miRNA profiles in maternal blood samples from our clinical gene-environment study. We identified 119 differentially expressed miRNAs, with 90 of these being stress-dependent. Notably, some of these miRNAs, such as miR-1224-5p and miR-331-3p, had previously been identified in stress-exposed offspring mouse brains by our group, suggesting common epigenetic alterations across species and tissues. The fact that these maternal miRNA changes were detectable years after pregnancy indicates a persistent epigenetic signature related to the gene-environment interaction in ASD. Pathway analysis of the identified miRNAs highlighted their targets within dopaminergic, glutamatergic, and GABAergic synapses, emphasizing key neurobiological systems implicated in ASD pathophysiology (Beverdorf et al., 2021).

Finally, our latest study involved a multistage approach to recapitulate the GxE interaction model of ASD, maternal blood collection and isolation, and microRNA analyses of maternal mice samples at E21 (on the day of giving birth) and then again 60 days later (Woo et al., 2023). Our mouse model replicated what we had reported previously (Jones et al., 2010; Matsui et al., 2018), with male offspring of SERT +/-, prenatally stressed dam exhibited decreased social preference compared to controls, but low in anxiety measures from the open field and elevated plus maze, further

contextualizing these behavioral patterns as social-specific anxiety, and the offspring also exhibited increased self-grooming bouts, characteristic of ASD animal models. From samples collected at E21, three miRNAs (mmu-miR-5622-3p, mmu-miR-6900-3p, and mmu-miR-7684-3p) were consistently upregulated in the SERT-het/stress condition across all comparisons (HS vs. WN, HS vs. WS, HS vs. HN). However, at PD60, a different set of six miRNAs (mmu-miR-16-5p, mmu-miR-1893, mmu-miR-6347 downregulated; mmu-miR-126a-3p, mmu-miR-340-5p, mmu-miR-3620-3p upregulated) were identified as consistently differentially expressed, thus highlighting the dynamic nature of the maternal miRNA profile in this epigenetic model (Woo et al., 2023).

We previously suggested the identified miRNA on E21 to be potential biomarkers, but we now seek to understand if the same miRNA that would have been theoretically exposed to the offspring can recapitulate synonymous altered social and repetitive behaviors. The three miRNAs selected were commonly upregulated in the prenatally stressed SERT-het mouse model compared to all genetic controls (HN & WN) and environmental controls (WS). We hypothesize that the administration of differentially expressed miRNAs at E6, corresponding to the beginning of the CVS paradigm in our previous work, and again at E14, will be sufficient to produce altered social and repetitive behavioral compared to non-exposed control offspring.

## 2. Materials and Methods

### 2.1 Animals

Dams used for injections were bred using C57BL/6J purchased from Jackson Laboratories (Maine, USA). The first leg of this experiment aimed to produce the injection naïve and miRNA cocktail injection groups. At eight weeks of age, 10 females

were paired with 5 males (in the recommended 2:1 sibling-sibling breeding scheme). Dams were introduced to respective breeding cages at the start of the dark cycle (at ~ 6 PM) and removed by 8 AM the following morning while experimenters determined the presence of a seminal plug. Vaginal plugs were checked between 0730-0800 each morning and the identification of copulatory plug was designated as embryonic day 0 (E0). All females were weighed prior to mating, once again when copulatory plugs were found and one day prior to gestational day 6. On E6, corresponding to the beginning of the chronic variable stress (CVS) paradigm on pregnant SERT<sup>+/-</sup> dams in our previous work (Woo et al., 2023), pregnant mice were injected via lateral tail vein with 100 uL of miRNA mimic cocktail while another cohort received no injection at all. Injections were repeated (100 uL of miRNA mimic) on E14, accommodating for miRNA's predicted half-life, as predicted by the manufacturer (IDT), and thus maintaining consistent concentration of the miRNA cocktail (Stevanato et al., 2016; Reschke et al., 2020). Additional groups composed of pregnant dams injected with negative control ( $n = 3$ ) and buffer solutions ( $n = 3$ ) have been completed, but behavioral data collection and analysis is still in progress.

Whole litters of injected dams were behaviorally tested to identify possible effects amongst the miRNA injected within and between litters. Animals were maintained in a temperature- and humidity-controlled room at  $25 \pm 2^{\circ}\text{C}$  on a 12-h light/dark cycle with food and water available *ad libitum*. All animals were housed in clear carbonate cages

provided with aspen shaving bedding. All procedures were in accordance with protocols approved by the University of Missouri Institutional Animal Care and Use Committee.

## 2.2 miRNA identification and mimic cocktail preparation

Following our previous studies of identified differentially expressed miRNA within the blood of SERT<sup>+/-</sup> pregnant mice (Woo et al., 2023), we purchased a miRNA mimic cocktail from Integrated DNA Technologies (IDT) including equal parts (33.3 nanomoles) of mmu-miR-7684-3p, mmu-miR-5622-3p and mmu-miR-6900-3p previously suspended in a IDTE (10 mM Tris, 0.1 mM EDTA) buffer (pH=8.0) total of 1000 uL. Each duplex was synthesized containing 2' O – Methyl RNA bases, previously found to increase resistance to nuclease degradation and increased stability in binding (Yoo et al., 2004; Y. Zhang et al., 2013). To dilute in preparation of injection, the total mass of miRNA content was calculated based on the molecular weights (13,631.4, 13,005.1, and 13,677.5 g/mol, respectively), yielding 454 µg, 433 µg, and 455 µg for each miRNA species, and a combined mass of 1,342 µg. The initial stock solution (1.342 µg/µL) was diluted with 1684 µL of IDTE buffer (pH = 8.0) to achieve a final concentration of 0.5 µg/µL (50 µg/100 µL) in a total volume of 2684 µL. The diluted solution was thoroughly mixed by gentle pipetting to ensure homogeneity. 8 individual aliquots of approximately 400 µL each (containing 200 µg total miRNA per aliquot) were prepared in sterile, nuclease-free microcentrifuge tubes. All aliquots were prepared simultaneously using calibrated pipettes under sterile conditions to maintain consistency across samples. The remaining volume was retained as quality control samples. Aliquots were immediately stored at -80°C until use to prevent degradation and minimal freeze-

thaw cycles.

### 2.3 Neurological exam battery

On postnatal day (PD) 35, all offspring underwent a comprehensive neurological assessment to identify deficits in sensory processing or gross motor development, adapted from Crawley (1999) and Jones et al. (2010). The purpose of this evaluation was to confirm that any observed autism-related behaviors could not be attributed to general neurological impairments. Previously, any failures of the following tasks would be grounds for exclusion, but due to the novel nature of this study, no mice were planned to be excluded. Prior to testing, each mouse was individually weighed to assess normal physical development. Mice whose body weight fell more than 1.5 standard deviations below the sex-specific mean would be classified as outliers as suggested in previous work (Crawley & Paylor, 1997; Crawley, 2000), but none were excluded.

To evaluate olfactory function, a small food pellet was randomly buried beneath the bedding in one of nine visually defined quadrants within a 72 cm × 40 cm × 28 cm chamber. The latency to uncover the food was recorded, with a maximum search duration of 5 minutes.

For neuromuscular assessment, each mouse was placed on the wire lid of its home cage, which was then gently inverted approximately 15 cm above the cage floor. The latency to fall was measured, with a max time allowed of 60 seconds; mice that fell within 10 seconds were given 2 tries before they would be excluded.

Acoustic startle response was tested by placing each mouse in a clean cage, and the experimenter would clap their hands approximately 2 feet away from the cage.

Another experimenter observed for flinch and eyeblink characteristic of the acoustic startle response, and those that failed to exhibit a response would be excluded from the study.

Finally, visual placing reflexes were assessed by slowly lowering each mouse by the tail toward a wire cage lid. A reaching response was taken as an indicator of intact visual function; mice that did not reach would also be excluded.

Only mice that passed all sensory and neurological assessments were retained for behavioral testing to ensure that observed phenotypes were specific and not confounded by broader functional deficits (Crawley, 1999). No mice required exclusion.

## 2.4 Behavioral assays

On PD 60, we assessed the behavior of the offspring using a battery of tests to evaluate sociability, repetitive and stereotyped behaviors, and generalized anxiety (Fig. 1). All behavioral tests were conducted during the light phase of the cycle with minimal or red bulb lighting, and each apparatus was cleaned with 70% isopropyl alcohol between subjects. The movement in each apparatus was recorded using video-tracking software, specifically Stoelting AnyMaze Software (Wood Dale, IL, USA).

### 2.4.1 Sociability Tasks

#### 2.4.1.a Social Approach

The sociability of mice was evaluated using the three-chamber test, which consists of a Plexiglas apparatus (54.5 cm x 41.5 cm x 22 cm) with three chambers (17.5 cm x 41.5 cm x 22 cm) separated by dividers (0.5 cm thick walls) with openings (10.25 cm x 2.5 cm) for free movement between chambers (Jones et al., 2010; Matsui et al., 2018; Nadler et al., 2004). Experimental mice were first familiarized with the apparatus after 10 minutes. An

age and sex matched stranger mouse was then placed in a wire cage (10.8 cm height, 10.16 cm bottom diameter, bars spaced 1 cm apart; Galaxy Cup; Spectrum Diversified Designs, Inc., Streetsboro, OH, USA) on one side of the apparatus to assess social approach with time with social stimulus (Ts), while the opposite chamber only held an empty wire cage (Tns). Sides were counterbalanced with each trial as the stranger mouse either began under the wirecup in the right or left chamber. The experimental mouse was allowed to explore the three chambers for an additional 10 minutes. Time spent interacting with this novel stranger is used and was designated as an interaction when the experimental mouse was recorded within a 2 cm circumference around the wire cage holding the novel mouse. Time spent in similar proximity to the opposite chamber's empty cage was also timed as the non-social stimulus. However, this region is only a portion of the entire chamber, thus durations spent in the chamber with the novel conspecific (Ts) and time in the chamber of the empty cage (Tns) were also recorded, alongside time spent within proximity to the available stimuli. The Social Preference Index (SPI) was calculated as an index of social ability ( $Ts/(Ts+Tns)$ ).

#### 2.4.1.b Social Novelty

A new stranger mouse was placed in a wire cage in the opposing chamber while the original mouse remained in its first chamber, and time spent with the novel stranger is indicative of exploratory behavior related to social novelty and a willingness to explore new social interactions. C57Bl/6J mice have previously been shown to spend more time with the novel conspecific, providing evidence of social recognition of the “familiar” stranger with which they had just spent the previous 10 minutes with and a preference for the newly introduced stranger (Moy et al., 2004; Silverman et al., 2010; Crawley, 2023). ASD-models, in contrast, exhibit more time with, and thus a preference for, the “familiar”

mouse, displaying the ability to discriminate between a previously encountered stranger compared to a newly introduced one (Moy et al., 2004). The experimental mouse was then placed in the three-chamber apparatus for a final 10 minutes. The video tracking software measured the duration of time spent by the experimental mouse in each chamber, as well as time spent investigating the newest stranger versus interacting with the “familiar” stranger that was also present in the previous stage. The number of exits and entries were used as a control parameter to look for locomotive differences between groups, as seen by previous groups, and as a control measure to observe these differences as possibly anxiety driven hypolocomotion resulting in decreased sociability or social novelty (Moy et al. 2004, Moy et al. 2009). Time spent in the center chamber was also assessed during the final stage to ensure avoidance of both the new novel stranger and the familiar stranger was also captured. The Social Novelty Index (SNI) was calculated as the duration in the chamber of the unfamiliar stranger (Tuf) as a proportion of the total time spent in the unfamiliar and the familiar stranger’s (Tfs) chamber ( $Tuf/Tuf+Tfs$ ).

#### *2.4.2 Restrictive and Repetitive Behaviors (RRB)*

##### *2.4.2.a Self-Grooming*

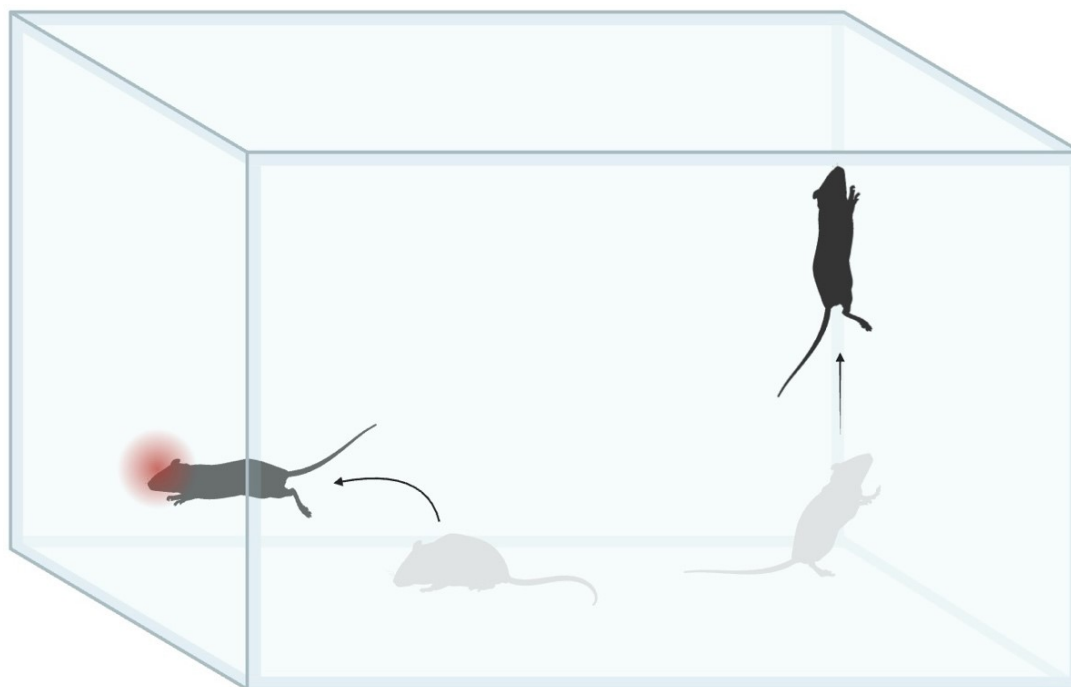
During the open field test, mouse models such as BTBR, Valproic acid exposure, and this lab’s SERT<sup>+/-</sup> × prenatally stressed model have displayed excessive grooming behavior, representative of a core behavioral domain of ASD (Kalueff et al., 2016). To assess self-grooming behavior, mice were observed and recorded the total time spent grooming and the frequency of grooming bouts during the 10-minute testing period. These

measures have been used in previous studies to assess repetitive behavior in mice (McFarlane et al., 2008; Matsui et al., 2018).

#### 2.4.2.b Repetitive Jumping

Bouts of jumping were hand-scored during the open field task. Like humans, RRB can vary, including based on differing mouse models of ASD and its comorbidities. Previously recorded in deer mice (Powell et al., 1999) in a model of obsessive-compulsive disorder (OCD), this stereotyped behavior has also been seen in mouse models of substance use disorders (Pang et al., 2016), acute environmental stressors (Harikai et al., 2004), as well as neurodevelopmental disorders such as ASD (Ryan et al., 2010). Jumping behavior has previously been recorded and defined as events in which the mouse rears to its hindlegs and braced against a wall or corner of the apparatus followed by jumping such that all 4 paws leave the ground simultaneously (also called “jackhammer jumping”). Wall-directed jumping behavior, in comparison, did not begin from a wall-supported rearing position, but rather a quadrupedal leap towards a wall of the enclosure, resulting in either head- or body-impact against the wall (Figure 1). Some mice showed these behaviors in a repetitive or stereotyped manner, defined as events

occurring within 5 seconds of one another. Total number of jumps combines all up-directed and wall-directed jumping events.



**Figure 1:** Representative diagram of jumping sequence differences between “up-ward directed” jumping (right) compared to “wall-directed” jumping (left).

#### 2.4.2.c Marble Burying

Marble burying was also used to assess repetitive behaviors (Chang et al., 2017; Crawley, 2007). The test was conducted in a cage with corncob bedding covering the bottom and then 20 glass marbles were placed in a 5 x 4 arrangement on top of the bedding. Mice were habituated to the testing room for 30 minutes, after which individual mice were introduced to their respective cages for 30 minutes. Marbles were considered buried if more than two-thirds of the marble was covered by bedding; marbles were subsequently counted by two independent experimenters after mice had been removed from the cage and reported by Cohen’s  $\kappa$ . It should be noted that marble burying has been used

previously as a repetitive behavior task in ASD and OCD animal models but was and still is used as a measure of general anxiety as well, though not without critique (Crawley 2007, Angoa-Perez 2013).

### *2.4.3 Generalized Anxiety Measures*

#### *2.4.3.a Open field*

The open field test was conducted on the following day after the 3-chamber test to evaluate general locomotor and anxiety behavior. The mice were placed in a Plexiglas enclosure measuring 45 cm x 45 cm x 22 cm, and each mouse was tested individually for 10 minutes (Jones et al, 2010). AnyMaze software was used to record the total distance traveled by the mice and the time they spent moving. A 4 x 4 grid centered in the apparatus was defined as the “center” of the field while all external area was considered the outer region. Time and distance within this outer ring of the open field was measured to quantify thigmotaxic behavior, a validated anxiety measure in mice which prefer to peripherally explore when placed in this type of apparatus (Simon et al., 1994). This time and distance can be compared to that of the inner-region, as a measure of exploratory behaviors and lower anxiety may be indicated by more distance or time spent in the center of the arena.

#### *2.4.3.b Elevated Plus Maze*

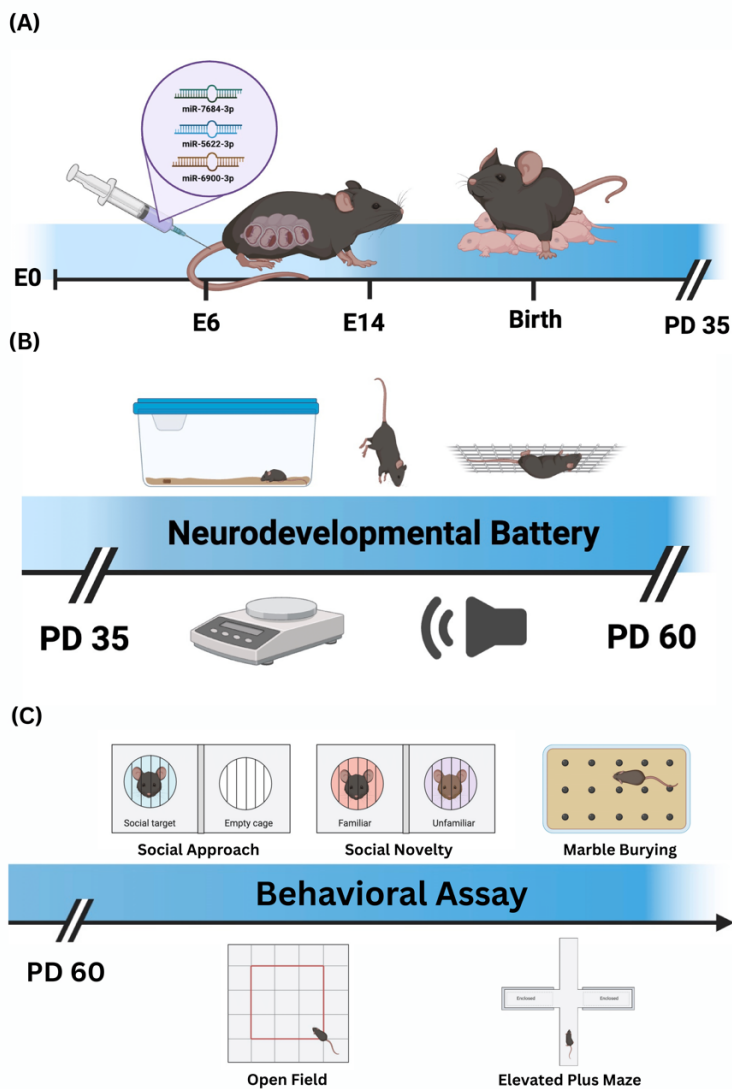
The elevated-plus maze measures anxiety-like behavior to determine if there are any effects due to general anxiety (Komada et al., 2008). The maze is raised off the ground (75 cm). The maze has two open arms (35 cm x 6.25 cm x 0.25 cm) and two closed arms (35 cm x 6.25 cm x 21 cm) which join at a platform (5 cm x 5 cm). Mice were placed in the center of the maze and recorded for 10 minutes each. The amount of time the mouse spent in the open arms and closed arms was recorded and used to calculate the open arm

ratio (Time in open arm/Time in open arm + Time in closed arm). The less time spent on open arms and thus a lower open arm ratio indicates increased anxiety observed in the mouse being measured.

## 2.5 Statistics

All statistical analyses were performed using R (R Core Team, 2024). For the three-chamber social interaction test, a repeated measures analysis of variance (ANOVA) was conducted with chamber as the within-subjects factor, following established protocols (Moy et al., 2004; Silverman et al., 2010). When significant main effects were detected, post hoc pairwise comparisons were performed with Bonferroni correction to adjust for multiple comparisons.

All other behavioral tests were analyzed using independent samples t-tests to compare group means. Where assumptions of homogeneity of variance were violated, Welch's correction was applied. Statistical significance was set at  $p < .05$  for all analyses. Data are reported as mean  $\pm$  standard error of the mean (SEM).



**Figure 2:** Experimental timeline: (A) Embryonic day 0 (E0) was designated by plug identification. 100  $\mu$ L of miRNA mimic mixture was injected into the pregnant dam on E6 and E14. (B) Neurodevelopmental battery was conducted on postnatal day 35 and included pellet detection (odor), reaching behavior above wire lid (visual), wirelid hold for a maximum of 60 seconds (neuromuscular), and acoustic startle response (auditory). (C) Behavioral testing began on PD60, including open field, three chamber's social approach and social novelty, marble burying, and elevated plus maze. Illustration created using Biorender.

### 3. Results

Out of 10 plugs identified, 6 (60%) resulted in a completed pregnancy. Half ( $n = 5$ ) of the possible dams were treated with miRNA mimic cocktail on E6 and E14 of gestation, while the other half ( $n = 5$ ) were undisturbed except for weight measurements. 3 litters of miRNA injected dams were born with a total of 25 offspring (Sex ratios (Males:Females): 4:4, 5:3, 4:5). A comparative 3 litters of injection naive mice (NI) were mated and produced a total of 22 mice (Sex ratios (M:F): 2:6, 3:4, 4:3). 2 males and 2 females were randomly selected from control litters to minimize litter effects. In the present study, independent t-tests were used to identify group differences between the Mimic Positive ( $n = 23$ ) and No Injection ( $n = 14$ ) conditions across several behavioral variables, and if homogeneity of variance was violated, then Welch's t-test was employed instead.

#### 3.1 Neurological exam battery at PD35

No offspring were excluded from the study. Injected and non-injected mice did not differ in weight at postnatal day 35 ( $p = 0.624$ ). All offspring were able to locate the food pellet within the 5 minutes allowed, but injected mice took longer (Mimic Positive:  $M = 143.09$ ,  $SEM = 17.5$ ; No Injection:  $M = 87.81$ ,  $SEM = 12.7$ ) to locate the pellet than did NI mice ( $t(36.46) = 2.55$ ,  $p = 0.01$ ), suggesting possible odor-specific deficits at this developmental timepoint. When slowly lowered to a wire lid, mice exhibit a "reaching" response as they can see an area to latch onto, but a visually-impaired mouse would not exhibit this behavior (Jones et al., 2010). All mice exhibited appropriate "reach" behavior, displaying normally developing visual acuity. After a sudden, loud sound (experimenter's hand clap), mice display a startle eyeblink and flinching behavior

indicative of the appropriate auditory startle response, and all offspring tested displayed the appropriate startle response.

## 3.2 Social Behaviors

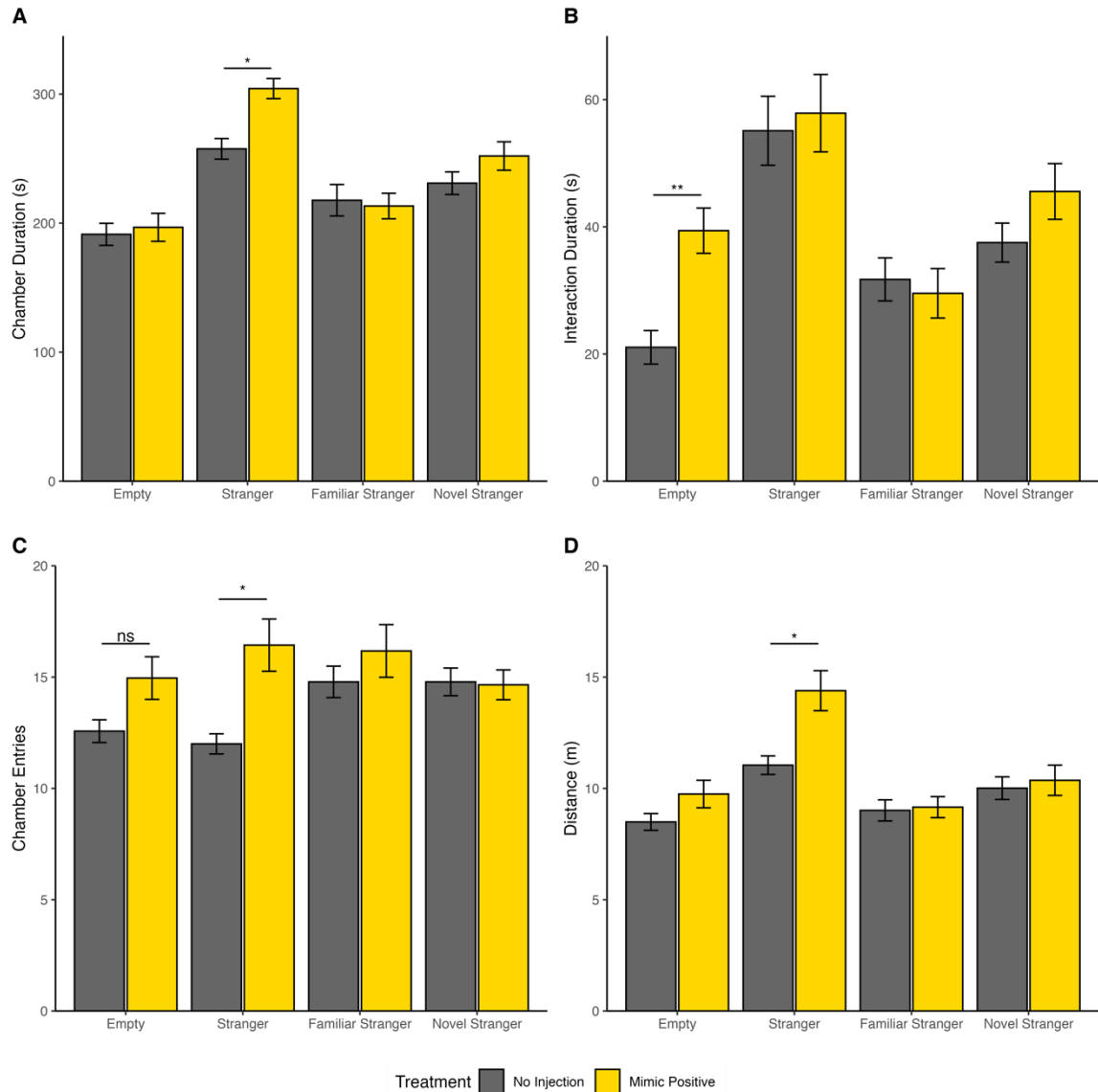
### 3.2.1 Social Approach

An analysis of social interaction behaviors revealed several significant differences between the offspring of mimic-injected and non-injected dams. Mimic Positive ( $M = 39.39$ ,  $SEM = 3.56$ ) animals spent significantly more time,  $t(35) = 3.35$ ,  $p = .002$ , than controls, ( $M = 21.05$ ,  $SEM = 3.86$ ), in proximity of the non-social stimulus (empty wire cup). However, no significant difference was found in the total duration spent in the empty cup chamber,  $t(35) = 0.32$ ,  $p = .751$  (Mimic Positive:  $M = 196.74$ ,  $SEM = 10.85$ ; No Injection:  $M = 191.31$ ,  $SEM = 12.51$ ). Similarly, the distance traveled in the chamber did not differ significantly,  $t(35) = 1.39$ ,  $p = .174$  (Mimic Positive:  $M = 9.75$ ,  $SEM = 0.62$ ; No Injection:  $M = 8.49$ ,  $SEM = 0.55$ ) between groups. No significant differences were found in time spent near the social stimulus (stranger mouse),  $t(35) = 0.28$ ,  $p = .783$  (Mimic Positive:  $M = 57.87$ ,  $SEM = 6.09$ ; No Injection:  $M = 55.10$ ,  $SEM = 7.94$ ). Yet, miRNA injected offspring spent significantly more time in the social chamber,  $t(35) = 3.46$ ,  $p = .001$  (Mimic Positive:  $M = 304.25$ ,  $SEM = 7.78$ ; No Injection:  $M = 257.56$ ,  $SEM = 11.67$ ), and traveled a greater distance within it,  $t(35) = 2.68$ ,  $p = .011$  (Mimic Positive:  $M = 14.39$ ,  $SEM = 0.90$ ; No Injection:  $M = 11.04$ ,  $SEM = 0.61$ ). These offspring also displayed increased entries into the social chamber ( $M = 16.4348$ ,  $SEM = 1.173$ ) compared to non-injected offspring,  $t(32.67) = 3.29$ ,  $p = 0.002$ ;  $M = 12$ ,  $SEM = 0.63$ ), and entries into the empty cup chamber trended toward significance,  $t(35) = 1.75$ ,  $p = .089$  (Mimic Positive:  $M = 14.96$ ,  $SEM = 0.96$ ; No Injection:  $M = 12.57$ ,  $SEM = 0.75$ ).

The social preference index did not differ between groups, with both displaying a preference (SPI > 0.50) for the social chamber over the non-social chamber.

### *3.2.2 Social Novelty*

During the final trial, the social novelty phase, the experimental mice are given the option of interacting with a familiar mouse, from the social approach trial, or to interact with a new novel mouse they have never interacted with previously. No significant group differences were observed in the time spent interacting with the familiar stranger or distance traveled in the familiar stranger's chamber, nor total duration in the familiar stranger's chamber (all  $p > 0.05$ ). Likewise, there were no significant group differences between offspring in interaction time with the novel stranger mouse, duration within the novel stranger's chamber, nor chamber distance metrics (all  $p > 0.05$ ). The social novelty index (SNI) was also not significant,  $t(35) = 0.54$ ,  $p = .593$  (Mimic Positive:  $M = 0.54$ ,  $SEM = 0.02$ ; No Injection:  $M = 0.52$ ,  $SEM = 0.03$ ). There were no differences in entrances to either the familiar social chamber nor the novel social chamber ( $p > 0.05$ ).



**Figure 3:** Measures from the three-chamber social approach and social novelty tasks. A) Offspring of injected dams spent more time in the chamber of the stranger mouse than did control offspring. B) Looking at proximal interaction duration, there were no differences in time proximal to the stranger mouse, but there was significantly more time spent investigating the empty cup by miRNA injected offspring. C) Injected offspring also entered the social chamber more times than did the non-injected, but did not show significantly more entries into the non-social chamber. D) Distance traveled within the chamber of the social stimulus, during the social approach phase, was higher in treated offspring, but this was not seen in other chambers or phases.

### 3.3 Restrictive and Repetitive Behaviors

#### 3.3.1 Grooming behaviors

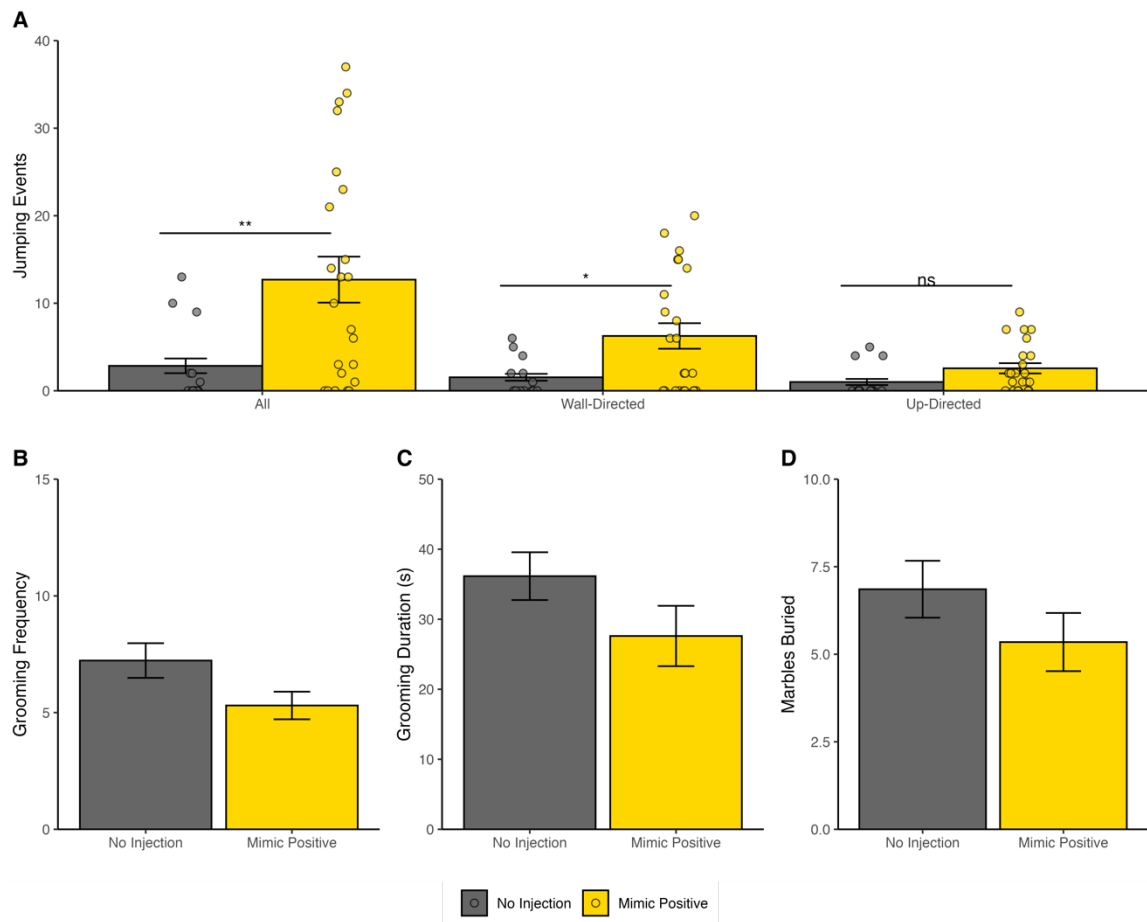
Grooming behaviors were reviewed using videos of open field testing. There were no significant differences between the Mimic Positive and No Injection groups, but injected offspring trended lower in measured grooming behaviors. Grooming frequency in the injected offspring did not differ from non-injected offspring (Mimic Positive:  $M = 5.30$ ,  $SEM = 0.59$ ; No Injection:  $n = 13$ ,  $M = 7.23$ ,  $SEM = 1.13$ ;  $t(34.0) = 1.67$ ,  $p = 0.104$ ) in frequency. Similarly, total grooming duration (Mimic Positive:  $M = 27.61$ ,  $SEM = 4.31$ ; No Injection:  $n = 13$ ,  $M = 36.15$ ,  $SEM = 5.18$ ) also did not significantly differ,  $t(34.0) = 1.23$ ,  $p = 0.226$ .

#### 3.3.2 Jumping behaviors

Mimic exposed animals showed significantly more jumping behaviors. The majority (18/23) of offspring from miRNA-injected dams displayed at least 1 jumping event, compared to the few occurrences seen in (5/22) control offspring. Total number of jumps observed were higher,  $t(30.48) = 3.37$ ,  $p = .002$ , among the injected offspring ( $M = 12.70$ ,  $SEM = 2.63$ ) than amongst injection naïve offspring ( $M = 2.85$ ,  $SEM = 1.28$ ). In particular, offspring exposed prenatally to the mimic miRNA showed significantly more wall-directed jumping,  $t(28.5) = 3.00$ ,  $p = .006$  (Mimic Positive:  $M = 6.26$ ,  $SEM = 1.46$ ; No Injection:  $M = 1.54$ ,  $SEM = 0.60$ ). Jumping from a wall-supported rearing position up-ward jumping showed a trend,  $t(34) = 1.78$ ,  $p = .084$  (Mimic Positive:  $M = 2.57$ ,  $SEM = 0.59$ ; No Injection:  $M = 1.00$ ,  $SEM = 0.53$ ), but wall-supported rearing was not significantly different between groups,  $t(34) = 1.73$ ,  $p = .093$ .

### *3.3.3 Marble Burying*

Offspring were individually placed in opaque cages already prepared with a 4x5 grid of novel navy marbles and covered by transparent covers to be recorded. Marble burying behaviors also showed no significant group differences, both in count,  $t(35) = 1.35$ ,  $p = .187$  (Mimic Positive:  $M = 3.17$ ,  $SEM = 0.87$ ; No Injection:  $M = 5.07$ ,  $SEM = 1.10$ ), and percentage,  $t(35) = 1.35$ ,  $p = .187$  (Mimic Positive:  $M = 15.87$ ,  $SEM = 4.36$ ; No Injection:  $M = 25.36$ ,  $SEM = 5.51$ ).

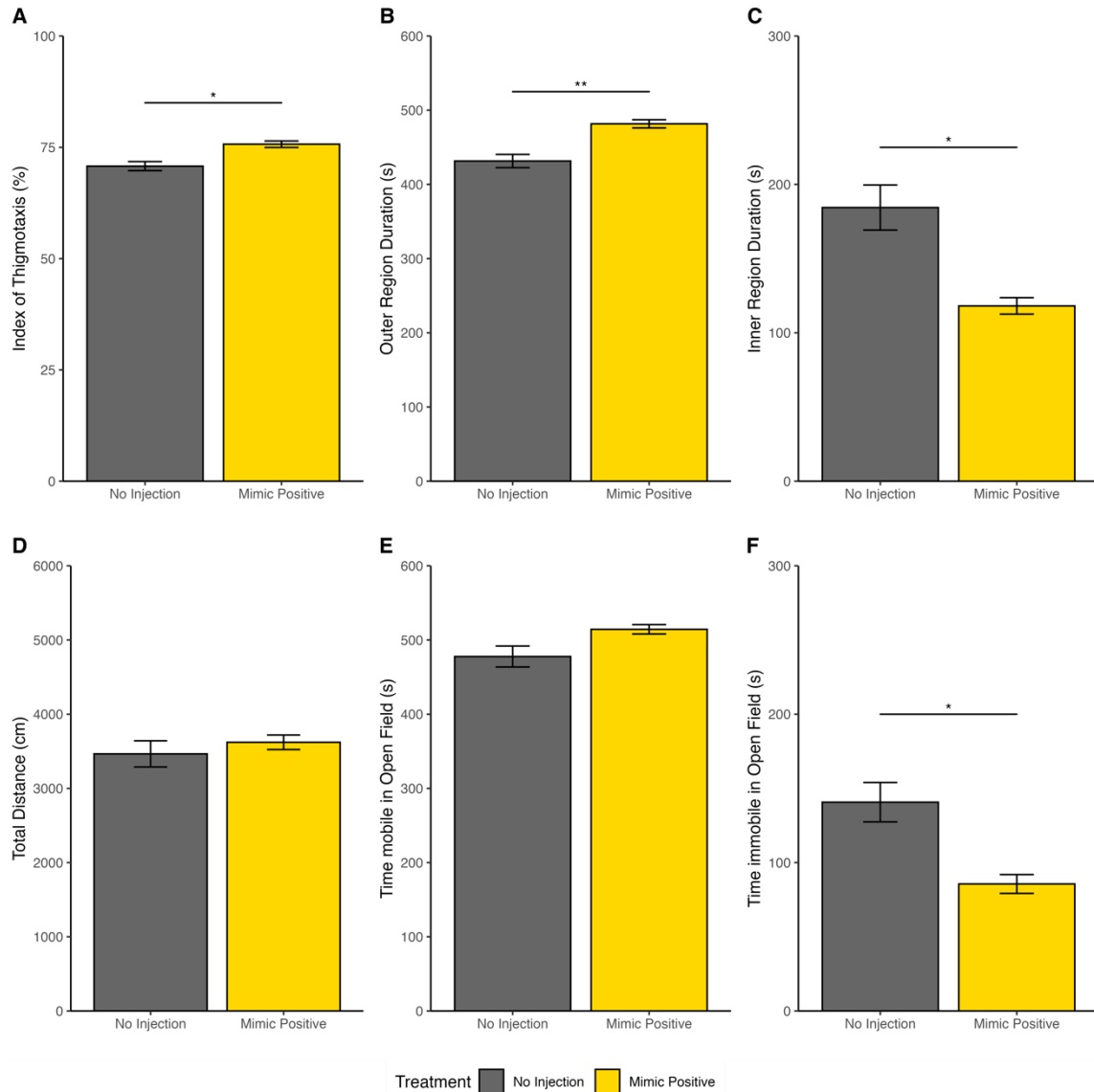


**Figure 4:** Repetitive behaviors were coded from open field trials and marble burying A) Jumping behavior was seen in injected offspring and were seen to be higher in the injected offspring in total jumping events and wall-directed jumping, but not up-directed (escape behavior) jumping B) Grooming frequency and C) total time grooming did not differ between groups. D) Marbles buried did not differ between groups.

### 3.4 Generalized Anxiety

#### 3.4.1 Open field

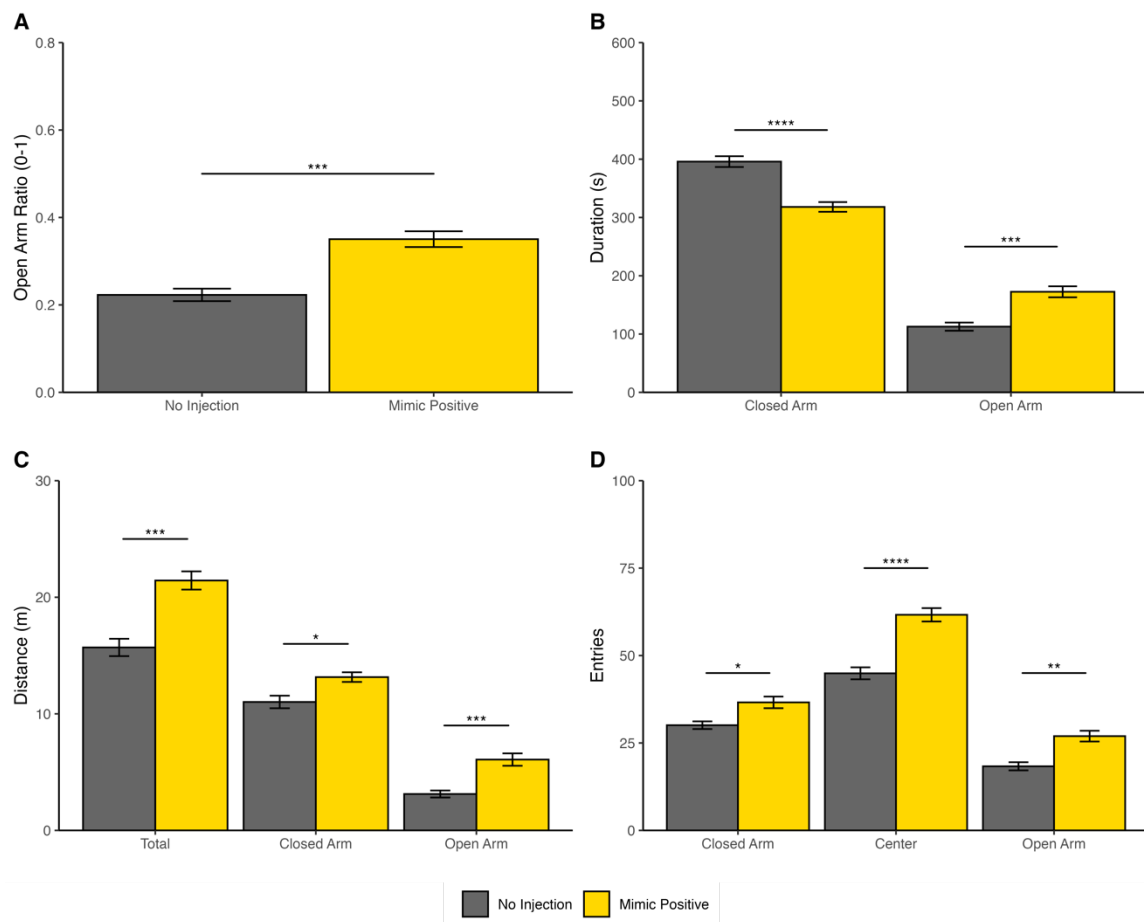
The index of thigmotaxis described by the ratio of distance travelled in the outer region (along the wall of the apparatus) to total distance expressed as a percentage was greater in the Mimic Positive group,  $t(33) = 3.23$ ,  $p = .003$  ( $M = 75.7\%$ ,  $SEM = 0.01$ ; No Injection:  $M = 70.8\%$ ,  $SEM = 0.02$ ). Offspring of injected dams spent more time in the outer region of the open field,  $t(17.71) = 3.53$ ,  $p = .002$  ( $M = 481.64$ ,  $SEM = 5.51$ ) compared to controls ( $M = 431.54$ ,  $SEM = 13.06$ ), and less time in the inner region ( $t(14.63) = 2.89$ ,  $p = 0.011$  ( $M = 118.1304$ ,  $SEM = 5.537$ ) than controls as well ( $M = 184.41$ ,  $SEM = 22.24$ ). Although the total distance in the open field and total distance travelled in the center were not significantly different between groups,  $t(13.77) = 0.53$ ,  $p = .605$  and  $t(35) = 1.66$ ,  $p = .106$ , respectively. This may be partially explained by their faster speed in the inner region of the open field,  $t(35) = 3.13$ ,  $p = .004$  (Mimic Positive:  $M = 7.6$  cm/s,  $SEM = 0.3$ ; No Injection:  $M = 5.9$ ,  $SEM = 0.5$ ) despite less time in the center of the open field, but total average speed in the open field was not significantly different between groups ( $t(35) = 0.33$ ,  $p = .744$ , Mimic Positive:  $M = 6.3$  cm/s,  $SEM = 0.4$ ; No Injection:  $M = 6.1$  cm/s,  $SEM = 0.5$ ). No significant group differences were observed in time mobile  $t(12.8) = 1.58$ ,  $p = 0.139$ , (Mimic:  $M = 514.4130$ ,  $SEM = 6.342$ ; Control:  $M = 477.7333$ ,  $SEM = 22.346$ ), the miRNA group displayed decreased immobility ( $M = 85.59$ ,  $SEM = 6.34$ ) compared to controls  $t(13.05) = 2.51$ ,  $p = 0.026$  ( $M = 140.71$ ,  $SEM = 20.98$ ).



**Figure 5:** Open Field measures included A) Thigmotaxis index indicated offspring of miRNA injections travelled proportionally more in the outer region (near the wall of the apparatus) compared to the inner region than did the control offspring. B) Offspring also spent significantly more time in the outer region of the open field C) Injected offspring consistently spent significantly less time in the center of the open field than controls. D) Total distance travelled did not differ between offspring. E) Total time spent mobile did not differ F) But mimic offspring did differ in total time spent immobile during open field testing.

### 3.4.2 Elevated plus maze

The open arm ratio provides an index of exploratory behavior as well as open-space invoked anxiety characteristic in mice. Mimic Positive animals exhibited a significantly higher open arm ratio,  $t(33) = 4.27$ ,  $p < .001$  (Mimic Positive:  $M = 0.35$ ,  $SEM = 0.02$ ; No Injection:  $M = 0.22$ ,  $SEM = 0.02$ ). On the elevated plus maze, miRNA offspring spent significantly more time ( $M = 172.50$ ,  $SEM = 9.45$ ) in the open arms,  $t(33) = 3.89$ ,  $p < .001$ , than controls ( $M = 112.71$ ,  $SEM = 11.12$ ), and in contrast, control offspring spent more time in the closed arms,  $t(33) = 4.98$ ,  $p < .001$ . Mimic Positive animals traveled significantly farther in the open arms,  $t(33) = 3.62$ ,  $p < .001$  (Mimic Positive:  $M = 6.08$ ,  $SEM = 0.53$ ; No Injection:  $M = 3.12$ ,  $SEM = 0.48$ ), and entered them more frequently,  $t(33) = 3.43$ ,  $p = .002$ . This high locomotor behavior was consistent in the closed arms as the miRNA group also travelled further in the closed arms  $t(33.0) = 2.54$ ,  $p = 0.016$ , ( $M = 13.1501$ ,  $SEM = 0.414$ ) and displayed a greater number of entries into the closed arm ( $t(33.0) = 2.48$ ,  $p = 0.018$ ;  $M = 36.61$ ,  $SEM = 1.67$ ) than non-injected dam offspring respectively ( $M = 11.02$ ,  $SEM = 0.85$ ;  $M = 30.08$ ,  $SEM = 1.73$ ). Finally, the miRNA-mimic group travelled more through the center,  $t(33) = 5.09$ ,  $p < .001$  (Mimic Positive:  $M = 61.65$ ,  $SEM = 1.91$ ; No Injection:  $M = 44.92$ ,  $SEM = 2.71$ ), and exhibited significantly greater average speed,  $t(33) = 4.13$ ,  $p < .001$  (Mimic:  $M = 3.6$  cm/s,  $SEM = 0.1$ ; Control:  $M = 2.6$  cm/s,  $SEM = 0.2$ ).



**Figure 6:** Elevated Plus Maze measures A) Open arm ratio was significantly higher in offspring of the B) Offspring also spent significantly more time in open arm of the apparatus and less time in the closed arm than the control offspring. C) Injected offspring consistently traveled more across both arms and total distance. D) Mimic offspring also entered both arms and the center of the elevated plus maze than controls.

## 4. Discussion

### 4.1 Main Findings

In this study, we investigated the causal role of three miRNAs found to be upregulated in postpartum samples after chronic variable prenatal stress exposure from E6 until giving birth, as reported in our previous study (Woo et al., 2023). The primary finding is that prenatal intravenous exposure to a mimic cocktail of mmu-miR-7684-3p, mmu-miR-5622-3p, and mmu-miR-6900-3p at E6 and E14 is sufficient to induce a complex and distinct behavioral phenotype in offspring, characterized by significant alterations in repetitive behaviors and activity levels, and a paradoxical anxiety profile, but not clear deficits in core sociability. These results suggest miRNAs identified from a prenatally stressed (SERT-het) dam at E21 are potent mediators of neurodevelopmental programming and can independently produce behavioral abnormalities absent from other genetic or other non-miRNA mediated stress factors.

We first aimed to evaluate autism-related behavioral domains, beginning with the three-chamber social approach task. The offspring of dams exposed to the miRNA cocktail did not show a reduction in social preference time, and instead spent comparable the stranger's chamber compared to controls . However, this can be attributed to increased locomotor behavior, as shown by the increased entries into the stranger's chamber, and the distance travelled within the chamber was significantly greater than control offspring. Similarly, the empty cup was investigated for a longer duration by treated offspring, but distance in the chamber and duration spent in the chamber were not different from controls. Again, this may be partially explained by increased entries into

the empty cup chamber, but this did not reach significance ( $p = .089$ ). Social novelty did not differ in any measure.

Repetitive behaviors, as measured by self-grooming and marble burying, did not differ between groups, but miRNA offspring did display more jumping events, including jumping from a wall-supported rearing position upward and wall-directed jumping starting not against the wall. While these behaviors were not observed in our previous work, repetitive jumping has been described in the C58/J ASD mouse model, an inbred strain from The Jackson Laboratory (Ryan et al., 2010; Moy et al., 2014). Originally bred from the same male origin as the C57BL strains widely used today, but a different female, they exhibit incredibly high rates of leukemia and are susceptible to aortic lesions. Other behavioral characteristics include low social preference and increased motor stereotypies that begin around 6 weeks of age. Critically, C58/J jumping behavior was only described as starting from a wall-supported rearing position along with “upright scrambling” and “back-flipping” in home cage behavior, but neither of which was witnessed during behavioral tasks.

In contrast to our previous study, miRNA mice displayed increased thigmotaxic behavior in the open field. Injected offspring spent increased time near the wall of the open field compared to offspring of non-injected dams, concurring with previous prenatal stress effects, but not aligned with all previous findings in the SERT-het model. Time immobile within the apparatus was also lower than control offspring, but time mobile did not differ. The OA ratio and total time spent on the open arm were higher amongst the offspring of injected dams. Similar to our three-chamber findings, indications of locomotor differences were seen in the EPM, as distance travelled in and number of

entries to both closed and open arms exceeded that of controls. While not observed in the study isolating the novel miRNA injected, our research group has described increased OA ratio alongside decreased time in the center of the open field, but its association with prenatal stress or the SERT gene/prenatal stress interaction remains unclear (Jones, Smith, et al., 2010b; Matsui et al., 2018). However, no locomotor differences were observed in either study.

## 4.2 Interpretations

Our findings do not recapitulate the complete behavioral profile seen from the offspring of prenatally stressed SERT<sup>+/-</sup> mice, but they have a few behavioral similarities and stark differences. The microRNAs introduced, while upregulated in our G x E model, may reflect an incomplete miRNA profile, void of necessary miRNA response elements (MREs), nucleotide sequences in the 3' UTR which increase the likelihood of miRNA-binding and can be used as decoy messengers deterring endogenous miRNAs from “normal” functions, and other mediating factors, such as hormonal and other exosomal regulators, thus reducing interactive processes by which endogenous miRNAs play a crucial role (O'Brien et al., 2018; Long et al., 2019; Riolo et al., 2020).

The characterized jumping behaviors identified predominantly in miRNA-exposed offspring are reminiscent of some escape behaviors previously described in other murine models. For example, mice exhibit jumping behaviors in naloxone-precipitated withdrawal models of morphine addiction, exposure to acute heat stress (Harikai et al., 2004) or CO<sub>2</sub> exposure, and a pituitary adenylate cyclase-activating polypeptide (PACAP) knock out (Hashimoto et al., 2001; Shintani et al., 2006). Morphine and heroin dependent, naloxone-precipitated withdrawal-related locomotor behavior was reduced

with a 5-HT<sub>2A</sub> receptor antagonist (MDL 11,939) and 5-HT<sub>2C</sub> receptor agonists (lorcaserin), including jumping behaviors (X. Wu et al., 2015; Pang et al., 2016). CO<sub>2</sub> exposure, but not hypoxia, induced “panic” jumping, and jumping was reduced when chronic (21 days) intraperitoneal (I.P.) injections of Fluoxetine at 5, 10, and 15 mg/kg, but only reduced jumping in 10 and 15 mg/kg acute doses (Spiacci et al., 2018). A full knockout model of *Adcyap1*, but not *Adcyap1*<sup>+/-</sup>, encoding the neuropeptide PACAP displayed explosive jumping behaviors by 6 weeks of age, and intraperitoneal treatment of fluoxetine and 5-HT precursor, 5-HTP, reduced the number of jumps observed over 90 minutes. However, timing of serotonergic exposure is critical as SSRIs can produce anxiolytic effects by and after 6 weeks of age, but fluoxetine exposure during development may instead result in an anxiogenic phenotype. Another study recently published showed jumping behaviors in the offspring of BTBR T+ Itpr3tf/J (BTBR) mice that were prenatally stressed and subcutaneously injected with Fluoxetine. Fluoxetine without restraint and restraint without fluoxetine was sufficient to produce jumping behavior in female BTBR mice, but not in males or either B6 offspring. However, restraint and fluoxetine administration during pregnancy resulted in jumping behavior in both male and female BTBR offspring (Arzuaga et al., 2025). According to TargetScan, the *Htr2c*, the gene coding for the 5-HT<sub>2C</sub> receptor is a predicted target for both miR-5622-3p and miR-6900-3p, but further work is required to understand the prenatal role of these miRNAs on specific gene targets.

C58/J, an inbred ASD mouse model mentioned previously, exhibits spontaneous jumping behavior in both the home cage and during open field testing as well as reduced social communication (Ryan et al., 2010). However, this strain also displays decreased

self-grooming and marble burying behavior, suggesting type of repetitive behavior may vary and not necessarily will correlate with other measures of RRB. C58/J mice also displayed “jackhammer” jumping in multiple behavioral tasks, while the miRNA-treated mice in this study only exhibited these behaviors in the open field task. Interestingly, this model also shows opposing anxiety-related behaviors, demonstrating more time in the center of a novel open field but decreased exploration into the open arms of an EPM. Lastly, hyperlocomotion was observed from post-natal day 6 in a small open field in C58/J mice, but jumping behaviors were not observed until PD 16/17. Future studies should similarly investigate the earliest age at which hyperlocomotion can be detected. In a similar vein, Ts65Dn mice, a mouse model for Down Syndrome, also exhibit repetitive jumping in home cage behavior, but differs due to decreased locomotor behaviors in the open field (Turner et al., 2001).

### 4.3 Limitations and Future Research

While behavioral changes were evident in miRNA-exposed offspring, the findings herein should be interpreted with caution. Intravenous injection of miRNA mimic introduces multiple confounding stressors, such as the needle entry during injection, introduction of a novel solution to the bloodstream, short-term restraint stress during the injection, and increased researcher handling not experienced by control dams. The stress of the injection procedure itself could be a confounding variable, and future studies should incorporate a vehicle-injected control group to isolate the effects of the miRNA cocktail from procedural stress. Intravenous injection protocols were undertaken by trained researchers, but no microarray analyses were conducted to verify fetal exposure of miRNA or differential distribution throughout the litter.

The neurological exam at PD35 revealed that injected mice took significantly longer to locate a buried food pellet, suggesting a potential olfactory deficit. As social recognition in mice relies heavily on olfactory cues, this deficit could have potentially confounded social behavior assessments, but both groups exhibited expected preference for the novel stranger, suggests intact social discrimination capabilities. This study only examined the effects of a specific cocktail of three miRNAs; future research should aim to disentangle the individual contributions of each miRNA to the observed behavioral phenotype, including further investigation of contributing factors within the maternal system and their subsequent interactions with these, or other, miRNAs.

#### 4.4 Conclusions

This study provides preliminary novel evidence that intravenous maternal injection of prenatal-stress-related miRNAs isolated from a stress-susceptible (SERT-het) mouse model can produce behavioral modifications in the offspring in comparison to injection naïve and minimally stressed control offspring. The findings point toward a complex phenotype characterized by hyperactivity and specific motor stereotypies. Future research should focus on confirming the hyperactivity phenotype and exploring the underlying neural mechanisms. We hope to further elucidate the role of the miRNA cocktail compared to the maternal stressors of injection and other confounds using negative controls of missense nucleotide and buffer intravenous exposure. Investigating the gene targets of these miRNAs within the developing brain could illuminate the pathways through which prenatal introduction of these specific miRNA are capable of producing this unique behavioral profile.

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