

**MATERNAL INFLUENCE ON EPIGENETICS AND OFFSPRING
BEHAVIOR OUTCOMES: EXPLORING UNDERLYING MECHANISMS
AND SEX-SPECIFIC SENSITIVITIES IN A GENE ENVIRONMENT
INTERACTION MOUSE MODEL**

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by
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MATERNAL INFLUENCE ON EPIGENETICS AND OFFSPRING BEHAVIOR
OUTCOMES: EXPLORING UNDERLYING MECHANISMS AND SEX-SPECIFIC
SENSITIVITIES IN A GENE ENVIRONMENT INTERACTION MOUSE MODEL

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Dedication

For Gloria,
Dwight and Alyssa
and Kaden.

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LIST OF ABBREVIATIONS

5-HT: 5- hydroxytryptamine

5-HTTLPR: 5- hydroxytryptamine transporter gene linked polymorphic region

ADHD: Attention deficit and hyperactivity disorder

AGO: Argonaute

ANOVA: Analysis of variance

ASD: autism spectrum disorder

CNS: central nervous system

CRH: Corticotropin releasing hormone

DA: dopamine

DBPS: Dulbecco Phosphate Buffered Saline

DE: differential expression

DOHaD: Developmental Origins of Health and Disease

DSM: Diagnostic and Statistical Manual of Mental Disorders

E 21: embryonic day 21

EPM: elevated plus maze

FXS: Fragile X syndrome

G x E: gene environment interaction

GABA: γ aminobutyric acid

GO: gene ontology

GWAS: Genome-wide associated study

HPA: Hypothalamic-pituitary axis

ID: Intellectual disability

L-allele: long allele

MB: marble burying

MDD: major depression disorder

miR: micro RNA

miRISC: microRNA induced silencing complex

mRNA: messenger RNA

NDD: Neurodevelopmental disorders

NE: norepinephrine

nt: nucleotide

OCD: obsessive compulsive disorder

OF: open field

PD: postnatal day

PPD-NOS: Pervasive disorder not otherwise specified

pre-miRNA: precursor miRNA

pri-miRNA: primary mRNA

PTSD: Post traumatic stress disorder

RNAi: RNA interference

RRBI: repetitive and restricted behaviors and interests

rRNA: ribosomal RNA

S-allele: short allele

SERT: serotonin transporter

SERT/het: heterozygous serotonin transporter genotype

SLC6A4: solute carrier family 6A4

tRNA: transfer RNA

UTR: Untranslated region

WBC: white blood cell

XPO: exportin

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ABSTRACT

Autism Spectrum Disorder (ASD) is a lifelong neurodevelopmental disorder characterized by impaired social communication, overly regimented adherence to routine, restricted interests, and repetitive behaviors. Even though early studies implicated genetic risk factors, recent evidence emphasize environmental factors, including experiencing adverse life events elevating increasing ASD risk, which is of greater importance in pregnancies of genetically stress susceptible individuals. We previously reported the *SLC6A4* gene, encoding the human serotonin transporter (SERT), along with the variant, the serotonin transporter gene linked polymorphic region (5-HTTLPR), playing a critical role in elevated stress responsivity and embryonic methylation dysregulation in our gene-environment interaction (GxE) mouse model. MicroRNA (miR) molecules negatively regulate gene expression and has been duly implicated in physiological and pathophysiological processes, including cell differentiation, neurodevelopment, and neuropsychiatric disorders and has recently demonstrated a critical role in stress- and behavior- related disorders

including ASD. Therefore, to examine maternal stress susceptibility's impact on underlying microRNA expression and behavioral outcomes in offspring, pregnant wild type and SERT (+/-) dams were exposed to a chronic variable stress (CVS) protocol, after which, maternal microRNA profiles were examined. Furthermore, cohorts of female and male offspring underwent a battery of behavioral tests beginning at postnatal day 60 (PD60) to examine SERT (+/-) x CVS effects on behaviors associated with ASD including, anxiety, elevated repetitive behaviors and sociability deficits. Maternal molecular profiling detected three upregulated microRNAs unique to SERT (+/-) x CVS cohort, *mmu-miR-5622-3p*, *mmu-miR-6900-3p* and *mmu-miR-7684-3p* with predicted genetic projections associated with stress and autism spectrum disorders with biological enrichment implicated in forebrain and central nervous system development. Moreover, offspring assays revealed selective, sex-specific behavioral differences with female offspring demonstrating maternal genotype and interaction mediated behaviors, while male offspring displayed maternal stress and interaction mediated behaviors, which suggests the combination of both or individual factors of SERT (+/-) x CVS can sufficiently impact offspring behavior outcomes. Taken together, the results of this study demonstrate the complexity and dynamism of the interplay between maternal genotype and adverse prenatal stress exposure on underlying regulators of ASD- and stress- associated gene networks. And furthermore, provides evidence of their effects on behavioral outcomes of offspring

Chapter 1

History of autism spectrum disorders

Autism Spectrum Disorders (ASD) refers to a group of neurodevelopmental disorders including autism, Asperger's syndrome (AS) and pervasive developmental disorder-not otherwise specified (PDD-NOS) (Sharma et al., 2018a). Originating from the Greek word *autos*, meaning *self*, the term *autism* was first used by Eugen Bleuler, a Swiss psychiatrist in 1911 (Maye et al., 2016) to describe symptoms of schizophrenia in which individuals existed within self-appointed social isolation. He notes: *'In their autism they can comport themselves in a crowded work-room as if they were alone; everything which concerns others does not exist for them... Sometimes patients are obtrusive, continue endlessly repeat the same thing over and over again, and are completely deaf to all objections. At other times, they comport themselves very disdainfully, curtly and rudely'* (Bleuler, 1911).

In 1943, Leo Kanner, an Austrian psychiatrist, presented evidence distinguishing autism as a separate disorder, independent of childhood-onset schizophrenia (COS). In his seminal report titled, *Autistic Disturbances of Affective Contact*, Kanner describes 11 case studies where children, predominantly male, between 2- and 8-years old exhibit traits previously reported in Bleuler's publication sans delusional and hallucinogenic schizophrenic ascriptions. Kanner notes, *'He paid no attention to persons around him. When taken into a room, he completely disregarded the people and instantly went for objects, preferably those he can spin*

(Kanner, 1943). Some patients displayed irrelevant speech utterances despite the absence of cognitive and linguistic impediments, failed to display appropriate reciprocal facial expressions (e.g., smiling, head nodding in assent) in social settings, restrictive interests with no utility (e.g., knowing a specific train schedule in a certain country, playing only with puzzles), ritualistic insistence on sameness when performing tasks or daily routines, interminable, and repetitive behaviors such as clapping or repeating idiosyncratic words or phrases (Maye et al., 2016). In the 8 decades since Kanner's groundbreaking study, the development and continuous enhancement of screening tools, and modification of diagnostic criteria, made it clear the autistic phenotype was divergent from schizophrenic manifestations. Consequently, *infantile autism* was officially acknowledged by the American Psychiatric Association and included as a distinct disorder in the 1980 revision of Diagnosis and Statistic Manual of Mental Disorders (DSM-IV).

During the infancy of autism's conceptualization, other similarly related neurobiological disorders emerged. In 1944, Austrian psychiatrist, Hans Asperger reported patients exhibiting atypical social and communicative deficits similar to those in the Kanner study. However, Asperger also noted distinct higher functioning cognition; symptomology that later became to be known as Asperger's Syndrome. When speaking to their intelligence, he notes, "*This becomes clearer when we look at the language production of autistic children. They, and especially the intellectually gifted among them, undoubtedly have a special creative attitude towards language. They are able to express their own original experience in a*

linguistically original form" (Asperger, 1943). Asperger's syndrome was classified as a distinct neurological disorder in the DSM-IV, (2000).

Pervasive Developmental Disorder Not Otherwise Specified (PPD-NOS) was another neurodevelopmental disorder diagnosis added to the DSM-IV to classify individuals who displayed symptoms that reflected the presence of restricted and/or repetitive behavior but did not meet the criteria for autistic disorder (Maye et al., 2016). In 2013, the American Psychiatric Association revised the DSM to include Autism Disorder, Asperger Syndrome and Pervasive Developmental Disorder Not Otherwise Specified (PPD-NOS) under a single clinical classification category, Autism Spectrum Disorders (APA, 2013). Affecting 1 in 59 births (Maenner et al., 2020), hallmark features include verbal and non-verbal social and communication impairments, and repetitive and restricted behaviors and interests (RRBI) (DSM-V, 2013), typically prominent by 36 months of age. However, there have been incidences in which infants have been reported to exhibit abnormal socialization and communicative deficits as young as 6 months of age (Maestro et al., 2002). Although both sexes are at risk for autism spectrum disorders, it is a general consensus that males are 4 times more likely than females to be affected with ASD (Sharma et al., 2018a), suggesting the possibility of ASD being a sex-biased neurodevelopmental disorder. Moreover, ASD demographic data further suggests its prevalence is similar across all racial, ethnic, and socioeconomic groups.

Although much is known about ASD, its etiology is not fully understood. Early reports initially implicated genetic components. However, recent population

studies have demonstrated the influence of environmental factors, thus changing the perspective of ASD pathology (Lipkin et al., 2023).

The Developmental Origins of Health and Disease (DOHaD)

The developmental origins of health and disease (DOHaD) is a theory which postulates the impact of the maternal environment influencing the development of offspring and health outcomes after birth and throughout its lifespan (Gage et al., 2016). Compelling evidence argues the effects of prenatal stress such as exposure to natural disasters (Kinney et al., 2008), environmental pollutants (Perera and Herbstman, 2011), and interpersonal discord (Schneider et al., 2002) significantly alters the trajectory of fetal neurodevelopment. Our lab's previous study show, when compared to control groups, maternal stressors experienced during the late 2nd - to mid-3rd trimesters result in an elevated incidence of autistic births (Beverdors et al., 2005). This suggests not only the potential contribution of environmental factors to autism development, but also the critical timing of adverse exposures. Furthermore, findings from our lab's previous study (Hecht, 2016) show pregnant women with the functional variant of *SLC6A4* allele, serotonin transporter gene linked polymorphic region, (*5-HTTLPR*) report a greater number of stressors during mid- 2nd to late 3rd trimester when compared to mothers. This finding provides evidence of genetic variance determining stress susceptibility thresholds in humans. Hence, leading our group to argue in favor of the significance of maternal gene-mediated stress susceptibility.

The role of prenatal stress in autism spectrum disorder

During pregnancy, the developing embryo is at a very vulnerable stage. Studies investigating the consequences of stressors while *in utero* have argued that prenatal stress exposure, for example maternal infection and adverse diet conditions such as malnutrition (Bale, 2015), can potentially alter the trajectory of fetal programming, which subsequently impose profound impact on future health, behavioral and cognitive outcomes. For example, a pivotal retrospective study investigated whether the abrupt changes in prenatal food intake of the Dutch population following Nazi blockade in 1944- 1945 (aka The Dutch Hunger Winter of 1944-1945), alters risk of schizophrenia diagnoses in children later in life (Susser ES, et al., 1992). The authors concluded, when compared to unexposed pregnancies with a normal diet, children *in utero*, exposed to limited food reported higher incidence of schizophrenia hospital admissions during adulthood. Additional obstetric- associated complications noted by the authors included higher prevalence of 'very low birth weights and stillbirths' and 'anomalies of the central nervous system such as spina bifida and hydrocephalus, cerebral palsy' (Susser and Lin, 1992). Another study looking into the effects of prenatal exposure to war on schizophrenic risk in offspring, also saw an increase in schizophrenic diagnoses in adult children (Malaspina et al., 2008). Taken together, these results support the plausibility of the effects of stress exposure during fetal development can influence the future psychiatric well-being of offspring.

In addition, published studies have reported the effects of environmental insults on endogenous stress pathways, such as the hypothalamic- pituitary-adrenal stress

axis (HPA stress axis), which, its dysregulation during pregnancy can negatively impact pregnancy, such as gestation length (Wadhwa et al., 1998), and placental growth (Cuffe et al., 2012), Interestingly, a study in 1999 by the Fujioka group demonstrated fetal vulnerability to maternal stress by observing elevated fetal corticotropin releasing hormone (CRH) expression in response to 30- minute maternal restraint stress exposure performed during embryonic day 15 relative to the fetus of non-stress exposed rats (Fujioka et al., 1999). This finding not only demonstrates that the stress systems of developing offspring are not only active during pregnancy, but also evinces their sensitivity to mount a response against external stimuli.

As previously inferred, gestational stress exposure has significant potential to alter offspring development. In addition, as several studies have reported, the consequences of environmental stressors can exert long-lasting effects on the future health and behavioral outcomes of offspring (Wadhwa et al., 2009). For example, studies using preclinical models have demonstrated a positive correlation between prenatal stress exposure and characteristics observed in individuals with affective and neurodevelopmental disorders, including depression (Alonso et al., 1991) and anxiety (Weinstock, 2017), and autism spectrum disorders (Petroni et al., 2022). Indeed, findings from epidemiological studies have demonstrated a correlation between ASD diagnoses and prenatal stress exposure. For instance, a study by Kinney and colleagues demonstrated higher ASD risk following exposure to hurricanes and tropical storms during pregnancy (Kinney et al., 2008). Additional factors include adverse life events such as marital discord,

involuntary job loss, (Beversdorf et al., 2005; Varcin et al., 2017), and maternal bereavement (Li et al., 2009). Taken together, the results of these studies provide evidence demonstrating the role of maternal stress in ASD etiology.

The underlying substrates of autism spectrum disorders

In recent years, animal studies have made it increasingly clear of the interplay between genes and stress and their roles in physiological and pathophysiological conditions. Although their precise effects are still unknown, studies are beginning to reveal the influence these factors play in biological (Padula et al., 2020), social (Dubow et al., 2012), (Jansen et al., 2010), psychological (Dick, 2011), (Weinstock, 1997), (McLean et al., 2018) and emotional (Lesch, 2004) (Marchette et al., 2018) states. As these studies suggest, the effects of stress are diverse; capable of affecting a wide range of domains relevant and essential to an individual's quality of life and survival.

One early study investigating the relationship between genes and stress, argued that an individual's ability or inability to cope and adapt to changes in the environment could be largely dependent upon genetic factors (Selye, 1950). Subsequent studies examined the stress reactivity in genetically stress susceptible models. For example, a study led by Bale and colleagues in 2000, examining stress reactivity in corticotropin-releasing hormone 2 (Crh2) mutant mouse model observed significantly reduced exploration of the exposed arms of the elevated plus maze (EPM), and central areas of the open field (OF) behavioral tests in stress exposed Crh2 mutants relative to the control group that were exposed to identical stress protocol. Moreover, molecular analyses uncovered elevated corticosterone levels in the Crh2 stressed group (Bale et al., 2000). In this case, these results suggest the potential role of genetic factors modulating behavioral impairments and underlying molecular dysregulation in response to adverse conditions.

Within the last few decades, technological advancements and improvements in methodological practices are providing a better understanding of the role of genetics during development. Recent published findings argue neurobiological architects of brain development and function such as, establishment of signaling pathways (Bulovaite et al., 2022), timely cell division (Bae et al., 2015) and neuronal migration (Geschwind, 2011), active neurotransmission systems (Valenzuela and Zucca, 2011), and proper arrangement of neuroanatomical structures (Müller and Fishman, 2018) are essential to proper brain development and function during pregnancy and throughout an individual's lifetime. This concerted involvement of cellular, molecular and biochemical processes suggests that neurodevelopment relies on distinct yet interconnected pathways. However, evidence is beginning to reveal the underlying genetic blueprint as a major driver of brain development and function in physiological and pathological states. For example, a seminal study examining the heritability of autism using samples of mono- and dizygotic twins argues that an increased incidence of autism among monozygotic twins when compared to dizygotic twins (Folstein and Rutter, 1977), which strongly suggests a heritable component for the disorder. This hypothesis may be supported by several retrospective studies with in which simplex and multiplex family (families with one or more than one autistic individual, respectively) samples have demonstrated an increase of first degree relatives (parent or sibling) with a psychiatric or neurodevelopmental disorder when compared to the general population (Jokiranta et al., 2013) (Liu and Takumi, 2014), (Jokiranta-Olkonieni et al., 2016), (Wilfert et al., 2021). However, several genome-wide associated studies

(GWAS) into ASD etiology have implicated single nucleotide polymorphisms (SNPs) (Wang et al., 2009), (Masini et al., 2020), insertion/deletions (indels), (Chan et al., 2019), (Wilfert et al., 2021), (Paulsen et al., 2022) copy number variants (CNVs) (Wilfert et al., 2021) and *de novo* mutations (de la Torre-Ubieta et al., 2016), (Courchet et al., 2018) thus lending to the heterogenous genetic and behavioral phenotypes in ASD individuals. Taken together, these studies provide insight into the dynamic nature of ASD and provide a better scientific and clinical understanding of complex neurodevelopmental disorders.

The serotonin transporter (SERT)

The serotonin transporter (SERT) protein, encoded by the *SLC6A4* gene, is responsible for regulating sleep, appetite, and emotion in mammalian systems (Figure 1). SERT's primary function is to return the neurotransmitter, serotonin, from the synaptic space to the presynaptic terminal of neurons. Its variant, the serotonin transporter-linked polymorphic region (*5-HTTLPR*) polymorphic short allele (S), is comprised of a 44-base pair deletion in the promoter region, (whereas the long allele (L) promoter is full length (Figure 2). Analyses of the *5-HTTLPR* has revealed how modifications within its genetic architecture can impose adverse downstream repercussions, including dysregulated transcription (Park et al., 2006), a reduction of presynaptic SERT expression (Murphy and Lesch, 2008), which significantly play roles critical to serotonergic signaling (Kim et al., 2005).

Researchers have long postulated the existence of underlying mechanistic pathways between genetic factors and the external environment- leading to their interaction and cumulative effects on development during pregnancy. For example, a study in 2017 from our group demonstrated maternal stress altering the methylome in the embryonic brains of offspring (Sjaarda et al., 2017). Overall, these studies provide compelling insight into the underlying mechanisms of G x E associated pathologies, and their potential influence during development (Beversdorf et al., 2018). Although these mechanisms have been studied previously, many remain unknown and less understood.

Figure 1: Localization and mechanism of serotonin transporter (SERT)

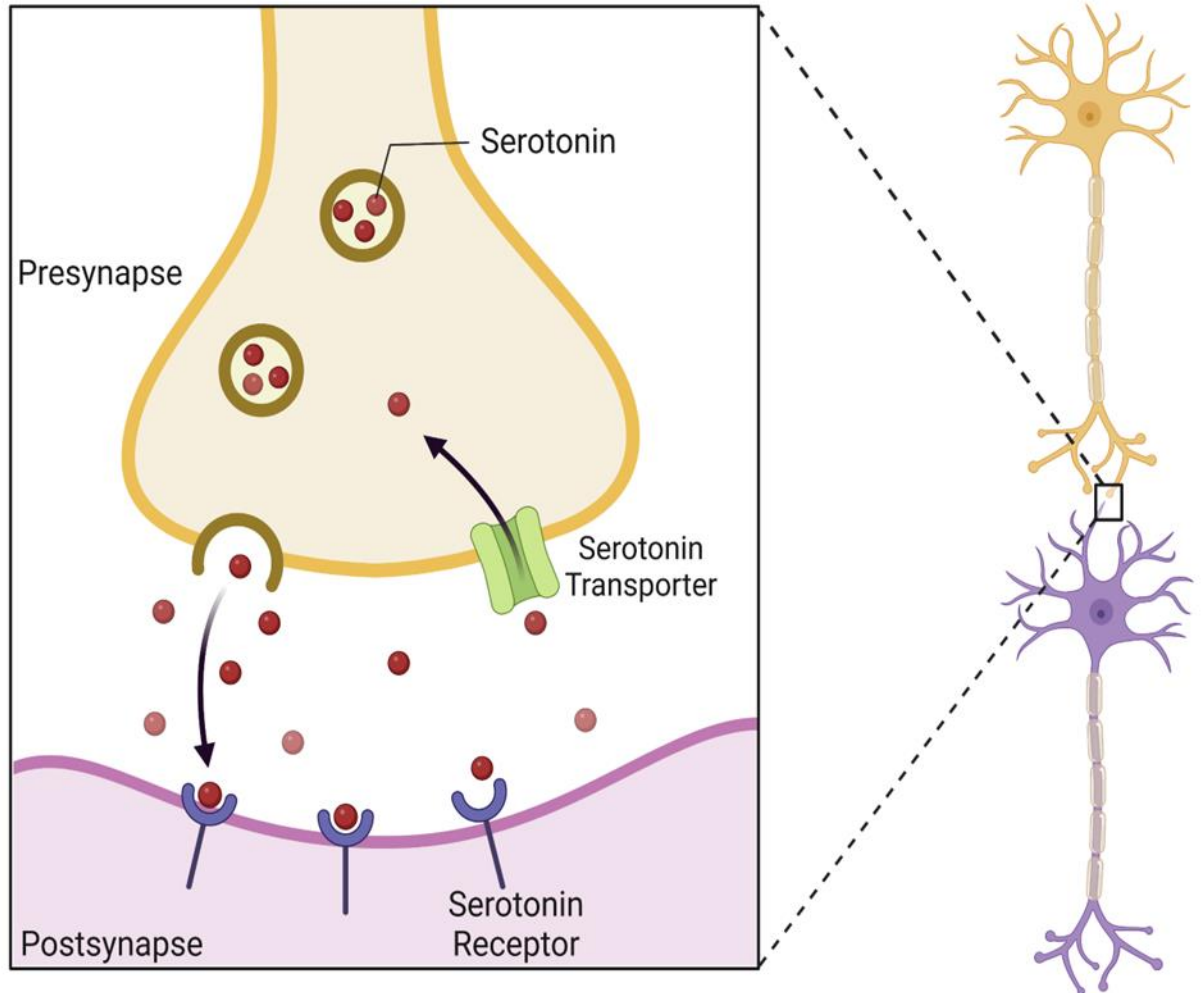


Figure 1. Predicted landscape and mechanistic function of mammalian serotonin 5-hydroxytryptamine, (5-HT). Vesicle-bound 5-HT (red circles) is trafficked to active zone of presynaptic compartment (yellow) and released into synaptic space unto awaiting postsynaptic 5-HT receptors (purple cups). Termination of serotonergic activity involves 5-HT binding to serotonin transporter (SERT) (green). Serotonin molecule undergoes retrograde trafficking (re-uptake) into presynaptic compartment for vesicular endocytosis and re-use. Illustration created using Biorender.

Figure 2: Schematic of long and short 5-HTTLPR

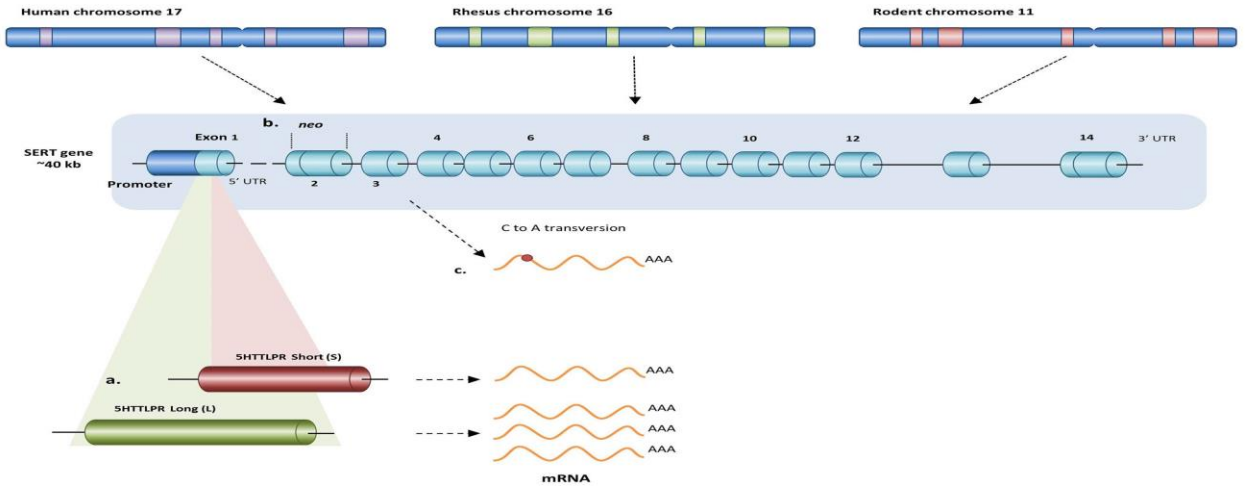


Figure 2. Illustration of *SLC6A4* and 5-HTTLPR genetic variants encoding normal (long) and (short) serotonin transporter (SERT) protein in humans, non-human primates, and rodents. Respective species chromosomal locations indicated above. 5-HTTLPR short allele (highlighted in red) is a result of ~ 44 base pair deletion in promoter region. Functional consequences of short allele genotype include reduction of SERT mRNA, subsequent decrease SERT expression at synapse. 5-HTTLPR genetic variant generation in C57BL/6J mouse is generated via insertion of *neomycin* cassette into exon 2 of mouse *Slc6a4*, thus producing similar transcriptional, translational and expression impairments and deficiencies of the short allele. Figure adapted from Houwing et al., 2017.

The role of the environment in autism spectrum disorders

Epidemiological surveys of the 1980's, estimated autism prevalence within the United States at 4 in 10,000 (Ritvo, 1989). Nearly 40 decades later, recent studies approximate nearly 1 individual in every 59 births (Maenner et al., 2020) are born with the disorder. This rise in prevalence can be attributed in part to increased public awareness, accessibility to sensitive evaluation tools, trained medical and clinical experts, and consensus of diagnostic criteria. Moreover, progress in research efforts over the years has provided invaluable genetic, behavioral, cognitive and molecular insight into potential factors. However, due to the heterogeneity of hallmark characteristics, a clear understanding of ASD etiology remains elusive. Nevertheless, findings from recent population studies are beginning to demonstrate environmental disturbances and stress, as potential risk factors (Lord et al., 2020). Another study providing evidence corroborating environmental contribution to autism risk is the use of valproate, a medication prescribed to primarily treat epilepsy (Sargar et al., 2015), and psychiatric disorders, including bipolar disorder (Beveridge and Cairns, 2012), and schizophrenia (Suzuki et al., 2009), during pregnancy. In addition, a study conducted by Kinney et. al., looking into whether prenatal exposure to stress, specifically, natural disasters, increasing subsequent ASD diagnoses in children. The authors of the study compared health records of pregnancies occurring during tropical storms and hurricanes against simultaneous, unexposed pregnancies over a 15-year period. Findings from the study observed an increased prevalence of autism diagnoses in children whose mothers were pregnant in areas exposed to

natural disasters (Kinney et al., 2008). Although Kinney's study did not examine other neuro- developmental or psychiatric disorders, similar studies including a 2008 Danish study (Khashan et al., 2008) also suggest maternal exposure to stressful life events during pregnancy can potentially detrimentally alter neurodevelopmental and psychiatric outcomes in future offspring.

Epigenetic regulation in neurodevelopment and stress

Evidence from animal and population-based studies have demonstrated the importance of the mechanisms underlying regulating genetic expression in eukaryotic (Holstege et al., 1998) and prokaryotic (Goldberger et al., 1976) organisms. Epigenetic mechanisms including methylation and acetylation of DNA and RNA molecules (Motorin and Helm, 2011), chromatin remodeling (Dong and Pandey, 2021), posttranscriptional events including messenger RNA (mRNA) alternative splicing (Millan, 2013) and activity of non-coding protein classes, i.e. microRNA (Moore et al., 2013), are diverse and sophisticated processes utilized by mammalian cells to enhance or suppress gene expressivity (Bale, 2015), and demonstrates the various endogenous layers of control on gene expression. Studies investigating the biological relevance of these mechanisms, argue that they play essential roles in a multitude of normal and diseased states, including, cell differentiation (Moore et al., 2013), immune responsivity (Allis and Jenuwein, 2016), stress tolerance (Griffiths and Hunter, 2014), diabetes (Shan et al., 2017), embryonic development (Gross et al., 2017) altered dendritic branching and spine density (Nithianantharajah and Hannan, 2006), and neurodevelopmental disorders (NDD) including Fragile X Syndrome, intellectual Disability (ID), and Phelan-McDermid Syndrome (Millan, 2013). Taken together, these studies indicate the underlying modulators of genetic expressivity playing an integral role in physiological and pathophysiological conditions.

Studies investigating neurodevelopmental disorders argue the implications of aberrant epigenetic mechanisms can play in social, psychological, emotional and

behavioral impairments at various stages in life. For example, intellectual disabilities (ID) (Liyanage, 2016), Fragile X Syndrome (Colak et al., 2014), autism spectrum disorders (ASD) (Waye and Cheng, 2018), major depressive disorders (MDD) (Engel et al., 2018), anxiety (Griffiths and Hunter, 2014), and schizophrenia (Hannon et al., 2016) are proposed to arise, in part, due to epigenetic dysregulation. In some instances, symptoms can be apparent from early childhood and persist or worsen throughout adulthood, thus implying the ever-present epigenetic- mediating contribution to the onset, development and severity of heterogenous and complex neurodevelopmental and psychiatric disorders. Moreover, identifying and delineating relevant epigenetic modifications for specific conditions can potentially highlight another layer of genetic control of neuropathological processes and functional defects, and most importantly, identify mechanisms for further scientific research and vulnerable as therapeutic targets. For example, DNA methylation is an epigenetic modification involving the addition of methyl groups to predominantly cytosine nucleotides of DNA (Feng and Lou, 2019) along loci enriched with phosphorylated cytosine/guanine aggregates (CpG islands). Published studies have reported CpG island dysregulation in clinical and animal models of pathologies associated with embryogenesis (Kuzmin et al., 2008), obesity (Perera and Herbstman, 2011), Rett Syndrome (Shah and Bird, 2017), and placental defects (Hemberger et al., 2020). Taken together, these studies suggests differential the involvement of epigenetic mechanisms play a fundamental role in disease onset in early development (Moore et al., 2013), (Masini et al., 2020). Similarly, our group observed epigenetic changes in

embryonic brain tissues. Using our SERT-Stress interaction paradigm, we compared embryonic brain methylation from prenatally stressed or non-stressed pregnant wild type dams to embryonic methylation from prenatally stressed or non-stressed pregnant heterozygous SERT dams. Our findings show extensive epigenetic changes in the embryonic brains of the stressed exposed SERT cohort, when compared to embryonic brains of other groups (Sjaarda et al., 2017). Therefore, this finding supports the potential presence of pathologically induced epigenetic dysregulation, which could potentially lay a mechanistic foundation for behavioral impairments associated with neurodevelopmental disorders, including ASD, as reported by other colleagues (Zucchi et al., 2013), (Bale, 2015), (Schaafsma et al., 2016) (Dong and Pandey, 2021), (Buthmann et al., 2022).

The placenta and its role in neurodevelopment and maternal exposures

The placenta is an organ found in eutherian organisms including, dogs, whales (Springer, 2004), sheep (Tesema et al., 2020), mice and humans (Sferruzzi-Perri et al., 2022). Its primary function is to provide protection and nutrients to the developing conceptus during gestation. Albeit a temporary stay, studies show the importance of a properly functioning placenta is extremely critical not only during all stages of development and pregnancy, but in addition, a significant factor impacting, cognitive (Bonnin et al., 2011), physiological, behavioral and health outcomes (Sferruzzi-Perri et al., 2022) which can impact the quality of life throughout the organism's lifespan.

As the sole source of nourishment and safety to the developing fetus, from conception to parturition, a well-functioning placenta will typically undergo constant morphological, biochemical, and molecular remodeling to accommodate the physical, energetic, and nutrient demands of the growing embryo (Sooranna et al., 1999), (Sferruzzi-Perri et al., 2022).

The underlying mechanisms governing placenta-associated factors such as placental adaptation, remodeling, maintenance and functionality are vulnerable to local and macro perturbations during gestation. Studies using both mouse (Higgins et al., 2016) and human models (Sferruzzi-Perri et al., 2022), propose mechanisms essential to placentation-the formation, type, structure or arrangement of the placenta- are susceptible to changes throughout gestation, which can potentially impact downstream gestation- associated processes and functions.

Another element critical to survival during pregnancy are the molecular communicative activity between the developing embryo and maternal environment (Arumugasaamy et al., 2020). Known as the 'maternal-fetal interface', findings from studies using human and mouse models demonstrate that multiple modes of communication exist. For example, diffusion spaces (Sferruzzi-Perri et al., 2022), facilitating the trafficking, metabolization of hormonal messengers such as estrogen (Bidarimath et al., 2014) and neurotransmission systems (Valenzuela and Zucca, 2011), microvesicles and exosomes (Ouyang et al., 2014) have also been observed traversing adjacent placental and maternal cell membranes (Rajagopalan and Long, 2018); which collectively, highlights the salience of maternal and fetal communication and their relevance to proper fetal development. Conversely, placental trafficking of pathological externally derived factors and their effects on programming and future health outcomes has been extensively reported in the literature. For instance, fellow colleagues have demonstrated a relationship between poor maternal diet, gestational infection and stress exposure with changes in placental DNA methylation (Babenko et al., 2015; Bale, 2015) and impaired neurodevelopment programming (Mueller and Bale, 2008).

Evidence of microRNA trafficking via the maternal-fetal interface has been well studied and observed to demonstrate physiological and pathological influences on complex pathways essential to the fetus during pregnancy. Reports of their presence and activity impacting pregnancy-related processes including placental development (Cai et al., 2017), modulation of maternal immune tolerance of the fetus (Liu et al., 2010), and preeclampsia (Cai et al., 2017), pre-term labor (Mayor-

Lynn et al., 2011) its role in angiogenesis (Suárez and Sessa, 2009), (Landskroner-Eiger et al., 2013), and finally, stress response (Zucchi et al., 2013) has been well documented in clinical and preclinical models. Taken together, these findings demonstrate an iota of the scope of the physiological networks under the influence of microRNA regulation. Findings from current research has identified microRNA localized within various tissues including, in part, the brain, heart, lungs (Kabekkodu et al., 2018), kidneys (Zhou et al., 2017) and present in body fluids such as, tears, saliva, breast milk, seminal fluid and blood (Cortez et al., 2011), which denotes microRNA's vast distribution, and utilization of body fluids and vascular conduits including blood and lymphatic vessels for systemic circulation, thereby facilitating microRNA trafficking and crosstalk across maternal and fetal compartments during pregnancy For example, a study by Vilella and colleagues using endometrial fluid of human origin shows the presence of maternally-derived microRNA *hsa-miR-30d* uptake within embryonic cells (Vilella et al., 2015). Overall, these studies support our previous postulation of placental transport playing an essential role in communicating stress susceptible elements from the maternal environment to the embryo (Sjaarda et al., 2017), and influencing mechanisms underlying fetal development and behavioral programming.

Animal models of autism

Approximately 30 years ago, research findings using family, twin and population-based studies began to reveal ASD may be linked to heritable and genetic origins (Liu and Takumi, 2014). However, despite having a strong genetic component, studies have shown, in some cases, aberrant genetics alone is not sufficient in the etiology of the disorder. This suggests the involvement of additional non-genetic elements (Nishimura and Kurosawa, 2022). To determine whether the environment plays a role in elevating risk of neurodevelopmental disorders such as ASD, studies such as Kinney et. al., provided just such evidence by demonstrating a higher reporting of ASD diagnoses in geographic regions susceptible to social economic hardship and vulnerable to the brunt of natural disasters (Kinney et al., 2008). Although these studies appear to support overlapping genetic and environmental elements as causal factors, investigating the underlying mechanisms in the clinical population is challenging.

With the recent advancements in the fields of genetics and molecular biology, modern research efforts led to the creation of animal models, specifically rodents, to investigate neurodevelopmental disorders, including ASD. Despite an evolutionary distance of approximate 65 million years, the genomes of *Mus musculus* and *Homo sapiens* share remarkable similarities, including, genetic organization (Yue et al., 2014). Findings from mouse model studies have provided valuable insight into the underpinnings of neurodevelopmental disorders, and increased our fundamental knowledge of disease development (Hahler and Elsabbagh, 2015). Furthermore, like humans, mice possess a complex nervous

system, specialized cellular components (Norton et al., 2020), and several classes of biogenic amines and neurotransmission systems, including, glutamate, dopamine (DA), GABA, and serotonin (-5-HT) (Valenzuela and Zucca, 2011), which would therefore, make them essential in translational medicine when investigating potential pharmacotherapeutic products (LeClerc and Easley, 2015) and intervention strategies (Estes et al., 2015), (Nicholas et al., 2018) of many disease states and disorders, including ASD.

Furthermore, the use of mouse model in ASD research has expanded our knowledge and understanding of the genes and mechanisms underlying ASD-associated genes and genomic regulation (Varghese et al., 2017), behavioral phenotypes (de la Torre-Ubieta et al., 2016), and therapeutic development (Bey and Jiang, 2014), thus providing examination and insight of ASD at multiple levels. The heterozygous SERT knockout model a relevant and valid genetic model to investigate the relationship between stress susceptibility, and underlying mechanisms of ASD as it replicates the short allele, 5-HTTLPR in humans, which our previous clinical and mouse studies demonstrate a relationship between SERT variant, 5-HTTLPR and increased stress responsivity in mothers of ASD individuals (Hecht et al., 2016), and methylation dysregulation (Sjaarda et al., 2017) and behavioral impairments associated with ASD (Jones et al., 2010; Matsui et al., 2018).

Moreover, current ASD diagnosis is mainly behavioral, with core and comorbid characteristics reported by clinical experts to include abnormal peer-to-peer interactions, communication repetitive movements (Kanner, 1943; Lord et al.,

1989), and elevated anxiety. Mice are a commonly used animal model in behavioral studies, as they inherently display a gamut of behaviors analogous to humans. They live in social hierarchies with adults and juveniles alike observed in socially engaging via sniffing (Weinstock, 2017), direct tactile contact, self-groom to maintain body fur condition and whiskers (Crawley and Paylor, 1997), or groom the fur of conspecifics (allogrooming) while socializing (Burrows et al., 2011), thus making mice an appropriate animal behavioral model. Therefore, tests exploring social interaction in familiar and novel contexts, (3 chamber), (Nadler et al., 2004), repetitive movement, (self-grooming, and defensive object burial), (Broekkamp et al., 1986; McFarlane et al., 2008) and anxiety (open field and elevated plus maze), (Hall and Ballachey, 1932; Lister, 1987) was used in this study to glean further insight into elucidate behavioral phenotypes.

Our previous works (Hecht et al., 2016) corroborate existing reports of the contribution of the 5-HTTLPR variant to heightened anxiety related traits and stress responsivity (Lesch et al., 1996) observed in individuals. Moreover, Hecht and colleagues demonstrated an association between the presence of the 5-HTTLPR genotype and children diagnosed with ASD, which has been supported in independent publications, (Cho et al., 2007; Devlin et al., 2005), which provide evidence of genetic factors elevating ASD risk. However, reports of adverse environmental exposure such as, pollution (Volk et al., 2014), medication (Bromley et al., 2008), and stress (Kinney et al., 2008), has been linked to ASD development. A potential gene-environment interaction (GxE), in ASD development has been reported in the literature (Nishimura and Kurosawa, 2022; Lipkin et al., 2023). Our

previous studies in both clinical and preclinical models have demonstrated supportive evidence of an association of GxE mediated ASD development. (Hecht et al., 2016; Matsui et al., 2018), the results of which provided an evidentiary framework of both genetic and environmental factors playing a role in ASD pathogenesis. Thus, the use of the GxE model was used in this study to glean greater insight into the underlying mechanistic mediators and behavioral outcome in offspring.

RNA interference

The discovery and characterization of microRNA and its role in gene silencing is credited to several research groups: In 1993 the group, Feinbaum, Lee and Ambros used the nematode *Caenorhabditis elegans* to demonstrate the importance of microRNA during development (Lee et al., 1993). Secondly, in 1998, two research groups, led by Fire and Mello and Waterhouse published findings from their respective studies, providing compelling experimental evidence of RNA-mediated genetic silencing, in *C.elegans* (Fire et al., 1998) and plant model *Nicotiana tabacum* (Waterhouse et al., 1998), respectively. Both reported the effect of the presence of a sequence-specific RNA molecule on animal development and plant viral resistance. The mechanism became known as RNA interference (RNAi) in animals, quelling in fungi and post-transcriptional gene silencing (PTGS) in plants (Waterhouse and Helliwell, 2003). Prior to this, the general consensus among the scientific community regarding plausible gene silencing regulation were attributed nucleic centered mechanisms such as promoter methylation and chromatin remodeling (Ashfaq et al., 2020). Therefore, the revelation of extra-nuclear gene modification systems challenged existent dogma and expanded the current conceptual framework of gene regulatory networks.

MicroRNA

MicroRNAs (miRNAs) are short non protein-coding RNA molecules primarily involved in regulation of gene expression in most eukaryotic organisms (Gebert and MacRae, 2019b). They belong to a larger class of RNAs identified predominantly by their small size, aptly named, short noncoding RNAs (short ncRNAs). Ranging from 18 to 200 nucleotides (nt) in length within plant (Yu et al., 2019) and animal (Bartel, 2009) cell systems, numerous reports demonstrate the effects of short ncRNAs in epigenetic, transcriptional and post-transcriptional regulation (Qureshi and Mehler, 2011) in both physiological and pathophysiological contexts ranging from seed germination (Das et al., 2015), genomic imprinting (Isakova et al., 2020), homeostatic mechanisms and stress response (Yu et al., 2019); suggesting these diminutive molecules modulate innumerable and essential biological functions across multiple cellular systems and within a variety of conditions. Unlike the lengths of the larger and more commonly known RNA molecules such as messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA), the predicted length of mature microRNA confirmations are approximately 15-25 nucleotides (nt) long (Hollins and Cairns, 2016). Mature miRNAs are generated from 2 independent sources (Figure 3) : the most commonly used, canonical pathway in which microRNAs are synthesized from their microRNA-encoded genes via RNA polymerase II (Bushati and Cohen, 2007), while an alternative, non-canonical pathway involves excision from intronic regions of larger messenger RNAs (mRNAs) via spliceosome machinery (Saliminejad et al., 2019). In either scenario, at this stage, nascent and unmodified

miRNAs, known as *primary miRNA* (pri-miRNA) can measure approximately 1000 nucleotides (nt) in length and form their characteristic bulge, known as a *stem-loop*. It is at this stage, canonical immature miRNAs undergo a cleaving process; Drosha and DiGeorge Critical Region 8 (DGCR8) respectively cleave and steady both 5' and 3' ends of primary miRNA, to form a new structure; precursor microRNA (pre-miRNA). This interim form, now approximately 70 nt long, hybridizes with the trans nuclear transporter protein, *exportin-5 (XPO5)*, and is shuttled out of the nucleus and into the cytoplasm for further processing (Saliminejad et al., 2019). While in the cytoplasm, it binds with the endonuclease, Dicer, and its characteristic stem loop is excised, leaving two hybridized, complementary mature microRNA strands known as a *duplex* (Hollins and Cairns, 2016). In this final confirmation, each strand is approximately 16-24 nucleotides long. Finally, the duplex is loaded within the cleaving protein, Argonaute (AGO) and undergoes a separation process. One strand, known as the *passenger strand*, is ejected into the cytoplasm, while the remaining AGO-bound *guide strand* is retained to form the miRNA-induced Silencing Complex (miRISC) (Yates et al., 2013). miRNAs predominant function is the provision of a sequence template, known as the *seed region* (nucleotides 2-8) which recognizes target messenger RNA by way of complementary binding along mRNAs 3' untranslated region (UTR) (Bartel, 2018). This binding effectively suppresses messenger RNA translation by one of two mechanisms; translation inhibition, or complete cleavage (Gebert and MacRae, 2019). Numerous reports have demonstrated the sensitive and persistent nature of miRNAs and their significant expression changes in response

to stressful factors and events, (Bartel, 2018), (Saliminejad et al., 2019). In some individuals, some of these changes can be long lasting. For example, a study conducted by (Wang et al., 2015) compared miRNA profiles of smokers to non-smokers, and observed novel and persistent smoking associated miRNA changes in individuals years after they stopped smoking. Similarly, our group also demonstrated findings of sustained stress-mediated microRNA changes collected from mothers of children years after receiving ASD diagnosis (Beverdors et al., 2021a). Taken together, these findings suggest miRNA levels can remain in a dysregulated state long after initial stress exposure.

Figure 3: MicroRNA biogenesis

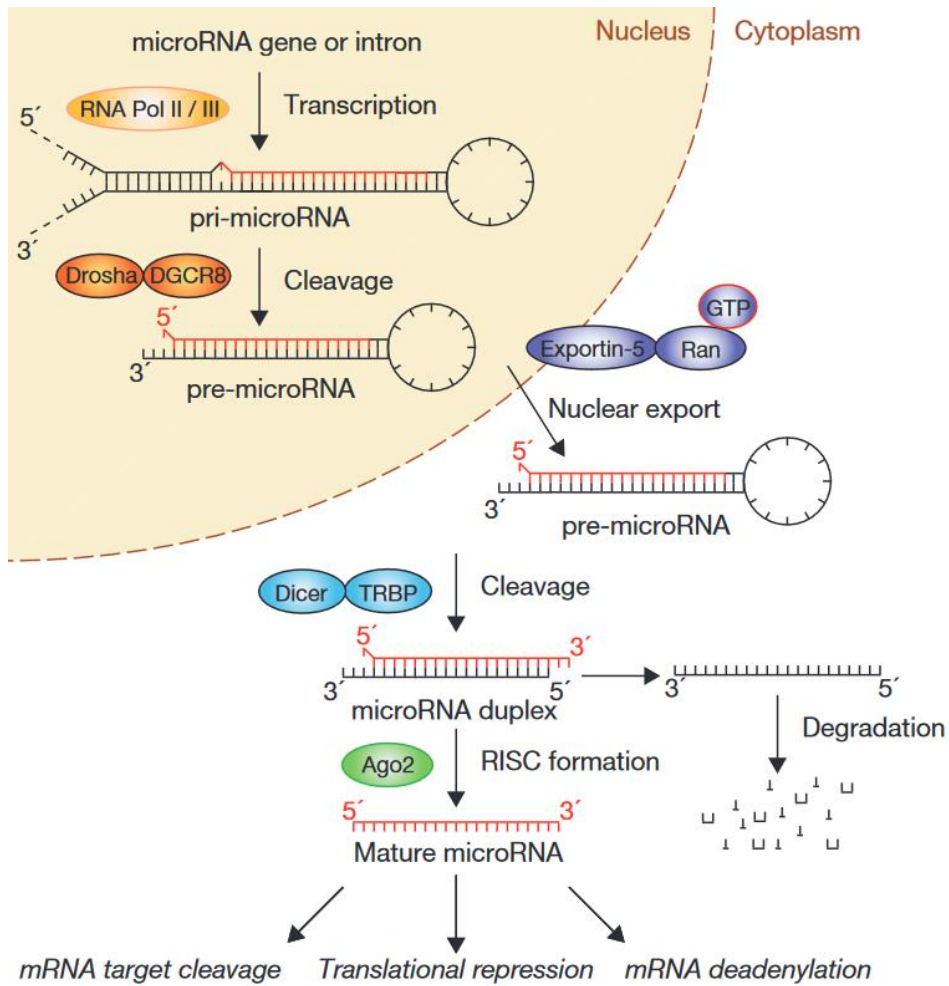


Figure 3. microRNA biosynthesis in mammalian cells: The microRNA gene is transcribed by RNA polymerase II or III to create primary microRNA (pri-microRNA). Next, cleavage by DROSHA and DGCR8 of 5' and 3' ends generate a ~70 nt long precursor microRNA (pre-microRNA) stem loop, which is shuttled across the nuclear membrane and into the cytoplasm by Exportin-5. The characteristic stem loop is cleaved by DICER, leaving the mature microRNA duplex (miR), which then binds to cleaving protein Argonathue (AGO). The duplex is separated into the guide strand (red) which will be retained by AGO while the passenger strand (black) is discarded. The miR of the AGO: miR complex serves as a template, binding unto the 3' untranslated region (UTR) of messenger RNA (mRNA), which initiates one of three silencing mechanisms: complete transcript cleavage, translational repression or deadenylation. Figure adapted from Winter et al., 2009.

Chapter 2

Materials and Methods

Colony housing and maintenance

All animals were housed according to policy of the Institutional Animal Care and Use Committee of the University of Missouri, within a standard Plexiglass cages lined with approximately one inch of Aspen pine bedding (Purina) temperature and humidity-controlled environment. Additionally, mice had ad libitum access to food (Lab Diet 5008, Purina) and water, and maintained under a 12hr/12-hr light/dark cycle (0730-1930). All animals in the colony were subjected to daily wellness checks to ensure normal development. Cages and bedding were replaced on a weekly basis.

Mating scheme

The first round of breeding generates female SERT (+/-) dams used for this study (Figure 5). Ten homozygous SERT knockout (KO) adult males (Jackson Laboratories, Bar Harbor, ME) (B6.129 (Cg)- *Slc6a4*^{tm1Kpl} /J #008355) on a C57BL/6J genetic background, were paired with 10 homozygous wild type (WT) adult females (Jackson Laboratories, Bay Harbor, ME) (#000664) on a C57BL/6J genetic background (Figure 4). Thereafter, 5 heterozygous SERT (SERT +/-) and 5 wild type SERT (SERT +/+) female mice were bred with 5 wild type SERT WT male mice to produce offspring used for this study. Female mice were inspected daily for vaginal plugs; indicative of successful mating and suspected pregnancy, upon observance of which, the experimental dams were removed from the

breeding cage and individually housed in a Plexiglas shoebox cage lined with Aspen bedding approximately 1-inch high with *ad libitum* access to water and food. Furthermore, several rodent nesting sheets (#K3510, Bio-Serv) and one cardboard rodent hut (#S3352-400, Bio-Serv) per cage was provided to all experimental dams. Finally, cages, water bottles, huts, nesting sheets and bedding were subjected to weekly cleaning and replacement as per laboratory protocol.

Figure 4: Heterozygous SERT knockout (+/-) mouse breeding scheme

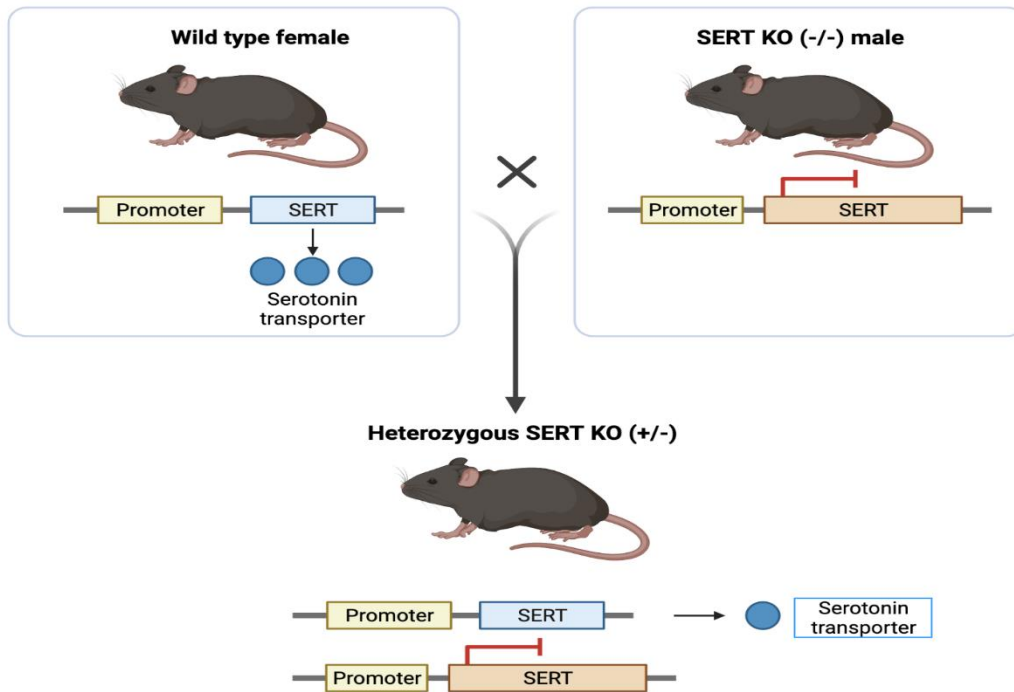


Figure 4. Breeding scheme for B6.129 (CG)-*Slc6a4*^{tm1Kpl}/J mouse strain to produce heterozygous SERT (+/-) dams. Initial round of breeding, wild-type female mice, with two long SERT copies (+/+) for the serotonin transporter gene are paired with SERT double knockout males (-/-), generating heterozygous progeny. Figure created in Biorender.

Chronic variable stress protocol (CVS)

8 wild type SERT and 8 heterozygous SERT dams were randomized into to chronic variable stress (CVS) groups adapted from (Matsui et al., 2018) as follows (Figure 5): beginning at embryonic day 6 (E6) until parturition, all pregnant experimental mice were segregated in a room separate from the rest of the mouse colony specifically designated for housing subjects undergoing CVS exposure. Each experimental dam was exposed to physical restraint for 10 minutes, fox scent exposure for 1-hour, multiple cage changes (which comprised of a newly sterilized cage, bedding, nesting sheets and hut) 3 times per day, constant overhead light exposure for 36 hours, overnight exposure to novel objects (20 uniformly colored marbles) and overnight exposure to novel noises (untuned radio), with one stressor presented per day in succession for 6 days, repeated approximately 2.5 times. Our control experimental, SERT WT and SERT (+/-) dams were housed individually as described previously and maintained among our mouse colony. However, they will not undergo CVS protocol described previously. Resulting offspring from all experimental dam cohorts remained with their respective mothers and weaned at postnatal day 21 (PD21). After which, all offspring were housed in sex-, gene- and age-matched groups at 4 animals per cage with ad libitum access to food (Lab Diet 5008, Purina) and water for the remainder of the study. Cage maintenance was performed as previously described.

Figure 5: Experimental workflow

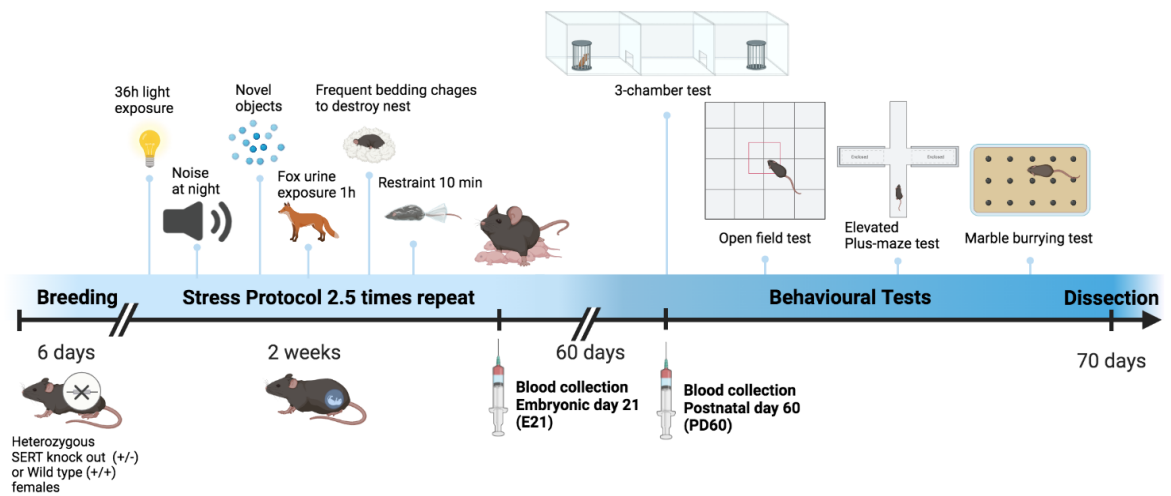


Figure 5: Schematic of experimental workflow of breeding, prenatal chronic variable stress (CVS) types and administration, sample collection and offspring behavioral assessments examining social approach and novelty seeking, anxiety, and repetitive behaviors. Illustration created on Biorender.com.

Chapter 3

Behavioral assays

As with our previous studies (Jones et al., 2010; Matsui et al., 2018), the following behavioral experiments were chosen due being highly well established in numerous rodent studies due to high construct validity in interrogating behaviors associated with autism spectrum disorders. The behavioral assessment schedule was conducted sequentially as follows in order to minimize the stress imposed upon the animal when undergoing behavioral battery of tests: Open field (OF) (Hall and Ballachey, 1932), spontaneous grooming (McFarlane et al., 2008), 3-chamber social approach/novelty seeking assay (Nadler et al., 2004), marble burying (Broekkamp et al., 1986) and elevated-plus maze (EPM) (Lister, 1987). Experiments were conducted during the hours of 0900 – 1500. Experimental animals were transported in transparent home cage systems (Thoren Caging, Hazelton, Pennsylvania, USA) to test rooms and allowed to acclimate to testing conditions for a minimum of 20 minutes prior to the beginning of test and had free access to food and water up to the beginning of test. Apparatus is thoroughly wiped with 70% ethanol solution prior to initial testing and between subjects. Monitoring of animals during test day was performed by at minimum one observer who remained out of visual range of animals during test. All assays were conducted in dedicated testing rooms at the Animal Science Resource Center at the University of Missouri- Columbia.

Open field

Adapted from Hall and Ballachey, the open field (OF) assay is used to observe the test animal's activity and evaluate signs of anxiety-like behaviors. During the 10-minute task, a single mouse is placed in the center of the arena. Although mice travel along the outermost sides and corners of the box, they are curious animals, and will thus naturally explore the more exposed central area of the box. Thus, a mouse with a greater exploration time along the arena's perimeter indicates greater anxiety levels, while greater time spent in the open exposed center demonstrates low anxiety (Hall and Ballachey, 1932). Testing subjects were placed in a 45 cm x 45 cm x 22 cm open top box made of black Plexiglas, and allowed to ambulate freely in the chamber, under dim lighting. ANY-maze© automated tracking software was used to overlay pre-configured outer and central zones (45cm x 45cm and 20.5 cm x 20.5 cm, respectively) within the apparatus to determine the spatiotemporal position of the animal subject during the task to be used for subsequent analysis. After 10 minutes, the animal is removed and returned to the cage. The apparatus is sanitized with 70% isopropyl alcohol between trials. The observer remained 2 meters away from the apparatus at the time of assay, out of the subject's visual field, and blinded to the test subject's genotype. Anxious animals will typically spend a greater duration in the corners and outer zone and avoid the central zone. Activity is scored by total distance traveled, time spent in the central zone, and the number of entries into the central zone.

Spontaneous grooming

Animals are placed in the central arena of an open field apparatus, and scored for time spent engaging in repetitive behaviors such as, self-grooming entire body, fur scratching, tail licking, washing snout, face, front paws, and hind feet during 10 minute assay (McFarlane et al., 2008). As individuals diagnosed with ASD tend to engage in heightened repetitive movements and behaviors, the animals observed spending a longer grooming time or increased number of grooming events are hypothesized to engaging in activities consistent in individuals with ASD. ANY-maze© automatic tracking software was used to record grooming activity for later analysis. The assay is scored for total time spent grooming and number of grooming events across all groups (SERT (+/-) stressed, SERT (+/-) non-stressed, WT stressed and WT non-stressed). Apparatus is cleaned with 70% isopropyl alcohol between test animals.

Social approach and novelty seeking

This assessment employs the 3-chambered apparatus to measure social approach preference and social novelty-seeking behavior (Nadler et al., 2004). The 62.87 cm x 42.55 cm apparatus is made of Plexiglas and evenly divided into 3 chambers. During the **social approach** phase, the subject initially underwent a 10-minute habituation phase, during which the subject can explore all three empty chambers. The mouse was sequestered within the central chamber while a stranger mouse is placed into an adjacent empty chamber (During the sociability assays, a weighted inverted metal cup cage separates all stranger mice from test subjects. This permits visual, auditory and olfactory communication while

minimizing the likelihood of physical overt displays of aggression). while the remaining unoccupied chamber contains a novel object (inverted cup cage). The test subject freely explored all three chambers for 10 minutes. During the **social approach** index is evaluated as the length of time spent with the stranger mouse or empty cup cage and scored as follows: $(\text{time with stranger mouse}) / (\text{time with stranger mouse} + \text{time with novel object})$. Animals displaying social preference will spend more time interacting with the stranger mouse than the empty cup (Rein et al., 2020). Finally, for the **novelty seeking** test phase, a second unfamiliar mouse is placed in the cup cage, and the test subject freely explores all 3 chambers for a final 10 minutes. ANY-maze© automated tracking software overlays pre-configured zones in all chambers and around each inverted cup cage to determine the spatiotemporal position of each test animal. The social novelty index is evaluated as the length of time spent with an unfamiliar mouse. Animals with socialization deficits typically spend more time interacting with the first stranger than with a new unfamiliar mouse. The apparatus is cleaned with 70% isopropyl alcohol at the conclusion of the novelty seeking phase. Social novelty index scored as follows: $(\text{time with a novel mouse}) / (\text{time with familiar mouse} + \text{time with the unfamiliar mouse})$.

Marble burying

Repetitive behavior (Broekkamp et al., 1986) in adult offspring using a marble-burying assay. A mouse is placed within a plexiglass chamber lined with corncob bedding and allowed to freely explore for 20 minutes. After this acclimation period, the mouse is removed to place 20 glass marbles are placed in a 5x4 grid

configuration within the chamber. The mouse is returned to the marble-filled chamber and permitted to freely explore for 30 minutes. ANY-maze© automated tracking software will be used to record experiments for subsequent analysis. The number of buried marbles (> 50% covered) is scored and recorded by an observer. Animals observed burying a greater quantity of marbles are hypothesized to be engaging in repetitive behaviors consistent in individuals diagnosed with ASD. Corncob bedding is discarded and replaced between trials. The apparatus is cleaned with 70% isopropyl alcohol between experiments.

Elevated plus maze

The apparatus is t-shaped, constructed of black plexiglass, elevated on a stand 100 cm above the floor. The maze (Lister, 1987) consists of two open arms (30 cm x 5 cm) and two enclosed arms (30 cm x 5 cm x 20 cm). This assay measures a mouse's innate and inquisitive nature to explore novel open areas. Therefore, animals observed spending a greater duration within the enclosed arms compared to duration spent exploring the open arms of the apparatus are hypothesized to be engaging in anxiety-like behavior (Lezak et al., 2017). ANY-maze© automated tracking software (Stoelting) overlaid pre-configured zones over open and closed arms, and a central area to determine the position of the mouse during the experiment. The mouse is placed in the area central to both open and closed arms and given 10 minutes to freely explore the apparatus. An observer is physically present during testing but not visible to test animals. To prevent any biases, animal genotypes were blinded to the observer. The apparatus is cleaned with 70% isopropyl alcohol between each test subject. Animals with elevated anxiety levels

typically avoid exploration of open arms and spend a greater duration within the enclosed arms. Anxiety index is scored as an open arm ratio (total time spent on open arm/ (time spent on open arm + time spent in the closed arm)).

Statistical analysis for behavioral assessments

GraphPad Prism statistical software (v 9.3.1). Unless otherwise indicated, a 2-factor Analysis of variance (ANOVA) of genotype x stress will be used to determine significance across groups. The appropriate post-hoc test was performed to determine significant main and interaction effects. Finally, the significance level is set at $P < 0.05$ for all experiments.

Animal behavior tracking and analysis

Behavioral assessments were recorded using ANY-maze© behavioral tracking software (Stoelting, Wood Dale, IL, USA). To avoid biases, assessors were blinded to animal genotype during behavioral tasks and statistical analyses portions of the study.

Sample collection, processing and analysis

Maternal blood samples were collected on embryonic day 21 (E21). After dams were anesthetized via gaseous Isoflurane inhalation, a lateral incision exposed chest cavity. Blood was collected via a heparin coated syringe, via cardiac puncture and immediately stored in tubes using RiboPure™-Blood Kit (Ambion, Foster City, CA, USA) according to manufacturer's instructions, temporarily stored

in dry ice containment until all samples were collected. Post collection, all samples were stored in -80°C until analysis.

Sample processing and isolation

Blood samples were grouped according to maternal genotype of WT and SERT (+/-) and presence or absence of CVS exposure (ST) and NS, respectively as follows: (5) WT/ NS, (5) WT/ST, (5) SERT (+-)/ NS, and (5) SERT (+-)/ST, where the number of replicates is indicated in the parentheses, and sent for processing, isolation and analysis at the microarray facility at the University of Kansas Medical Center, Kansas City, Kansas, USA.

Statistical analysis for microRNA analysis

Analysis to examine differential expression across groups was performed using Affymetrix® Transcriptome Analysis Console (TAC) software (Version 3.1.0.5). Statistical test of 1-way Analysis of variance (ANOVA) with fold change thresholds set to +/- 1.2 and significance at $p \leq 0.05$ and default FDR settings. Venn diagram was used to visualize overlapping microRNAs across all groups to identify uniquely upregulated microRNAs in group of interest, SERT (+-)/ ST.

Red blood cell lysis

Samples were collected into evacuation tubes containing K-EDTA (Becton Dickinson Vacutainer Systems) and kept refrigerated until processing. After a brief centrifuge at 1200x g for 20 minutes at 4°C, the top layer (plasma) was removed and stored in 2ml tubes on ice. At last stored at -20°C. Buffy coat was transferred

to a 15ml centrifuge tube containing 12ml of red blood cell lysis buffer (150 mM NH_4Cl , 10 mM NaHCO_3 , 1 mM EDTA, pH 7) via a disposable transfer pipette, vortexed and incubated at room temperature for 5 minutes. Samples were then centrifuged at 300x for 10 minutes at 4°C to wash and pellet the white blood cells (WBC). Finally, supernatant was discarded WBC pellet was washed twice (as described above) first using 5ml of red lysis buffer, and second using 5ml of ice-cold 1X Dulbecco Phosphate Buffered Saline (DBPS). After which supernatant was immediately discarded and WBC pellet was stored at -80°C.

Trizol and RNA isolation

1ml of Trizol was added to each sample, then transferred whole solution to a 1.7ml Eppendorf tube.

0.2 ML of chloroform and 1ml of Trizol were added to each sample and shaken by hand vigorously for 15 seconds then left to stand at room temperature for 2-3 minutes. Samples were then centrifuged at 12000x g for 15 minutes at 4°C (Eppendorf Centrifuge 5417R). The upper aqueous phase, containing RNA, was removed with a pipette and placed in new tubes for RNA precipitation.

RNA precipitation

Admixture of 1ml Trizol and 100% isopropanol was mixed with each sample, mixed thoroughly, inverted upside down, incubated at room temperature for 10 minutes, and centrifuged at 1200 x g for 10 minutes at 4°C. A gel-like pellet (RNA) is formed post centrifuge. Finally, samples proceed to RNA washing procedure.

RNA wash

After discarding supernatant of all samples, tubes containing RNA pellets were resuspended with 1ml 75% ethanol and 1ml Trizol, then briefly vortexed at 6500x g for 5 minutes at 4°C. Supernatant is discarded, and wash is repeated with 75% ethanol. Tubes are left to air dry for 5-10 minutes (or until liquid disappears), then proceed to RNA resuspension.

RNA pellets are resuspended in 60µl of sterile MiliQ water, then left to incubate in a heat block set at 55-60°C for 55-60 minutes. Samples are placed on dry ice, a 3µl aliquot is removed from each sample for concentration measurement via spectrophotometer (Nanodrop). Remaining solutions were stored at -80°C. RNA quality was determined via electrophoresis and/or fragment analyzer.

1µl of each sample was used to measure RNA quality, which was performed 2-3 times using Nanodrop (ND-1000 V.3.8.1). Sensor was washed with 2µl sterile MiliQ, and blank with 1µl sterile MiliQ. Sensor was washed with 2µl of sterile MiliQ water after all samples were measured.

Microarray

Total RNA was isolated from 1ml of the RNAlater blood samples using the Mouse RiboPure-Blood RNA Isolation kit from Ambion. Quality control of the total RNA isolates was performed using the TapeStation RNA ScreenTape assay (Agilent 5067-5576). Biotin labeling of Total RNA (1ug) was performed using the FlashTag Biotin HSR RNA Labeling Kit (Life Technologies 901910). The FlashTag labeling system employs the 3DNA dendrimer signal amplification technology. 3DNA

dendrimer is a branched structure of single and double stranded DNA conjugates which incorporates numerous biotin labels for ultrasensitive expression detection. The Genomics Core used the GeneChip system for processing the miRNA expression arrays. Using the GeneChip 645 Hybridization Oven, labeled target RNA is hybridized overnight (16 hours, 48C, 60rpm) to interrogating oligo probes contained in the GeneChip miRNA 4.0 expression cartridge array (Life Technologies #902412). Hybridized Genechip arrays undergo low and high stringency washing and R-Phycoerythrin-Streptavidin staining procedures using the GeneChip Fluidics Station 450 running the FS450_0002 Fluidics profile. After washing, GeneChip arrays are processed using a single scan on the Genechip Scanner 3000 7G with autoloader. Fluidics and scan functions are controlled by Affymetrix GeneChip Command Console software (AGCC). Raw expression data is loaded onto the Microarray Data Management System (MDMS) for access by the Investigator and further data analysis.

Chapter 4

Aim 1: Characterization of miRNA expression profiles in prenatally stressed SERT (+/-) dams

Our previous study demonstrated significantly differentially expressed methylation in embryonic brains of progeny of prenatally stressed heterozygous *Sert* females, relative to a non-stressed exposed wild type group (Sjaarda et al., 2017). Moreover, findings from our retrospective study, in which blood samples of normal and 5-HTTLPR mothers of autistic children (collected and analyzed approximately 6 years after birth), uncovered significantly differentiated microRNA in the heterozygous *SLC6A4* maternal cohort. Furthermore, these mothers also reported higher stress events during pregnancy. Therefore, I hypothesized an association between the maternal effects of *SLC6A4* heterozygosity, heightened stress susceptibility and altered miRNA expression profile in *Slc6a4* (+/-) dams when compared to other experimental groups.

MicroRNA profiling

Characterization of differential expression (DE) of microRNA profiles across all groups were analyzed via microarray analysis. Of the total 3,077 non-coding RNA products detected, approximately 1,908 (~63%) and 1,109 (~36%) displayed linear (mature) and stem-loop (immature) motifs. Due to the latter species functionality in the microRNA RISC (miRISC) complex, stem-loop microRNAs were not considered for further analysis.

Next, a hierarchical clustering heat map was constructed to visualize the microRNA profiles across all groups at embryonic day 21 (E21), (Figure 6). To determine whether heterozygous SERT dams exhibited stress-associated differentially expressed microRNA expression, comparison of the average \log_2 fold change values of the (SERT (++)/ non-CVS) cohort was used as the control, to be measured against the remaining experimental groups. Microarray analysis revealed upregulation of 3 distinct microRNAs in the maternally stressed heterozygous SERT cohort at embryonic day 21: *mmu-miR-7648-3p* ($p=0.030$), *mmu-miR-5622-3p* ($p=0.005$), and *mmu-miR-6900-3p* ($p=0.038$). Database search of mirBase, (Griffiths-Jones, 2006), Mouse Genome Informatics (MGI) (Blake et al., 2021), and National Center for Biotechnology Information (NCBI) (Brown et al., 2015) databases reveal the genomic loci of each microRNA on chromosomes 15, 2, and 1, respectively.

Next, to identify whether factors of the gene-environment interaction model influence maternal microRNA dysregulation, 2-way ANOVA analysis was performed. Results revealed an association between microRNA 5622-3p, 6900-3p and 7684-3p upregulation and factors of the gene- environment paradigm. Specifically, 5622-3p demonstrated interaction related upregulation, ($F(1,14) = 5.71$; $p= 0.0320$, no significant difference was detected in 6900-3p, while 7684-3p exhibited main effects of genotype and stress, ($F (1,15) = 7.99$; $p = 0.013$ and $F (1,15) = 5.859$; $p = 0.029$), respectively. Taken together, these findings reveal these microRNAs are susceptible to the interaction or independent factors of the gene-environmental model. Furthermore, to identify putative stress- and ASD-

associated targets for each microRNA, a network map was generated (Figure 7). The genes were verified using microRNA Target Prediction Database (miRBD), (Chen and Wang, 2020), TargetScan (Agarwal et al., 2015), (McGeary et al., 2019), and GeneCards.org (Stelzer et al., 2016). ASD-associated genes were cross referenced with Simons Foundation Autism Research Initiative (SFARI) Gene databases (Abrahams et al., 2013). Results included, regulator of nonsense mediated mRNA decay, (UPF3B), ankyrin repeat domain- containing protein 17 (ANKRD17), (Stessman et al., 2017), scaffolding protein, the calcium/calmodulin dependent serine protein kinase, (CASK) (Lord et al., 2020), and gamma-aminobutyric acid type B receptor subunit 2 (GABAR2), (Fatemi et al., 2009), lysine (K)-specific methyltransferase (KMT2C); a gene implicated in the developmental disorder, Kleefstra Syndrome (Sanders et al., 2015), and transcription factor, activity-dependent neuroprotector (ADNP) (Waye and Cheng, 2018), pre-messenger splicing genes, SRSF11 (Stamova et al., 2013), and SON (Dingemans et al., 2022), kinase mTOR (Tang et al., 2014), helicase CHD7 (Stessman et al., 2017).

MicroRNA biosynthesis is essential in genetic regulation of homeostatic processes (Hollins and Cairns, 2016). However, studies have provided evidence of microRNA dysregulation in a variety of pathological conditions, including, schizophrenia (Beveridge and Cairns, 2012), cancer (Esquela-Kerscher and Slack, 2006), autism spectrum disorders (Nakata et al., 2019), and stress (Rodgers et al., 2015), (Pegler et al., 2019). Furthermore, studies have also shown genes concomitant to microRNA biosynthesis including cleaving proteins DOSHA, DICER, Argonaute-4

(AGO4), and translocating protein Exportin-5 (XPO-5) are not exempt from dysregulation under adverse conditions (Beveridge and Cairns, 2012), (Nazer et al., 2022), (Wu et al., 2018). The effects of which consequently modulate the bioavailability, and abundance of functional microRNA (Bartel, 2009), (Gebert and MacRae, 2019) which can in turn, impact post-transcriptional activity of target genes (Gebert and MacRae, 2019). Therefore, analysis was performed to determine whether genes related to microRNA biosynthesis were targeted as well. Subsequent analysis of the E21 maternal blood samples from the SERT (+/-)/ CVS cohort cross-referenced against TargetScan database (Agarwal et al., 2015) revealed potential targets to include microRNA-associated genes AGO and XPO families. This suggests a reduction in mechanisms critical in microRNA biosynthesis and functionality: the exportation of precursor microRNA from the nucleus into the cytosol and decrease AGO: miR binding, the consequences not only affect *mmu-miR-7648-3p*, *mmu-miR-5622-3p* and *mmu-miR-6900-3p*, but potentially production and function of all microRNAs.

To elucidate the underlying biological relevance of the predicted gene targets of *mmu-miR-7648-3p*, *mmu-miR-5622-3p* and *mmu-miR-6900-3p* at embryonic day 21, the ShinyGO 0.77 toolkit was used to identify the most enriched biological pathways. Analysis revealed categories related to CNS, head and brain development (Figure 9). Additional functional classes included a substantial proportion of genes implicated in pathways, including positive regulation of biosynthetic processes, cellular protein localization, and neurogenesis. This finding demonstrates targets of *mmu-miR-7648-3p*, *mmu-miR-5622-3p* and *mmu-*

miR-6900-3p play vital roles in both small- and large-scale networks essential to development.

Figure 6: Hierarchical clustering of maternally derived microRNAs at embryonic day 21 (E21)

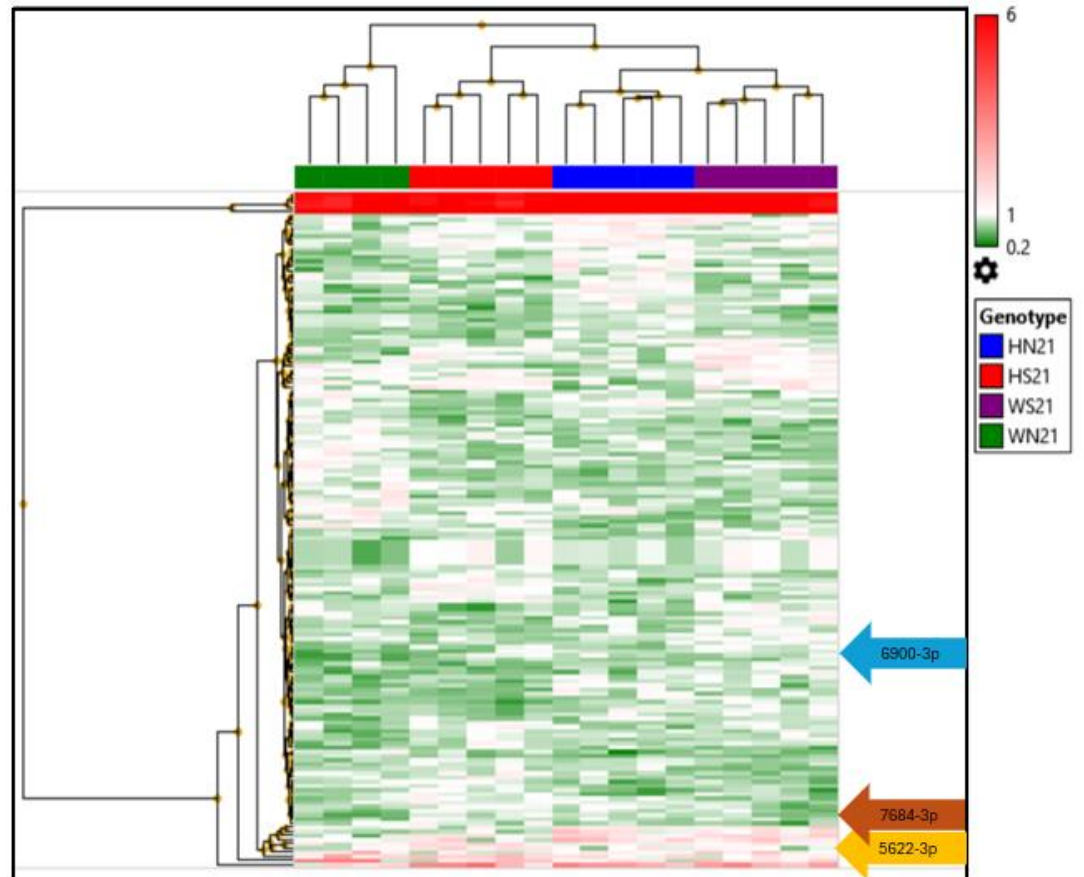


Figure 6. Heat map and hierarchical clustering and of endogenous differential expression of mature microRNA in maternal blood samples of prenatally stressed and non-stress exposed dams at embryonic day 21 (E21) with upregulated microRNAs 5622-3p, 6900-3p and 7684-3p detection in heterozygous SERT stress exposed dams (HS21). Top row: green: (5) wild type non-stressed exposed, (WN21), red: (5) heterozygous SERT stress exposed (HS21), blue: (5) SERT non-stress exposed (HN21), purple: (5) wild type stress exposed (WS21). Upregulated microRNAs are indicated red. Downregulated microRNAs are indicated green. MicroRNAs with no expression change indicated white. Adjusted $p < (0.05)$.

Figure 7: Gene x environment mediated E21 microRNAs

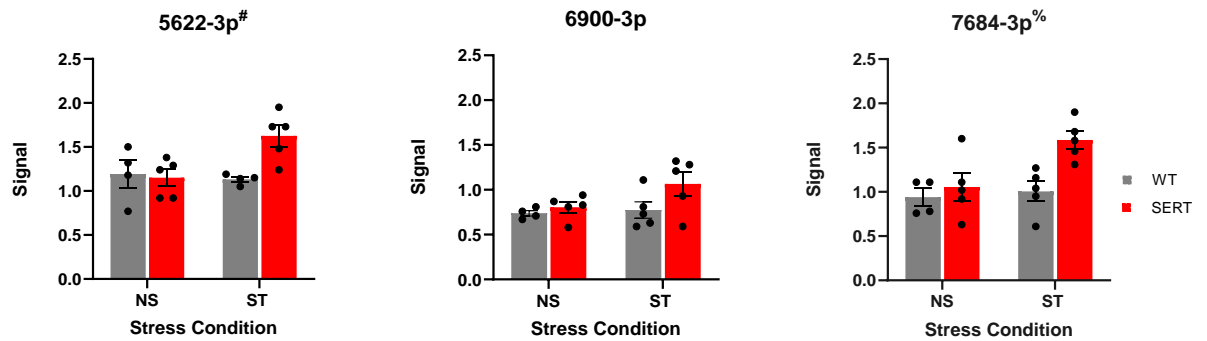


Figure 7: 2-way ANOVA analysis of gene x environment mediated signal intensities of microRNAs 5622-3p, 6900-3p and 7684-3p collected from wild type (gray) and heterozygous SERT (red) dams at embryonic day 21 (E21) in the presence (ST) or absence (NS) of CVS exposure beginning at E6. Dots indicate the number of samples per group. # indicate gene-environment interaction mediated dysregulation, % indicate main effects of gene and stress. Values are means \pm SEM. P values of main and interaction effects: <0.05 .

Figure 8: Predicted targets of top 3 upregulated microRNAs of prenatally stressed SERT dams at embryonic day 21

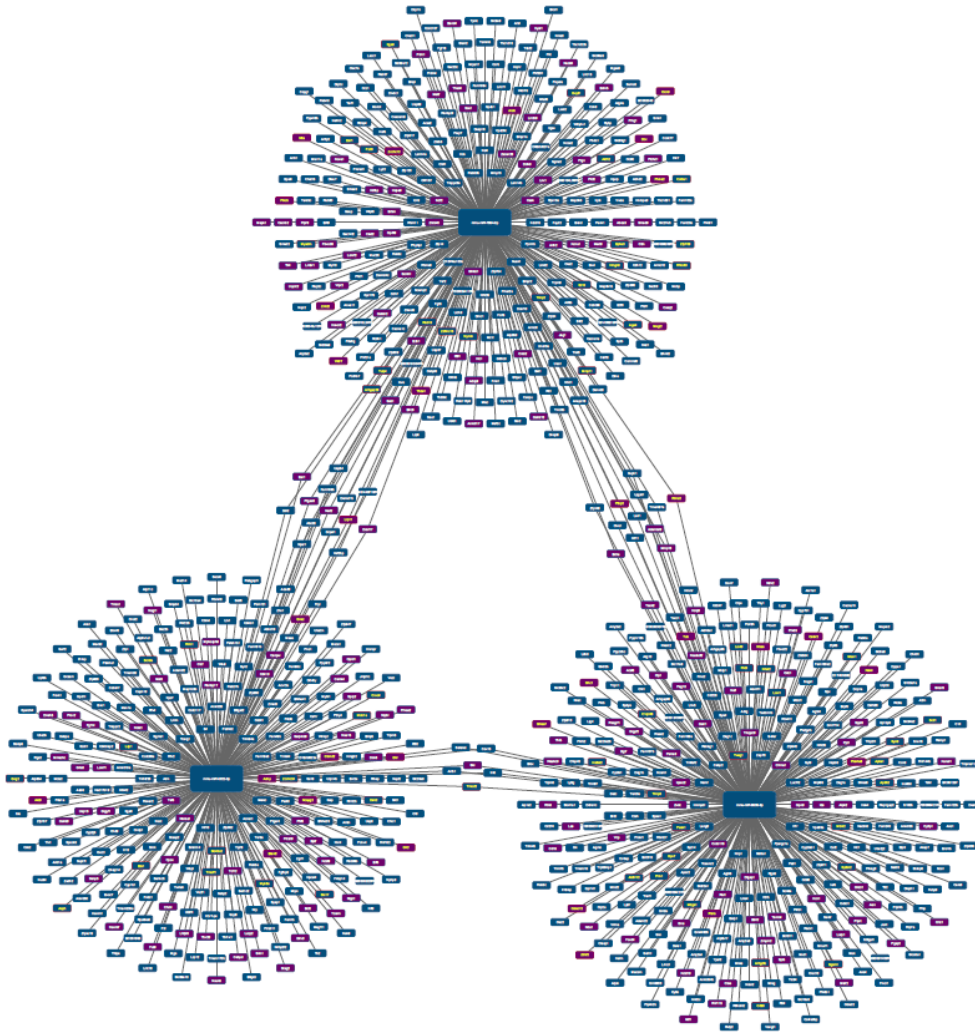


Figure 8. Network map illustrating top 3 upregulated mature microRNAs (center rectangles) ($p < 0.05$) of prenatally stressed SERT dams at embryonic day 21 and their predicted genetic targets. ASD associated genes indicated by (purple rectangles). Stress associated genes indicated by (blue rectangles). Genes regulated by more than one microRNA are also shown. Gene predictor database: miRD.

Figure 9: Dot plot of enriched terms of upregulated maternally derived microRNA targets at embryonic day 21 (E21)

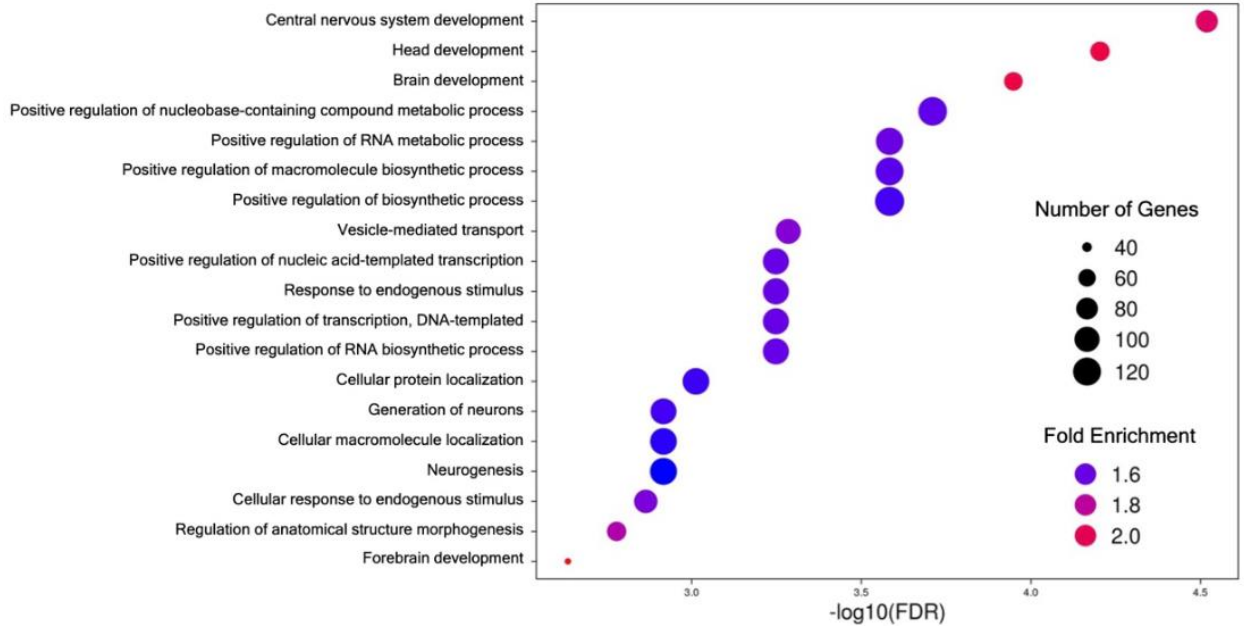


Figure 9: Enriched functional terms of predicted genetic targets of maternally derived microRNAs mmu-miR-7684-3p, mmu-miR-6900-3p and mmu-miR-5622-3p at embryonic day 21 (E21). Dot size indicates the number of genes, color of dots indicates fold enrichment.

Discussion

The autistic phenotype is inherently complex. With published findings pointing to the combinatorial effects of genomic dysregulation (Liu and Takumi, 2014), exposure to adverse environmental stressors (Kinney et al., 2008) and psychiatric illness (Jokiranta-Olkonemi et al., 2016) increasing ASD risk and pathophysiology. Moreover, the substantial clinical heterogeneity adds an extra layer of complexity of ASD diagnosis and risk prediction.

At the time of this study, there are no biomarkers in clinical practice to predict the risk of neurodevelopmental impairments and disorders, such as, intellectual disabilities (ID), Attention Deficit and Hyperactivity Disorder (ADHD), developmental speech and autism spectrum disorders (ASD) prior to birth. However, with an incidence of approximately 1 in every 59 births (Maenner et al., 2020), there is a clear and dire need to provide clinicians and medical professionals with tools which predict individual risk, facilitate development of efficacious interventions and relevant treatment options to improve the affected individual's quality of life. Therefore, the exploration, discovery and understanding of the biological mechanisms underlying autism etiology would potentially provide substantially critical insight of the genetic, molecular, and neurobiological contributors to the architecture of neurodevelopmental disorders and ASD-associated pathologies.

The biological relevance and functionality of microRNA has been extensively studied and documented since its discovery over 30 years ago (Lee et al., 1993; Fire et al., 1998). And yet, more recent findings from colleagues continue to reveal

novel characteristics, mechanisms, and targets, which suggests much of these non-coding molecules remains unknown and unexplored.

Recent evidence provided by (Wu et al., 2016) examined post-mortem brain samples of ASD and neurotypical subjects to elucidate whether ASD-associated genes are sensitive to microRNA targeting. Their analysis reveals a greater degree of microRNA: mRNA binding (miRNA: mRNA) with ASD-associated transcripts in samples of individuals diagnosed with ASD, relative to non-ASD samples. Furthermore, this group also reported greater downregulation in ASD-related genes in ASD samples when compared to the control cohort. Hence, their findings demonstrate the interplay between dysregulated microRNA profiles and ASD-specific genes can potentially exert deleterious consequences.

In this study, the molecular profiling of maternally derived microRNA revealed an admixture of stem-loop and linear motifs, which demonstrates feasibility and sensitivity at which distinct microRNA species can be detected and distinguished in biological fluids. This is an essential characterization as studies have demonstrated the stem-loop immature precursor as the non-functional species, and unlikely to participate in microRNA: mRNA binding (Bartel, 2004, 2018). Indeed, all isoforms of microRNAs 5622, 6900, and 7684 were detected across all samples at embryonic day 21. However, only the maternally stressed heterozygous SERT cohort expressed upregulated, *mmu-miR-5622-3p*, *mmu-miR-6900-3p*, and *mmu-miR-7684-3p*, which appears to suggest the combination of heightened stress susceptibility associated with SERT heterozygosity (Lesch et al., 1996; Jansen et al., 2010) may play a critical role in microRNA dysregulation.

Moreover, detection of these microRNAs may just reflect their abundance, and not necessarily capture biological activity. Typically, 'active' microRNA hybridization with Argonaute (AGO) proteins, which was not examined in this study, is required for transcript cleavage or degradation (Schirle and MacRae, 2012; Phan et al., 2018). Therefore, further experiments are required to determine whether the overexpressed microRNAs identified in this study are AGO bound, thus corroborating their abundance with biological activity.

The predictive genetic targets of *mmu-miR-5622-3p*, *mmu-miR-6900-3p*, and *mmu-miR-7684-3p* demonstrated the genomic breath of their influence. For example, projections populated genes implicated in both stress- and ASD-associated pathways distributed across all chromosomes (data not shown). However, closer examination revealed predominant X Chromosome occupation of 10 out of 41 'syndromic' ASD-related genes such as *CASK*, *GRIA3*, *NEXMIF*, and *AP1S2*, which previous studies implicate their involvement in neurodevelopmental and intellectual disability phenotypes including, autism spectrum disorders (Srivastava et al., 2016), X-linked mental retardation (Ghosh et al., 2013), and seizures (Cacciagli et al., 2014). Finally, functional annotation also revealed the involvement of these genes in critical pathways associated with brain and neural development, metabolic processes, and protein localization. These results suggest the elevated microRNAs contribute to posttranslational suppression of ASD-related genes, whose protein products play distinct molecular, cellular and biological functions.

Next, the data was examined to determine whether the upregulated microRNAs *mmu-miR-5622-3p*, *mmu-miR-6900-3p*, and *mmu-miR-7684-3p* reside in a clustered arrangement, that is, 2 or more microRNAs that are <10kb apart. The genomic distribution of human microRNAs is scattered along all chromosomes (Kim and Nam, 2006; Kamanu et al., 2013), and some have a higher number of microRNAs than others. For example, approximately 30% of all human microRNA reside within Chromosomes 1, 19, X and 2 (Kabekkodu et al., 2018; Gregorova et al., 2021), while 2 microRNAs have been mapped to the Y chromosome (Ghorai and Ghosh, 2014). Published studies have demonstrated clustered microRNAs share regulatory elements, such as promoters, and thus, can be expressed simultaneously under the same conditions (Kabekkodu et al., 2018). Therefore, the location of miR genes *mmu-miR-5622-3p*, *mmu-miR-6900-3p*, and *mmu-miR-7684-3p* were revealed to be located on chromosomes 2, 1, and 15 respectively. This finding suggests the upregulation of these microRNAs in stress exposed SERT heterozygous (SERT) dams at E21 are due to the independent activity of distinct regulatory regions.

Functioning microRNA: AGO-mediated gene modulation relies upon for the most part, partial complementary bonding of the microRNA's seed region with the 3' UTR loci of target mRNAs (Bartel, 2009), with an average length of approximately 1000 and 400 nucleotides long in humans and mice, respectively (Hong and Jeong, 2023). Furthermore, published reports postulate a positive correlation between 3' UTR length and increased binding sites for regulatory elements including microRNAs (Ji et al., 2009). Therefore, it may stand to reason that the

respective targets of *mmu-miR-5622-3p*, *mmu-miR-6900-3p*, and *mmu-miR-7684* may have longer 3'UTRs to accommodate the abundance of overexpressed microRNAs found at embryonic day 21 in the maternally stressed SERT heterozygous cohort. Shiny GO analysis revealed when compared against the mouse genome, the 3' UTRs of transcript targets of upregulated microRNAs *mmu-miR-5622-3p*, *mmu-miR-6900-3p*, and *mmu-miR-7684-3p* appear to have longer 3'UTRs (data not shown), hence increasing the potential of multiple binding sites for the aforementioned microRNAs. This finding suggests a probable greater degree of translational inhibition by the microRNAs detected in prenatally stressed SERT heterozygous dams.

With studies demonstrating bidirectional microRNA trafficking at the maternal-fetal interface (Chang et al., 2017), it raises the question of whether the overexpressed microRNAs detected at E21 being of either maternal or embryonic in origin. Indeed, gene expression analyses report differential tissue expression of *mmu-miR-5622-3p*, *mmu-miR-6900-3p*, and *mmu-miR-7684-3p* across embryonic, parturition and adult stages (Brunskill et al., 2014; Huntley et al., 2016; Zhao et al., 2023). These findings attest to the dynamic nature of microRNA expression throughout all stages of development and adulthood, thus making distinguishing between maternal or embryonically derived microRNA challenging. Nevertheless, their upregulation within this specific subgroup demonstrates perhaps a G x E-driven epigenetic signature unique to individuals with a genetic predisposition to environmental stress.

Aim 2: Behavioral characterization of offspring

I hypothesized behaviors of adult offspring of prenatally stressed SERT (+/-) dams to display behaviors similar to individuals diagnosed with autism spectrum disorders (ASD). To test this, SERT (+/-) dams will undergo a chronic variable stress (CVS) protocol from mid- 2nd trimester until late 3rd trimester or parturition. After which, resulting male and female adult offspring were assessed in order to identify hallmark phenotypes consistent within the ASD, including social avoidance, and elevated repetitive and anxiety-like behaviors.

Anxiety-related assays

Elevated plus maze (EPM)

Anxiety is a common comorbidity affecting the ASD population (Hahler and Elsabbagh, 2015). Thus, we were interested in whether offspring of gestationally stressed dam would display characteristics analogous to anxiety. Therefore, we subjected a total of 68 males, wild type non-CVS exposed (WT/NS (n=13)), wild type CVS exposed (WT/ST (n=15)), heterozygous SERT non-CVS exposed, (SERT/NS (n=21)), heterozygous SERT CVS exposed (SERT/ST (n=17)) to the elevated plus maze (EPM) assay (Lister, 1987) and scored against the number of entries and duration of time spent in either open or closed arms of the apparatus elevated approximately 36 inches high (See 'behavioral assays'). Mice spending a greater duration of time within closed arms relative to open arms are said to exhibit elevated anxiety-like behavior. Analysis of adult male offspring (Figure 10)

revealed no significant effects in genotype ($F(1,62) = 1.236$; $p = 0.2705$), stress ($F(1,62) = 1.304$; $p = 0.2579$), and interaction domains ($F(1,62) = 1.317$; $p = 0.2556$). Moreover, evaluation of open arm duration of 63 adult female offspring, wild type non-CVS exposed, (WT/NS ($n=14$)), wild type CVS exposed, (WT/ST ($n=15$)), heterozygous SERT non-CVS exposed (SERT/NS ($n=17$)), heterozygous SERT, (SERT/ST ($n=16$)) was conducted to observe exhibition of any anxiety-like characteristics (Figure 11). ANOVA analysis detected gene x stress interaction effects, ($F(1,58) = 4.228$, $p = 0.0443$), but was unable to detect main effects, of stress, ($F(1,58) = 0.5107$, $p = 0.4777$), or genotype ($F(1,58) = 0.1756$, $p = 0.6767$).

Figure 10: Male offspring elevated plus maze (EPM)

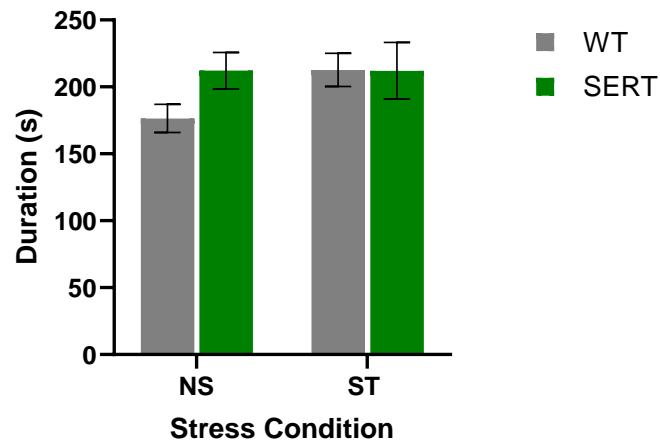


Figure 10: Average duration spent in open arms of elevated plus maze (EPM) of adult male offspring of non-stress exposed (NS) and prenatally stress exposed (ST), wild-type (WT: grey), and heterozygous (SERT: green) dams. Two-way ANOVA. Values are means \pm SEM * $p < 0.05$ (WT/NS (n=13), WT/ST (n=15), SERT/NS (n=21), SERT/ST (n=17)).

Figure 11: Female offspring elevated plus maze (EPM)

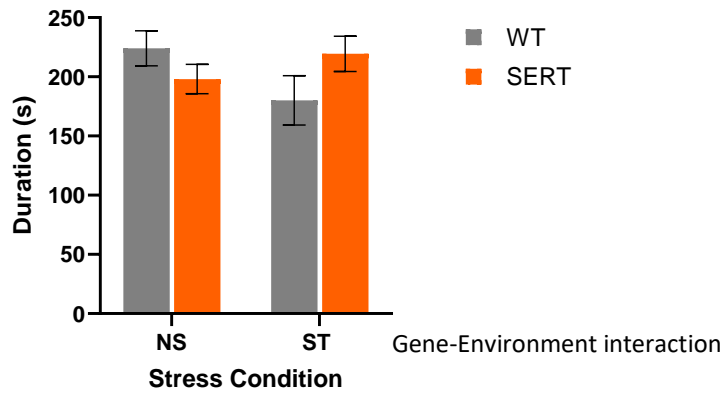


Figure 11: Average duration spent in the open arms of elevated plus maze (EPM) of adult female offspring of non-stress exposed (NS) and prenatally stress exposed (ST), wild-type (WT: grey), and heterozygous (SERT: orange) dams. Two -way ANOVA. Values are means \pm SEM. (WT/NS (n=14), WT/ST (n=14), SERT/NS (n=16), SERT/ST (n=16). Pairwise, main and interaction effects, * $p < 0.05$).

Open field (OF)

Offspring were singularly placed within a Plexiglass arena which they were allowed to freely explore for a duration of 10 minutes. This test measures the subject's innate behavior to explore open areas and assess the subject's general overall condition. Therefore, an offspring is considered to exhibit elevated anxiety-like behavior if it spends a greater amount of time exploring the corners or alongside the arena's perimeter relative to more central, unexposed areas of the arena.

We assayed 68 males offspring, (WT/NS (n=13), WT/ST (n=15), SERT/NS (n=21), SERT/ST (n=17) and observed no significant main genotype ($F(1,63) = 0.8875$; $p=0.3498$), stress ($F(1,63) = 1.927$; $p=0.1700$), and interaction effects ($F(1,63) = 1.994$; $p= 0.1629$) across all groups, (Figure 12)

ANOVA analysis of the female groups, (WT/NS (n=14), WT/ST (n=15), SERT/NS (n=17), SERT/ST (n=16) was unable to detect any significant differences or main effects within genotype, ($F(1,48) = 1.057$; $p= 0.3091$), stress ($F(1,48) = 0.297$; $p=0.5880$), or interaction ($F(1,48) = 0.551$; $p= 0.4615$) domains, (Figure 13).

Figure 12: Male offspring open field (OF)

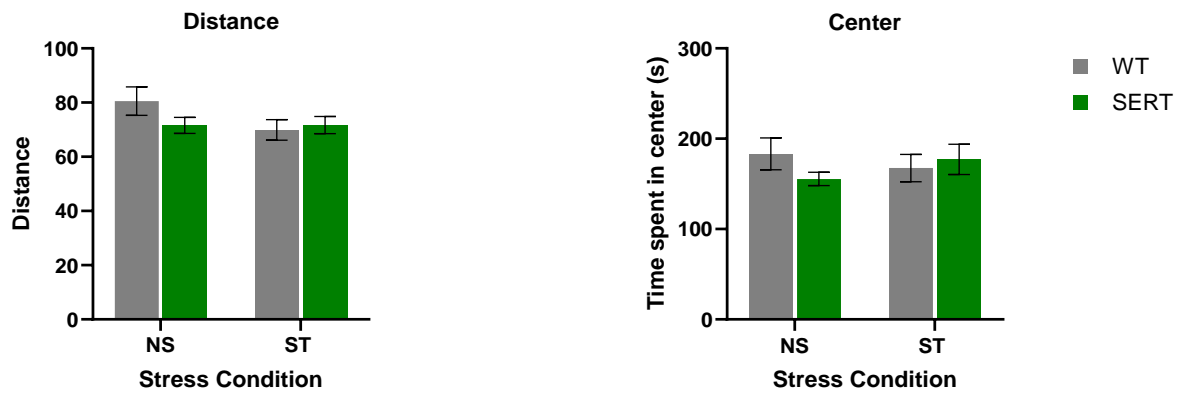


Figure 12: Average total distance and time in center of adult male offspring of non-stress exposed (NS) and prenatally stress exposed (ST), wild-type (WT: grey, and heterozygous (SERT: green) dams in open field assay. Two -way ANOVA. Values are means \pm SEM. * $p < 0.05$ (WT/NS (n=15), WT/ST (n=15), SERT/NS (n=21), SERT/ST (n=16)).

Figure 13: Female offspring open field (OF)

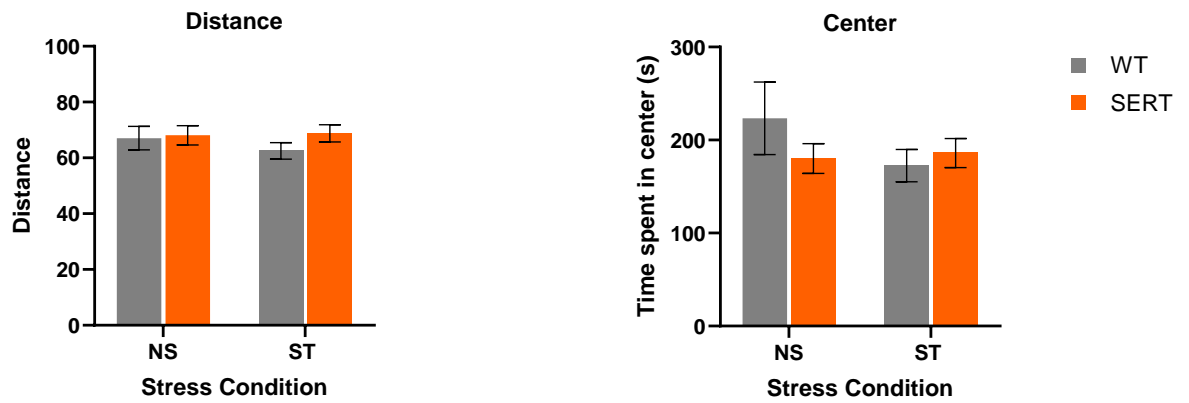


Figure 13: Average total distance and time spent in center of adult female offspring of non-stress exposed (NS) and prenatally stress exposed (ST), wild-type (WT: grey), and heterozygous (SERT: orange) dams in open field assay. Two-way ANOVA. Values are means \pm SEM. * $p < 0.05$. (WT/NS (n=14), WT/ST (n=14), SERT/NS (n=15), SERT/ST (n=16)).

Sociability assay

Social approach and novelty seeking

A hallmark characteristic of autism spectrum disorders (ASD) includes a low interest in initiating socialization with unfamiliar individuals and engaging in seeking novel activities. Thus, we sought to determine whether adult offspring of CVS exposed SERT (+/-) dam would exhibit impaired social approach and/or novelty seeking behaviors. To test this hypothesis, we first evaluated all adult offspring using 3 chamber social approach/ novelty seeking assay. To eliminate housing effects, animals are typically group housed, thereby reducing isolation-related stress, which studies demonstrate could affect the subject's performance on sociability tests (Rein et al., 2020).

Social approach

Analysis of 68 males, (WT/NS (n=15), WT/ST (n=15), SERT/NS (n=21), SERT/ST (n=17) demonstrated no main effects of genotype ($F(1,64) = 2.112$; $p = 0.1510$), or stress ($F(1,64) = 3.850$; $p = 0.0541$). However, an interaction effect was detected ($F(1,64) = 8.842$; $p = 0.0042$). Furthermore, an unpaired t-test was performed on the social performance ratio to address the hypothesis of SERT/ST offspring spent less time socializing relative to the WT/NS cohort. Indeed, the t-test did detect wild type non-stressed group spent more time with the initial stranger, ($t_{(28)} = 3.559$; $p = 0.0014$) than offspring of the prenatally stressed SERT group. (Figure 14).

Analysis of 62 virgin female offspring (WT/NS (n=14), WT/ST (n=15), SERT/NS (n=17), SERT/ST (n=16) determined significant main genotype ($F(1,58) = 5.074$, $p = 0.0281$), and interaction ($F(1,58) = 6.751$; $p = 0.0119$) effects. Furthermore, to address our hypothesis that offspring of prenatally stressed dams would exhibit low social approach, t-tests were performed between WT/NS and SERT/ST groups did not detect any significance, ($t_{(28)} = 0.748$; $p = 0.4605$), and no significant stress effects were detected for ($F(1,58) = 1.579$; $p = 0.2139$), (Figure 15).

Novelty seeking

Next, an examination of novelty seeking was conducted to determine whether adult male offspring exhibited limited interaction with novel individuals, (Figure 14). 2-way ANOVA analysis detected no significant differences among genotype, $F(1,64) = 0.0737$; $p = 0.7868$), stress $F(1,64) = 2.343$; $p = 0.1308$), or interaction $F(1,64) = 0.366$; $p = 0.5468$).

In addition, 2-way ANOVA of adult female offspring detected no significance in genotype ($F(1,58) = 2.051$; $p = 0.1575$), stress ($F(1,58) = 0.8342$; $p = 0.3649$) or interaction ($F(1,58) = 0.5723$; $p = 0.4524$), (Figure 15).

Figure 14: Male offspring social approach and novelty seeking

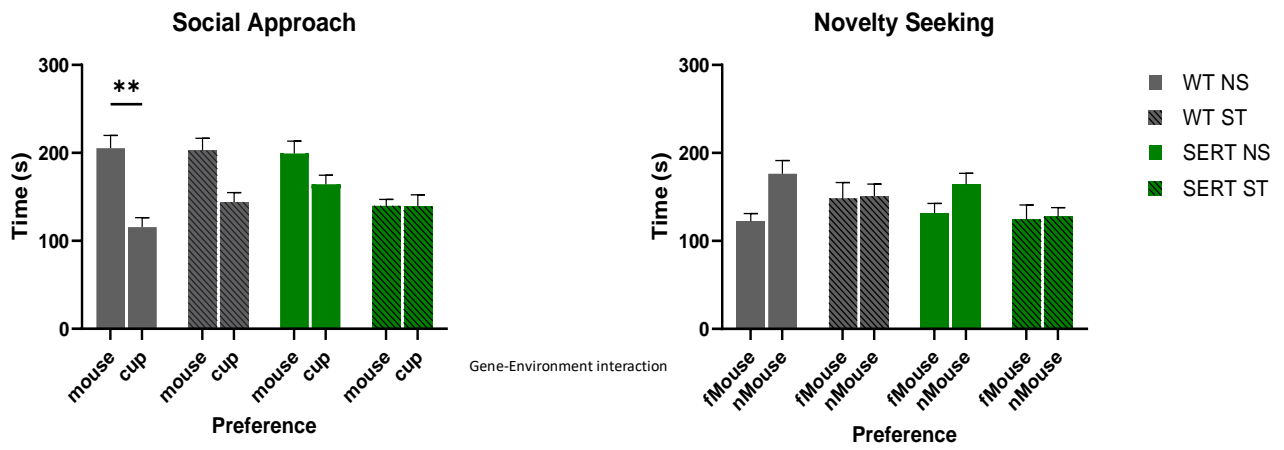


Figure14: Average interaction time of adult male offspring of non-stress exposed wild type (WT NS), prenatally stress exposed wild type (WT ST), non-stress exposed SERT (SERT NS), and prenatally stress exposed (SERT ST) dams during social approach (mouse vs. cup) and novelty seeking (fMouse vs. nMouse) phases of 3 chamber assay. Striped bars indicate offspring born to prenatally stressed exposed dams. Plain bars indicate offspring born to non-stressed exposed dams. Two-way ANOVA. Values are means \pm SEM. (WT/NS (n=14), WT/ST (n=15), SERT/NS (n=21), SERT/ST (n=16). Pairwise, main and interaction effects, *p<0.05.

Figure 15: Female offspring social approach and novelty seeking

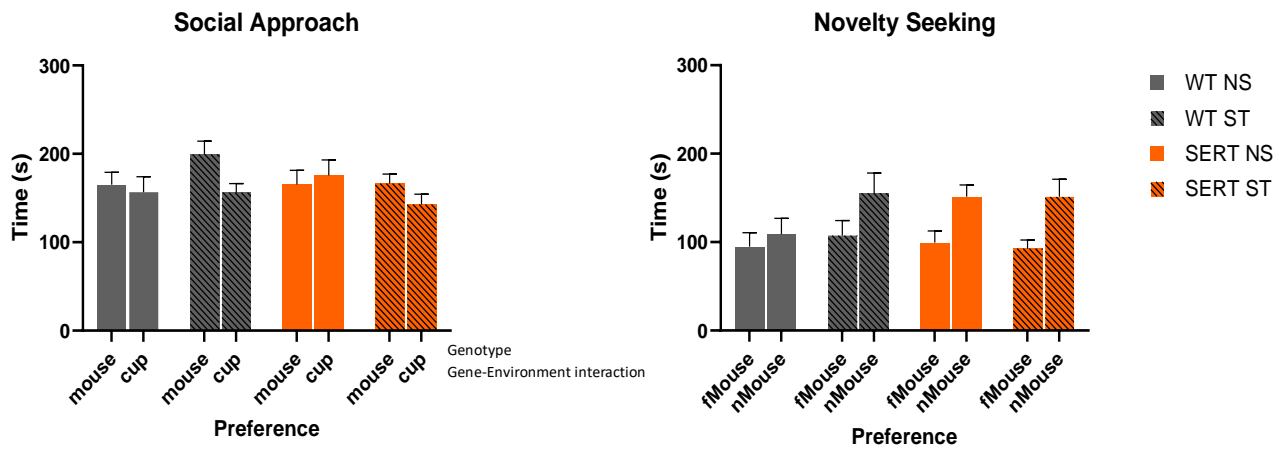


Figure 15: Average interaction time of adult female offspring of non-stress exposed wild type (WT NS), prenatally stress exposed wild type (WT ST), non-stress exposed SERT (SERT NS), and prenatally stress exposed (SERT ST) dams during social approach (mouse vs. cup) and novelty seeking (fMouse vs. nMouse) phases. Striped bars indicate offspring born to prenatally stressed dams. Plain bars indicate offspring born to non-stressed dams. Two-way ANOVA. Values are means \pm SEM. * $p < 0.05$ (WT/NS (n=14), WT/ST (n=14), SERT/NS (n=16), SERT/ST (n=16). Pairwise, main and interaction effects, * $p < 0.05$).

Social discrimination

Studies have reported mice spending more time socially investigating novel conspecifics, and less time with familiars (de la Zerda et al., 2022). Thus, the social discrimination index was calculated from the animal's behavior during the social approach phase and the animal's behavior during the novelty seeking phase. Thus, in the context of low sociability in ASD, this behavior was examined here to determine whether offspring of the GxE model would exhibit an altered social discriminatory phenotype. Paired t-testing was conducted across all experimental groups. The findings show no significance was detected in male offspring cohorts, WT/NS ($t_{(13)} = 1.492$; $p = 0.1594$), WT/ST ($t_{(14)} = 1.062$; $p = 0.306$), SERT/NS ($t_{(20)} = 0.678$; $p = 0.505$), and SERT/ST ($t_{(15)} = 0.571$; $p = 0.575$), (Figure 16).

Examination of adult female offspring of SERT non-stresses dams (SERT/NS) preferred interacting with an unfamiliar conspecific aka the novel mouse, ($t_{(15)} = 2.396$; $p = 0.030$), relative to other groups, WT/NS ($t_{(13)} = 0.256$; $p = 0.801$), WT/ST ($t_{(13)} = 0.551$; $p = 0.590$), SERT/ST ($t_{(14)} = 1.601$; $p = 0.131$), (Figure 17).

Figure 16: Male offspring discrimination index

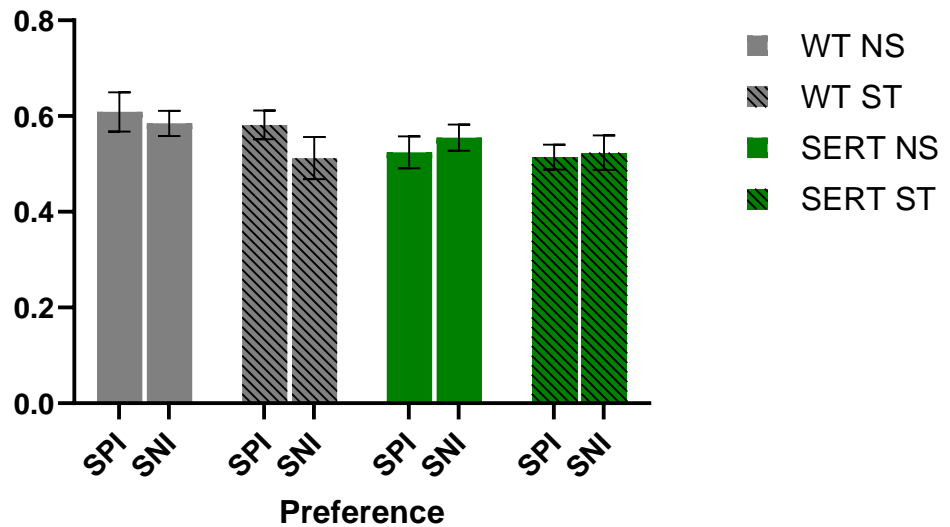


Figure 16: Discrimination index of adult male offspring of non-stress exposed (NS) and prenatally stress exposed (ST), wild-type (WT: grey), and heterozygous (SERT: green) dams in social approach and novelty seeking 3 chamber assay. Striped bars indicate offspring born to prenatally stressed dams. Plain bars indicate offspring born to non-stressed dams. Two-way ANOVA. Values are means \pm SEM. * $p < 0.05$. (WT/NS (n=14), WT/ST (n=15), SERT/NS (n=17), SERT/ST (n=16). SPI: social performance index; SNI: social novelty index.

Figure 17: Female offspring discrimination index

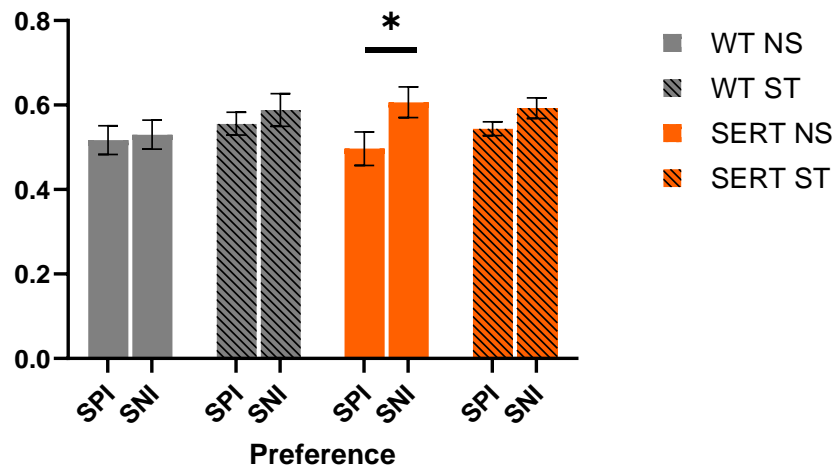


Figure 17: Discrimination index of adult female offspring of non-stress exposed (NS) and prenatally stress exposed (ST), wild-type (WT: grey), and heterozygous (SERT: orange) dams in social approach and novelty seeking 3 chamber assay. Striped bars indicate offspring born to prenatally stressed dams. Plain bars indicate offspring born to non-stressed dams. Two -way ANOVA. Values are means \pm SEM. (WT/NS (n=14), WT/ST (n=14), SERT/NS (n=16), SERT/ST (n=16). *p<0.05.

Repetitive behavior assays

Spontaneous self-grooming

Another commonly observed trait exhibited by individuals diagnosed with ASD, included stereotyped and repetitive movements and behaviors including incessant rocking back-and-forth, hand flapping, and aimless pacing. Therefore, to determine whether adult offspring of gestationally stressed heterozygous SERT dams would exhibit repetitive behaviors analogous to those observed in ASD, we subjected adult male and female offspring to our spontaneous self-grooming assay (McFarlane et al., 2008), in which subjects were scored by duration of grooming and total number of grooming events. First, we assessed the grooming duration of 68 males, (WT/NS (n=15), WT/ST (n=15), SERT/NS (n=21), SERT/ST (n=17). Our results did not reach significance across all groups in either genotype, ($F(1,64) = 0.1556$, $p = 0.694$), stress, ($F(1, 64) = 3.185$, $p = 0.090$) or gene x stress interaction, ($F(1, 64) = 1.537$, $p = 0.219$), (Figure 18).

We then assessed total grooming times of adult female offspring (WT/NS (n=14), WT/ST (n=15), SERT/NS (n=17), SERT/ST (n=16) and found no statistically significant differences in their total grooming times, nor any main stress, ($F(1,53)=0.251$, $p = 0.618$), genotype, ($F(1,53)= 0.802$, $p = 0.374$), or interaction effects ($F(1,53)= 2.185$, $p = 0.145$) was detected during analysis, (Figure 19).

Although total grooming duration appeared to be similar across all groups, interest shifted to determine whether there would be differences in the number of grooming events. ANOVA analysis of adult male offspring (WT/NS (n=15), WT/ST (n=15), SERT/NS (n=21), SERT/ST (n=17) demonstrated significant, main stress- effect

in the number of spontaneous self-grooming events ($F(1,64) = 9.152, p = 0.0036$), with SERT/ST adult males groomed more frequently than WT/NS group, ($t_{(30)} = 2.966; p = 0.0059$). However, no main genotype ($F(1,64) = 2.932, p = 0.0917$), or interaction ($F(1,64) = 0.2527, p = 0.6169$), effects were detected, (Figure 18).

Results from the adult female offspring group (WT/NS (n=14), WT/ST (n=15), SERT/NS (n=17), SERT/ST (n=16) demonstrated main genotype effect ($F(1,50) = 5.468, p = 0.0234$), with the SERT/ST group exhibiting a greater number of grooming events when compared to the WT/NS group, ($t_{(24)} = 2.702; p = 0.0125$). However, no significance was detected in stress ($F(1,50) = 1.766, p = 0.1899$) and interaction main effects ($F(1,50) = 0.2720, p = 0.6043$), (Figure 19).

Figure 18: Male self-grooming frequency and duration

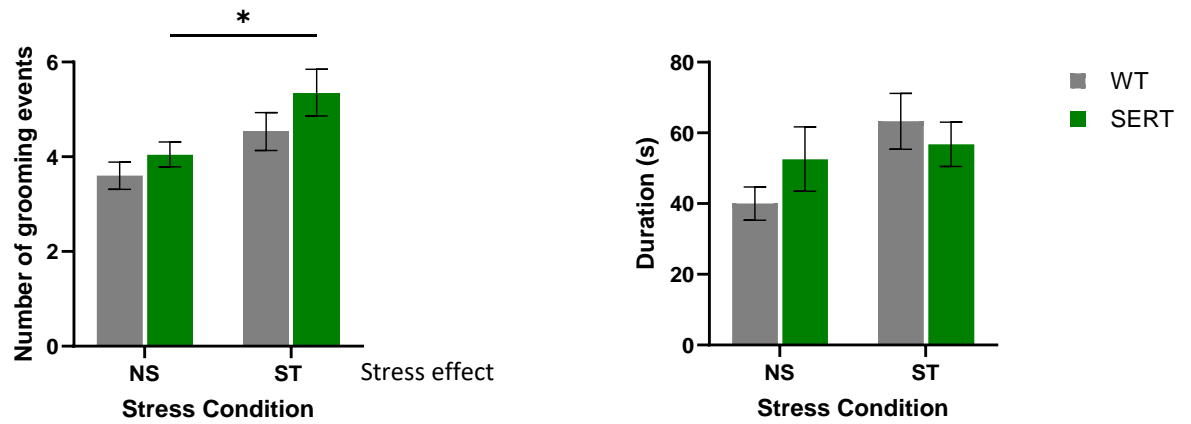


Figure 18: Average number grooming events and duration exhibited by adult male offspring of prenatally stress exposed (ST), and non-stress exposed (NS) wild-type (WT: grey), and heterozygous (SERT: green) dams. Two -way ANOVA. Values are means \pm SEM. * $p < 0.05$ (WT/NS ($n=15$), WT/ST ($n=15$), SERT/NS ($n=21$), SERT/ST ($n=17$)). Pairwise, main and interaction effects, * $p < 0.05$.

Figure 19: Female self-grooming frequency and duration

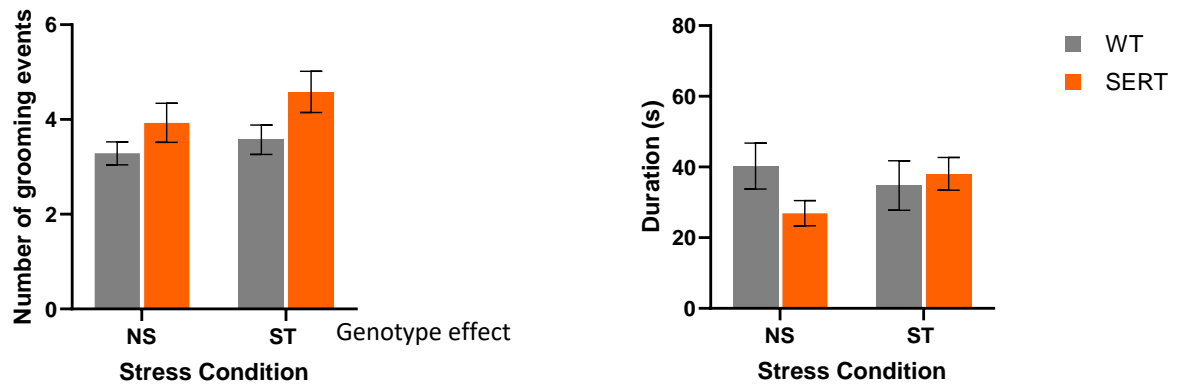


Figure 19: Average number grooming events and duration exhibited by adult female offspring of prenatally stress exposed (ST), and non-stress exposed (NS) wild-type WT (grey), and heterozygous SERT (orange) dams. Two -way ANOVA. Values are means \pm SEM. * $p < 0.05$; WT/NS ($n=14$), WT/ST ($n=15$), SERT/NS ($n=14$), SERT/ST ($n=16$). Pairwise, main and interaction effects, * $p < 0.05$.

Marble burying

We evaluated repetitive behavior in adult male and female offspring of gestationally stressed and non-stressed dams using the marble burying assay (Broekkamp et al., 1986). A total of 68 males, (WT/NS (n=15), WT/ST (n=15), SERT/NS (n=21), SERT/ST (n=17) were examined. 2- way ANOVA detected significant main effect of stress ($F(1,64) = 15.61, p = 0.0002$). Interestingly, *t* testing revealed when comparing the wildtype cohort, offspring of stress and non-stressed group, the former, (WT/NS) buried more marbles than the wild-type stress group (WT/ST), ($t_{(28)} = 3.761; p = 0.0008$). This observation was not detected between SERT/NS and SERT/ST groups ($t_{(36)} = 1.909; p = 0.0642$). Moreover, analysis did not detect any significant main genotype ($F(1,64) = 2.427; p = 0.1242$) or interaction ($F(1,64) = 1.640; p = 0.2049$) effects, (Figure 20).

Finally, analysis of adult female offspring cohorts (WT/NS (n=14), WT/ST (n=15), SERT/NS (n=17), SERT/ST (n=16) revealed a significant main effect of genotype, ($F(1,58) = 5.713; p = 0.0201$). However, interaction, ($F(1, 58) = 0.0359; p = 0.8502$), and main stress, ($F(1, 58) = 0.6252; p = 0.4324$) effects did not reach statistical significance, (Figure 21).

Figure 20: Male offspring marble burying

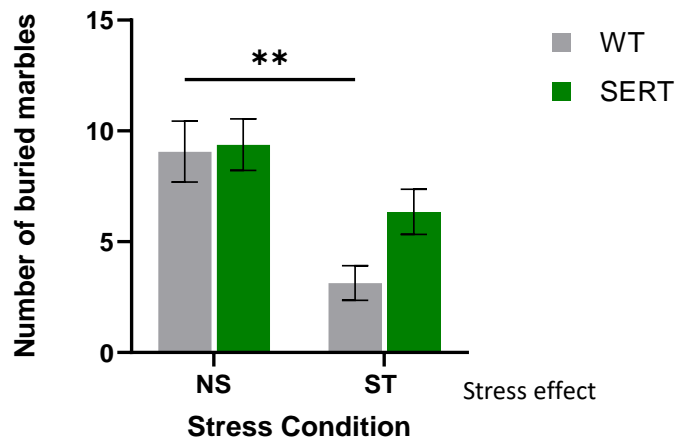


Figure 20: Average number of marbles buried by adult male offspring of prenatally stress exposed (ST), and non-stress exposed (NS) wild-type (WT: grey), and heterozygous (SERT: green) dams. Two-way ANOVA. Values are means \pm SEM. (WT/NS (n=15), WT/ST (n=15), SERT/NS (n=21), SERT/ST (n=17). Pairwise, main and interaction effects, *p<0.05.

Figure 21: Female offspring marble burying

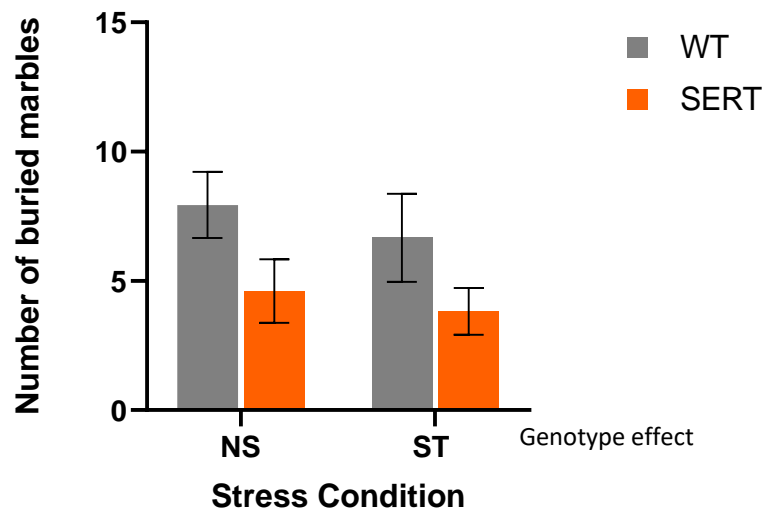


Figure 21: Average number of marbles buried by adult female offspring of prenatally stress exposed (ST), and non-stress exposed (NS) wild-type (WT: grey), and heterozygous (SERT: orange) dams. Two -way ANOVA. Values are means \pm SEM. * $p < 0.05$ (WT/NS (n=14), WT/ST (n=15), SERT/NS (n=15), SERT/ST (n=15). Pairwise, main and interaction effects, * $p < 0.05$.

Discussion

The objective of these assays was to determine whether environmental stressors experienced by stress susceptible individuals during pregnancy increases the likelihood of impaired behavioral manifestations in progeny. Unravelling the underlying mechanisms of complex neurodevelopmental disorders is further complicated by the impact of events experienced during pregnancy on fetal development. Longitudinal studies have implicated adverse external stressors to significantly increase the incidence of psychiatric and neurobehavioral disorders, including schizophrenia (Malaspina et al., 2008), generalized anxiety disorder (McLean et al., 2018), attention deficit hyperactivity disorder (ADHD) (Grizenko et al., 2012), and autism spectrum disorder diagnoses in progeny (Hecht et al., 2016). Although more research is needed to fully understand how genes and the external environment interact, the findings from this aim provide evidence of the impact of the maternal effects of gene-mediated stress response on the behavior of future offspring.

Social impairments are hallmark characteristics of autism spectrum disorders. Previous reports show, when compared to the neurotypical population, ASD individuals are more likely to exhibit unusual eye contact (Folstein and Rutter, 1977), (Lord et al., 1989), limited social awareness and reciprocity (Lord et al., 1989) (Huerta and Lord, 2012), and more likely to avoid social interaction; preferring to interact with inanimate objects rather than their peers (Kanner, 1943) (Bleuler, 1950). Given the salience of social deficits in ASD phenotype and diagnostic criteria, it was deemed fundamental to determine whether adult

offspring of our SERT- stress mouse model would exhibit similar behavioral characteristics. The findings of this study suggests that adult males appear to spend less time seeking new social interactions. (An et al., 2011), which according to previous reports, is indicative of low social preference (Balaan et al., 2019). Thus this cohort's social avoidant phenotype appears to be driven by the effects of our interaction model.(Klin et al., 2007), (de la Torre-Ubieta et al., 2016), (Lord et al., 2020), (Duncan et al., 2022).

Similar preference time spent interacting with a conspecific and inanimate object in the female cohort could be indicative of low social approach behavior; a hallmark ASD-like characteristic reported in mouse (Ricceri et al., 2008), (Hsu et al., 2020) and clinical observations (Amir et al., 1999) (Naidu and Johnston, 2011). Moreover, another interesting finding is the behavior displayed by adult male offspring of prenatally stressed SERT dam cohort, in which no obvious preference for the familiar mouse nor the empty cup was detected. This social impairment is consistent within genetic ASD mouse models, MeCP2 (Sato et al., 2012), PTEN (Napoli et al., 2012), and TSC (Reith et al., 2013). Moreover, this impairment is also a prevalent and salient behavioral characteristic in the clinical ASD population (Ritvo, 1989), (Schaafsma and Pfaff, 2014), (Lord et al., 2020).

Findings from our previous work examining GxE-related effects on offspring behavioral outcomes show both female and male progeny exhibiting robust preference for the novelty mouse (Matsui et al., 2018). However, in this study heightened novelty avoidance was observed only in the adult male offspring in this study. Although there are no differences in mouse strain used and stress protocol

between studies, the contrasting findings regarding offspring social interaction could be attributed to differences in testing environment (Lai, 1990), experimenter's sex (Sorge et al., 2014), and individual handling procedures (Georgiou et al., 2022), all of which have been shown to impact experimental results. Further analysis examining main and interaction effects, revealed an interaction effect of maternal genotype and chronic variable stressors on the average time spent engaging with conspecifics versus an inanimate object, which supports a finding in one of our previous studies (Jones et al., 2010). Overall, this study, like our previous works demonstrates that maternal stress during pregnancy can potentially increase the risk of ASD-associated behaviors in offspring (Jones et al., 2010; Matsui et al., 2018), thus supporting the concept of gene-environment interaction and heightened risk of ASD development. Furthermore, identifying gestationally related microRNA dysregulation using the same experimental paradigm provides evidence of a potential association between differentially expressed microRNA and core ASD-related behaviors.

During the novelty seeking phase of the sociability assay, in which interaction times between familiar and novel mice were measured, adult female offspring overall performed as expected and, appeared to spend more time interacting with the novelty mouse than their familiars relative to adult male offspring (Figure 15 & 16). Interestingly again, adult male offspring born to prenatally stressed SERT dams trended to spend similar times interacting with both familiar and novel mice (Figure 15), suggesting this cohort is engaging in low novelty seeking behavior (Rein et al., 2020).

In clinical populations, repetitive behavior is a core autism characteristic. Individuals diagnosed with ASD exhibit a suite of abnormal repetitive patterns of behaviors and movements (Lord et al., 2012), (Modabbernia et al., 2017). Moreover, studies using genetic animal models examining ASD-related behavior deficits also report diverse repetitive behaviors including compulsive burial of objects (Huynh et al., 2015), abnormal movements such as, intense tail-chasing, jumping, cage-top twirling, and compulsive face and body grooming (Lewis et al., 2007).

Spontaneous self-grooming in mice is a behavior often used as a metric when interrogating repetitive movements as it is analogous to repetitive and stereotypic movements observed in individuals diagnosed with ASD (Crawley, 2007). Therefore, this study investigated whether the effects of our gene x environment paradigm would alter repetitive behavior of future offspring. First, looking at total grooming time across all groups, all experimental animals engaged in self-grooming behaviors regardless of genotype, sex and stress conditions. ANOVA analysis was unable to detect any significant differences in total grooming time across all groups in both female and male cohorts. These results support our previous finding (Matsui et al., 2018), suggesting gene and/or environmental perturbances, was ineffective on total grooming time.

However, when analyzing whether animals exhibited differences in the number of grooming events, as significant main effects were observed in both cohorts. First, adult female offspring revealed main genotype effect on the number of grooming events, as a trend of increased grooming episodes was observed in adult female

progeny born to SERT dams. In addition, adult male offspring born to stress exposed wild-type and SERT dams exhibited main stress effects of increasing the number of grooming events. Another finding supporting this are the results of the marble burying tests which reveal a main effect of genotype playing a role in the number of marbles buried by adult female offspring, as female offspring born to SERT dams burying on average less than their wild-type counterparts, regardless of stress exposure conditions, while the main effects of stress affecting male offspring marble burial regardless of genotype. The latter finding recapitulates results from our previous study (Matsui et al., 2018), thus providing strong supporting evidence of male sensitivity to maternal stress exposure during pregnancy, and its subsequent effects on the behavior of male progeny (Cuffe et al., 2012; Babenko et al., 2015) Collectively, the results of the repetitive assays potentially demonstrate the sex-specific impact each factor of our gene-environment model exerts on offspring behavior

Potential explanation for *in utero* male stress sensitivity could be related to sex-specific differences of stress related pathways. For example, the hypothalamic adrenal pituitary axis (HPA) is a neuroendocrine network that controls physiological processes such as, digestion, and hormonal responses to internal and external changes in the environment (Heck and Handa, 2019), (Mbiydzennyuy et al., 2022). Although HPA functioning has been observed in fetal human and mouse during the second trimester and embryonic day 10 (E10), respectively, (Mbiydzennyuy et al., 2022), (Jafari et al., 2017), fetal HPA activation is responsive to placental and exogenously derived factors (Gunn et al., 2013) including

microRNA (Rodgers et al., 2013; Zucchi et al., 2013), however, the exact mechanistic involvement is not clear.

Expectations of the marble burying assay were that offspring of prenatally stress exposed dams would bury more marbles. However, the findings revealed that not to be the case, as some groups buried less than expected. A possible explanation could be that the mice were not simply unbothered by the presence of the marbles, even though their spatial distribution in the chamber appeared to interfere with locomotion paths of the experimental mouse during the experiment. Furthermore, another reason for the low burial scores could be due to the mice engaging in other potentially pathological repetitive behavior. That is, the mice were probably so fixated on grooming themselves, marble burial was not a prioritized behavior or activity.

Finally, the results of the repetitive behaviors in this study provide supporting evidence of outcomes regarding the interplay of genetic and non-genetic factors that contribute to core features of neurodevelopmental disorders.

Results of the open field (OF) assay did not reveal any differences or main effects among adult female offspring, regardless of maternal genotype and stress condition, as they appeared to spend similar time exploring the arena. Although there was a slight decrease detected in adult female offspring born to wild type stressed dams, the total distance travelled by all other adult female offspring were not significantly different, nor were any main effects detected via analysis. This suggests all adult female offspring demonstrated no locomotive impairments. Adult male offspring however, with the exception of adult male offspring born to wild type

non-stressed mothers, performed similarly to female offspring, as analysis detected no significance or main effects during the open field assay, suggesting no locomotive impairment among these groups. Furthermore, all groups were administered a second anxiety-related task via the elevated plus maze (EPM). Interestingly, analysis of adult female offspring detected an interaction effect in which offspring of prenatally stressed wild type and SERT dams respectively spent less and longer duration exploring the open arm of the maze. These findings support the initial hypothesis that progeny of prenatally stressed SERT dams would spend less time exploring the open arm of the maze, which would indicate high anxiety-like behavior. All other groups spent similar times exploring the open arms of the apparatus. The contrasting results between female and male progeny could be indicative of the behavior heterogeneity seen in clinical populations, as females are more likely to be diagnosed with anxiety-related disorders than males (Rubinow and Schmidt, 2019). Although not a core symptom of ASD, anxiety has an estimated prevalence as high as 80%, and is one of the most prevalent co-occurring neurological conditions in individuals with ASD (Sharma et al., 2018b).

Chapter 5

Study limitations

While the results of the study provide valuable insight into the effects of maternal 5-HTTLPR and stress on underlying molecular mechanisms and offspring behavior outcomes, it is important to acknowledge its limitations.

First, although the primary focus of the study was examining the effects of maternal genotype SERT (+/-) on offspring behavior, the genotype of the offspring, (SERT (+/+) and SERT (+/-)), was not considered. Published reports are inconclusive regarding the impact of embryonic genotype on development. While studies provide evidence supporting offspring genotype influence (Barkley and FitzGerald, 1990), other colleagues report embryo genotype play little to no role during pregnancy and development (Moler et al., 1980), with the maternal being the more predominant determinant (Côté et al., 2007; Pallares and Gonzalez-Bulnes, 2010). Therefore, to reconcile this, future experiments should include genotyping all resulting offspring to exclude offspring with confounding genotypes.

Furthermore, collection of maternal samples at embryonic day 21 (E21) detected changes in ASD- and stress- associated microRNA. However, collecting samples only during this time in gestation may not capture critical microRNAs that potentially affect future behavior in offspring. MicroRNA expression is dynamic throughout the lifespan an organism, including pregnancy, collecting samples at different times during gestation would fully capture changes in microRNA expression, hence, providing a more comprehensive profile of microRNAs that coincide with specific developmental milestones. However, repeated blood draws

will impose an additional confound of stress upon dams and could potentially affect maternal microRNA profiles and embryonic development.

Finally, while a unique microRNA signature was detected in our group of interest, (SERT (+/-) x CVS exposed group), which holds promise as a useful tool for identifying underlying epigenetic dysregulation, due to the small sample size, profiling experiments should be repeated to validate the results of the current study.

Future directions

Findings from this study provided evidence of microRNA dysregulation within a gene-environment framework, thereby highlighting the potential involvement of a posttranslational regulatory mechanism during pregnancy. Although adult offspring of maternally stressed heterozygous SERT dams demonstrated ASD-associated characteristics, the mechanistic role of microRNA in the programming of those behaviors remains unresolved. Therefore, to determine microRNA's role in neuropathogenesis, wild type pregnant dams can receive direct administration of *mmu-miR-5622-3p*, *mmu-miR-6900-3p*, and *mmu-miR-7684-3p* via intravenous injection, after which, adult female and male offspring can be behaviorally assessed for ASD-like characteristics. Doing this will validate the biological relevance of microRNAs and provide mechanistic insight into the integration of posttranslational gene regulation in the gene-environment interaction paradigm.

In addition, one limitation to our findings could be recapitulating the behavior associated miRNA profile of stress exposed SERT (+/-) mice in human samples. Our previous study uncovered candidate miRNAs implicated in maternal stress pathways in human blood and saliva samples, however, behavior associated miRNA expression was not explored. Therefore, I propose acquiring blood and saliva samples from individuals affected with autism spectrum disorders to examine and compare their miRNA profiles to those found in this study.

Finally, although differentiated microRNA profiles were detected in our maternally stressed animal model, further experiments may be necessary to determine whether the overexpressed microRNAs captured are actively inhibiting gene

expression. To accomplish this, a pull-down assay selecting for Argonaute (AGO) protein, thereby simultaneously capturing miRISC elements including mature passenger miR templates and messenger RNAs targets would be ideal. This method would eliminate the capture of passenger microRNA strands targeted for degradation, and other similarly sized non-coding (nc) RNAs including, small nuclear RNA (snRNA), subsequently selecting for functional AGO: miR: mRNA complexes potentially involved in regulating stress- and ASD- associated targets with greater resolution.

Another proposed experiment would be to assess whether maternally derived microRNAs can transect the maternal-fetal barrier in our gene-environment model. To accomplish this, we can intravenously administer fluorescently tagged microRNAs via tail vein injection to gestating dams and euthanize at embryonic day 21 (E21) to collect maternal blood, placental and offspring samples for microRNA profiling and microscopy to visualize localization of tagged microRNAs to provide a better understanding of mechanism of microRNA trafficking via the maternal-fetal interface, which would ultimately lead to developing early intervention tools.

Epilogue

As it stands, current studies are providing compelling evidence demonstrating the influence of an individual's genotype playing a major role in stress susceptibility, disease onset and behaviors such as fearfulness, and anxiety. For example, clinical studies led by researchers including Klaus-Peter Lesch and colleagues in 1996, show evidence of the effects of the 5HTTLPR variant on behavioral phenotypes associated with elevated stress response. Their study, using the NEO personality Inventory (NEO-PI-R)- a self-reporting personality test which examines personality traits including agreeableness, openness to experience, extraversion, conscientiousness, and neuroticism (Costa Jr. and McCrae, 1995) demonstrated individuals with the 5HTTLPR variant scored higher in areas associated with aggression, neuroticism, and anxiety-related personality traits. These findings, along with others, demonstrate an association between genes and behavioral output (Lesch et al., 1996).

Although research has shown that genes are capable of influencing behavior, the mechanisms underlying this relationship remain unknown. Our initial foray into identifying potential mechanisms sought to provide an explanation regarding the maternal effects of genetic stress vulnerability on the behavior of subsequent progeny (Sjaarda et al., 2017). A follow-up study using a preclinical model, presented experimental evidence of a gene x environment interaction playing a role in modulating epigenetic mechanisms, (Meaney, 2010) which, studies show play critical roles during fetal development (Soutschek and Schrott, 2023). These results provided a potential mechanistic explanation of our previous study, which

demonstrated the impact of the combined effect of maternal 5-HTTLPR variant and gestational stress on behavioral trajectory of offspring (Jones et al., 2010).

Further investigation using our clinical model revealed an association between gestationally stressed maternal 5-HTTLPR carriers and increased risk of ASD (Hecht et al., 2016), thus supporting the hypothesis of a gene x environment framework in ASD pathogenesis, which can allow for early identification for individuals with an elevated risk for the disorder. Thus, a follow up study sought to delineate differential microRNA expressivity in blood samples of mothers with at least one autistic child, to demonstrate potential ASD-specific microRNA signatures (Beverdors et al., 2021b). Our findings revealed stress mediated changes in microRNA expression were more robust in mothers carrying the heterozygous SERT genotype. Therefore, we posit the role of microRNA as a predictive epigenetic ASD- associated risk indicator within individuals genetically vulnerable to stress. This present study, to our knowledge, the first of its kind to identify microRNA alterations within a prenatally stressed SERT mouse model.

In conclusion, this study provides evidence of the effects of maternal stress susceptibility on underlying epigenetic mechanisms and offspring behavior outcomes in a gene-environment interaction paradigm.

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Appendix

Table 1: List of significant differentially expressed microRNAs collected from stressed heterozygous SERT (+/-), (SERT +/-) and wild type non-stressed exposed dams at embryonic day 21 (E21). MicroRNAs of interest are indicated in bold.

| MicroRN A | SERT (+/-) Stressed | Wild Type Non-Stressed | SERT (+/-) Stressed vs Wild Type Non-Stressed Fold Change | SERT (+/-) Stressed vs Wild Type Non-Stressed P Value |
|------------------------|-----------------------------|-------------------------------|--|--|
| mmu-let-7c-2 | 0.65 | 1.08 | -1.35 | 0.0005 |
| mmu-miR-7221-5p | 0.9 | 0.5 | 1.32 | 0.0005 |
| mmu-mir-875 | 0.68 | 0.9 | -1.16 | 0.002 |
| mmu-miR-3103-3p | 0.62 | 0.96 | -1.27 | 0.0023 |
| mmu-miR-1943-5p | 0.67 | 0.95 | -1.21 | 0.0023 |
| mmu-mir-7027 | 0.69 | 0.95 | -1.19 | 0.0025 |
| mmu-miR-7684-3p | 1.57 | 0.94 | 1.55 | 0.0026 |
| mmu-mir-486 | 0.81 | 1.08 | -1.21 | 0.0028 |
| mmu-mir-362 | 0.66 | 0.83 | -1.13 | 0.0031 |
| mmu-mir-1895 | 0.79 | 1.11 | -1.24 | 0.0032 |
| mmu-mir-467a-2 | 1.04 | 0.68 | 1.28 | 0.0033 |
| mmu-mir-467a-4 | 1.04 | 0.68 | 1.28 | 0.0033 |
| mmu-mir-467a-5 | 1.04 | 0.68 | 1.28 | 0.0033 |
| mmu-mir-467a-7 | 1.04 | 0.68 | 1.28 | 0.0033 |
| mmu-mir-467a-8 | 1.04 | 0.68 | 1.28 | 0.0033 |
| mmu-mir-467a-9 | 1.04 | 0.68 | 1.28 | 0.0033 |
| mmu-miR-7680-5p | 0.93 | 0.71 | 1.16 | 0.0039 |
| mmu-miR-873b | 0.7 | 0.91 | -1.16 | 0.0045 |
| mmu-mir-7578 | 0.93 | 0.42 | 1.42 | 0.0051 |

| | | | | |
|-----------------|------|------|-------|--------|
| mmu-mir-470 | 0.89 | 0.73 | 1.12 | 0.0051 |
| mmu-mir-6942 | 1.01 | 0.72 | 1.22 | 0.0052 |
| mmu-mir-370 | 0.89 | 0.56 | 1.26 | 0.0053 |
| mmu-mir-6481 | 0.69 | 0.86 | -1.13 | 0.0054 |
| mmu-mir-365-1 | 0.57 | 1.07 | -1.42 | 0.0057 |
| mmu-miR-7064-5p | 0.73 | 1.12 | -1.32 | 0.0061 |
| mmu-miR-7028-3p | 0.77 | 1.04 | -1.21 | 0.0068 |
| mmu-mir-499 | 0.88 | 0.61 | 1.21 | 0.0073 |
| mmu-let-7c-2 | 0.7 | 0.93 | -1.18 | 0.0079 |
| mmu-mir-1942 | 0.78 | 1.14 | -1.28 | 0.0082 |
| mmu-miR-328-5p | 3.09 | 1.75 | 2.54 | 0.0084 |
| mmu-miR-363-3p | 0.69 | 0.46 | 1.18 | 0.0085 |
| mmu-miR-1a-1-5p | 0.96 | 0.63 | 1.26 | 0.0096 |
| mmu-miR-491-5p | 1.08 | 0.76 | 1.25 | 0.0098 |
| mmu-miR-30f | 0.63 | 0.87 | -1.19 | 0.0098 |
| mmu-miR-466l-3p | 1.02 | 0.71 | 1.24 | 0.01 |
| mmu-miR-6951-3p | 0.81 | 0.71 | 1.08 | 0.0102 |
| mmu-miR-467d-3p | 0.71 | 1.07 | -1.28 | 0.0104 |
| mmu-miR-7224-5p | 1.08 | 0.71 | 1.29 | 0.0105 |
| mmu-miR-7219-5p | 0.76 | 1.04 | -1.21 | 0.0106 |
| mmu-miR-141-3p | 1.17 | 0.76 | 1.33 | 0.0106 |
| mmu-mir-7069 | 0.66 | 1.08 | -1.34 | 0.0109 |

| | | | | |
|-----------------|------|------|-------|--------|
| mmu-miR-199a-3p | 0.76 | 0.56 | 1.14 | 0.0113 |
| mmu-miR-199b-3p | 0.76 | 0.56 | 1.14 | 0.0113 |
| mmu-miR-691 | 1.19 | 0.77 | 1.34 | 0.0114 |
| mmu-miR-211-3p | 0.84 | 1.38 | -1.46 | 0.0117 |
| mmu-miR-367-3p | 0.84 | 0.72 | 1.09 | 0.0117 |
| mmu-mir-6419 | 0.59 | 0.98 | -1.31 | 0.0121 |
| mmu-mir-5128 | 0.78 | 1.09 | -1.24 | 0.0122 |
| mmu-miR-6358 | 0.86 | 0.48 | 1.3 | 0.0131 |
| mmu-miR-496b | 0.8 | 1.13 | -1.25 | 0.0132 |
| mmu-miR-8116 | 0.74 | 1.34 | -1.52 | 0.0134 |
| mmu-mir-493 | 0.84 | 0.91 | -1.05 | 0.0136 |
| mmu-mir-7067 | 0.83 | 0.52 | 1.24 | 0.0138 |
| mmu-mir-3095 | 0.95 | 0.78 | 1.12 | 0.0138 |
| mmu-mir-32 | 0.88 | 0.67 | 1.16 | 0.014 |
| mmu-miR-3620-3p | 0.81 | 1.14 | -1.26 | 0.0144 |
| mmu-miR-6998-5p | 1.04 | 0.8 | 1.19 | 0.0146 |
| mmu-let-7k | 0.83 | 1.12 | -1.21 | 0.0147 |
| mmu-mir-7040 | 0.98 | 0.71 | 1.21 | 0.0151 |
| mmu-miR-378a-3p | 0.72 | 1 | -1.22 | 0.0157 |
| mmu-miR-6339 | 0.65 | 0.93 | -1.21 | 0.0159 |
| mmu-mir-7055 | 0.71 | 1 | -1.22 | 0.0162 |
| mmu-miR-1943-3p | 0.79 | 1.02 | -1.17 | 0.0164 |

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|------------------------|-------------|-------------|-------------|---------------|
| mmu-miR-6962-5p | 1.21 | 0.85 | 1.28 | 0.0174 |
| mmu-mir-467b | 0.99 | 0.58 | 1.33 | 0.0176 |
| mmu-miR-6917-5p | 0.65 | 1.03 | -1.3 | 0.0182 |
| mmu-miR-130a-5p | 0.66 | 0.87 | -1.16 | 0.0184 |
| mmu-miR-3069-3p | 0.89 | 0.67 | 1.16 | 0.0185 |
| mmu-miR-362-3p | 1.09 | 0.55 | 1.45 | 0.0187 |
| mmu-mir-181b-1 | 1.15 | 0.89 | 1.2 | 0.0191 |
| mmu-miR-6936-3p | 0.84 | 0.6 | 1.18 | 0.0192 |
| mmu-miR-5622-3p | 1.65 | 1.25 | 1.33 | 0.0194 |
| mmu-miR-1983 | 0.61 | 0.96 | -1.28 | 0.0204 |
| mmu-miR-190a-5p | 0.8 | 0.54 | 1.2 | 0.0206 |
| mmu-miR-7073-5p | 0.8 | 1.07 | -1.2 | 0.0208 |
| mmu-miR-6338 | 0.55 | 0.91 | -1.29 | 0.0214 |
| mmu-miR-6948-3p | 0.7 | 0.92 | -1.16 | 0.0226 |
| mmu-miR-216c-5p | 0.74 | 0.84 | -1.07 | 0.0231 |
| mmu-miR-7037-3p | 0.9 | 0.63 | 1.21 | 0.0232 |
| mmu-miR-6372 | 0.64 | 0.97 | -1.26 | 0.0238 |
| mmu-miR-7a-5p | 0.8 | 1.18 | -1.3 | 0.0251 |
| mmu-miR-190a-3p | 1.69 | 3.65 | -3.88 | 0.0253 |
| mmu-miR-6930-5p | 0.66 | 1.06 | -1.32 | 0.0254 |
| mmu-miR-297a-5p | 1.5 | 0.9 | 1.52 | 0.0259 |

| | | | | |
|------------------------|-------------|-------------|------------|---------------|
| mmu-miR-7018-3p | 0.94 | 0.72 | 1.16 | 0.026 |
| mmu-mir-375 | 1.02 | 0.81 | 1.16 | 0.0261 |
| mmu-miR-1940 | 1.09 | 0.73 | 1.29 | 0.0271 |
| mmu-miR-7230-3p | 1.06 | 0.73 | 1.26 | 0.0272 |
| mmu-miR-6929-3p | 0.75 | 1.03 | -1.21 | 0.0272 |
| mmu-miR-6392-5p | 0.97 | 0.8 | 1.12 | 0.0273 |
| mmu-mir-3070b | 0.78 | 0.91 | -1.09 | 0.0275 |
| mmu-mir-6981 | 0.97 | 0.81 | 1.12 | 0.0275 |
| mmu-miR-8107 | 1.85 | 1.12 | 1.65 | 0.0277 |
| mmu-miR-9-3p | 0.72 | 0.92 | -1.16 | 0.028 |
| mmu-mir-219c | 0.94 | 1.21 | -1.21 | 0.0287 |
| mmu-mir-190b | 0.71 | 1.04 | -1.26 | 0.0289 |
| mmu-miR-3057-5p | 0.58 | 0.93 | -1.27 | 0.0292 |
| mmu-miR-6900-3p | 1.22 | 0.73 | 1.4 | 0.0299 |
| mmu-miR-344e-5p | 0.64 | 0.84 | -1.15 | 0.0301 |
| mmu-miR-344h-5p | 0.64 | 0.84 | -1.15 | 0.0301 |
| mmu-miR-7220-5p | 0.88 | 0.62 | 1.2 | 0.0307 |
| mmu-miR-6946-3p | 0.77 | 1.01 | -1.18 | 0.0312 |
| mmu-mir-7648 | 0.9 | 0.68 | 1.17 | 0.0315 |
| mmu-mir-222 | 0.6 | 0.83 | -1.17 | 0.0326 |
| mmu-miR-7004-5p | 0.72 | 0.97 | -1.19 | 0.0346 |
| mmu-miR-3108-5p | 0.83 | 0.56 | 1.2 | 0.0349 |

| | | | | |
|-----------------|------|------|-------|--------|
| mmu-miR-7678-5p | 1.29 | 0.82 | 1.39 | 0.035 |
| mmu-miR-682 | 0.64 | 0.52 | 1.09 | 0.0355 |
| mmu-miR-32-3p | 1.35 | 1 | 1.28 | 0.0359 |
| mmu-miR-762 | 4.43 | 2.88 | 2.93 | 0.0361 |
| mmu-miR-6992-5p | 0.96 | 0.71 | 1.19 | 0.0361 |
| mmu-miR-3544-3p | 0.89 | 0.71 | 1.13 | 0.0362 |
| mmu-miR-667-5p | 2.01 | 1.44 | 1.48 | 0.0364 |
| mmu-miR-7658-5p | 1.61 | 1.33 | 1.21 | 0.0364 |
| mmu-miR-505-5p | 0.81 | 1.08 | -1.2 | 0.0367 |
| mmu-miR-99b-3p | 0.85 | 1.11 | -1.2 | 0.0367 |
| mmu-miR-7077-3p | 0.65 | 0.83 | -1.13 | 0.0371 |
| mmu-mir-6951 | 0.89 | 1.25 | -1.29 | 0.0378 |
| mmu-miR-3066-5p | 0.82 | 1 | -1.14 | 0.0378 |
| mmu-mir-302b | 1.02 | 0.6 | 1.33 | 0.0379 |
| mmu-miR-3079-3p | 0.85 | 0.57 | 1.22 | 0.038 |
| mmu-miR-7059-3p | 0.55 | 0.82 | -1.21 | 0.0382 |
| mmu-mir-680-1 | 0.79 | 1 | -1.16 | 0.0385 |
| mmu-miR-6956-5p | 1.2 | 1.03 | 1.12 | 0.0385 |
| mmu-miR-342-5p | 0.79 | 0.98 | -1.14 | 0.0387 |
| mmu-mir-598 | 1.11 | 0.84 | 1.2 | 0.0389 |
| mmu-miR-590-5p | 0.77 | 0.98 | -1.16 | 0.039 |

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|------------------|------|------|-------|--------|
| mmu-mir-6919 | 0.97 | 0.83 | 1.1 | 0.039 |
| mmu-miR-3104-5p | 1.15 | 0.63 | 1.43 | 0.039 |
| mmu-mir-378d | 0.66 | 0.95 | -1.22 | 0.0391 |
| mmu-mir-487b | 0.71 | 1.08 | -1.29 | 0.0392 |
| mmu-miR-7057-5p | 0.78 | 0.92 | -1.1 | 0.0398 |
| mmu-mir-6931 | 0.98 | 0.76 | 1.16 | 0.0399 |
| mmu-mir-7688 | 0.8 | 0.74 | 1.04 | 0.0399 |
| mmu-miR-6943-3p | 0.75 | 1.02 | -1.21 | 0.0399 |
| mmu-mir-5113 | 0.66 | 0.98 | -1.25 | 0.0412 |
| mmu-mir-3086 | 0.64 | 1.1 | -1.38 | 0.0414 |
| mmu-miR-187-5p | 0.66 | 0.98 | -1.25 | 0.0422 |
| mmu-mir-137 | 0.73 | 0.89 | -1.12 | 0.0427 |
| mmu-miR-409-5p | 0.75 | 0.96 | -1.15 | 0.0432 |
| mmu-miR-103-1-5p | 0.7 | 0.96 | -1.2 | 0.0433 |
| mmu-mir-5121 | 0.84 | 0.56 | 1.22 | 0.0442 |
| mmu-mir-6373 | 1 | 0.83 | 1.13 | 0.0444 |
| mmu-miR-148a-3p | 0.52 | 0.76 | -1.19 | 0.0446 |
| mmu-miR-6973b-5p | 1.13 | 0.78 | 1.28 | 0.0448 |
| mmu-miR-6920-3p | 0.64 | 0.85 | -1.16 | 0.0449 |
| mmu-mir-3105 | 0.92 | 0.82 | 1.08 | 0.045 |
| mmu-miR-23a-5p | 0.84 | 1.04 | -1.14 | 0.0453 |
| mmu-miR-7086-5p | 0.72 | 1.04 | -1.25 | 0.0453 |

| | | | | |
|-------------------|------|------|-------|--------|
| mmu-miR-879-5p | 0.97 | 0.61 | 1.29 | 0.0455 |
| mmu-miR-6933-3p | 0.93 | 0.62 | 1.24 | 0.0464 |
| mmu-mir-491 | 0.87 | 0.68 | 1.14 | 0.0465 |
| mmu-miR-30a-3p | 0.77 | 1.03 | -1.19 | 0.0467 |
| mmu-mir-3085 | 1.2 | 0.68 | 1.43 | 0.048 |
| mmu-miR-137-5p | 0.97 | 0.69 | 1.21 | 0.0482 |
| mmu-miR-493-3p | 0.77 | 1.07 | -1.23 | 0.0482 |
| mmu-mir-21a | 0.96 | 0.6 | 1.28 | 0.0483 |
| mmu-miR-186-3p | 0.94 | 1.46 | -1.44 | 0.0495 |
| mmu-miR-669d-2-3p | 0.93 | 1.17 | -1.18 | 0.0496 |
| mmu-miR-344d-2-5p | 0.72 | 1.05 | -1.26 | 0.0497 |
| mmu-miR-6715-5p | 0.91 | 0.56 | 1.27 | 0.0499 |

Table 2: List of significant differentially expressed microRNAs in heterozygous SERT, (SERT (+/-)) non-stressed and SERT (+/-) stressed dams at embryonic day 21 (E21). MicroRNAs of interest are indicated in bold.

| MicroRN A | SERT (+/-) Non-Stressed | SERT (+/-) Stressed | SERT (+/-) Stressed vs SERT (+/-) Non-Stressed Fold Change | SERT (+/-) Stressed vs SERT (+/-) Non-Stressed P Value |
|------------------|--------------------------------|----------------------------|---|---|
| mmu-miR-1943-5p | 1.06 | 0.67 | -1.31 | 3.95E-05 |
| mmu-miR-3968 | 0.73 | 1.05 | 1.25 | 0.0007 |
| mmu-mir-875 | 0.91 | 0.68 | -1.17 | 0.0008 |
| mmu-miR-7224-5p | 0.69 | 1.08 | 1.3 | 0.0012 |
| mmu-miR-7062-3p | 0.48 | 0.92 | 1.36 | 0.0015 |
| mmu-miR-8093 | 1.33 | 0.94 | -1.3 | 0.0015 |
| mmu-mir-3100 | 0.67 | 0.87 | 1.14 | 0.0021 |
| mmu-mir-365-1 | 1.1 | 0.57 | -1.45 | 0.0022 |
| mmu-mir-2136 | 0.63 | 1.03 | 1.32 | 0.0022 |
| mmu-mir-222 | 0.88 | 0.6 | -1.21 | 0.0024 |
| mmu-mir-7029 | 1.06 | 0.79 | -1.21 | 0.0026 |
| mmu-mir-362 | 0.86 | 0.66 | -1.15 | 0.0027 |
| mmu-mir-6942 | 0.72 | 1.01 | 1.22 | 0.0027 |
| mmu-mir-3962 | 0.94 | 0.73 | -1.16 | 0.0032 |
| mmu-mir-5120 | 1.03 | 0.72 | -1.24 | 0.005 |
| mmu-mir-434 | 1.03 | 0.61 | -1.34 | 0.005 |
| mmu-miR-568 | 0.86 | 0.99 | 1.09 | 0.0055 |
| mmu-miR-188-5p | 1.42 | 0.85 | -1.48 | 0.0057 |
| mmu-mir-6355 | 0.91 | 0.64 | -1.21 | 0.0058 |
| mmu-mir-6340 | 0.79 | 1.04 | 1.19 | 0.0062 |
| mmu-miR-5128 | 7.21 | 7.63 | 1.33 | 0.0066 |

| | | | | |
|------------------------|-------------|-------------|-------------|---------------|
| mmu-miR-7684-3p | 1 | 1.57 | 1.49 | 0.0068 |
| mmu-miR-7021-5p | 0.82 | 0.63 | -1.14 | 0.007 |
| mmu-mir-218-1 | 1.01 | 0.69 | -1.25 | 0.0072 |
| mmu-miR-6365 | 0.72 | 0.97 | 1.19 | 0.0076 |
| mmu-miR-5622-3p | 1.18 | 1.65 | 1.38 | 0.008 |
| mmu-miR-381-5p | 0.56 | 0.96 | 1.32 | 0.0084 |
| mmu-miR-691 | 0.83 | 1.19 | 1.28 | 0.0087 |
| mmu-miR-702-3p | 0.67 | 1.23 | 1.48 | 0.0091 |
| mmu-miR-7003-5p | 1.18 | 1.68 | 1.41 | 0.0092 |
| mmu-miR-135a-1-3p | 1.08 | 0.96 | -1.09 | 0.0092 |
| mmu-mir-6336 | 0.61 | 1.08 | 1.39 | 0.0092 |
| mmu-miR-216b-3p | 0.89 | 0.52 | -1.29 | 0.0101 |
| mmu-miR-1306-5p | 0.62 | 1.15 | 1.44 | 0.0103 |
| mmu-miR-216c-5p | 0.92 | 0.74 | -1.13 | 0.0106 |
| mmu-mir-26a-2 | 0.95 | 0.77 | -1.13 | 0.0113 |
| mmu-mir-7040 | 0.72 | 0.98 | 1.2 | 0.0115 |
| mmu-mir-6481 | 0.85 | 0.69 | -1.12 | 0.0116 |
| mmu-mir-6905 | 0.88 | 0.66 | -1.17 | 0.0116 |
| mmu-mir-466q | 0.81 | 1.05 | 1.18 | 0.0121 |
| mmu-miR-674-3p | 0.85 | 1.08 | 1.17 | 0.0122 |
| mmu-miR-7035-3p | 0.63 | 0.99 | 1.29 | 0.0122 |

| | | | | |
|-----------------|------|------|-------|--------|
| mmu-miR-15a-3p | 0.9 | 0.62 | -1.22 | 0.0123 |
| mmu-miR-7066-3p | 0.93 | 0.74 | -1.14 | 0.0123 |
| mmu-miR-7680-5p | 0.78 | 0.93 | 1.11 | 0.0126 |
| mmu-miR-326-3p | 0.55 | 0.84 | 1.22 | 0.013 |
| mmu-let-7c-1-3p | 0.93 | 0.63 | -1.23 | 0.0131 |
| mmu-miR-106a-5p | 1.5 | 0.79 | -1.64 | 0.0131 |
| mmu-miR-6386 | 0.92 | 0.65 | -1.21 | 0.0136 |
| mmu-miR-19a-5p | 0.76 | 0.6 | -1.11 | 0.0138 |
| mmu-mir-1960 | 0.9 | 0.66 | -1.18 | 0.014 |
| mmu-miR-1224-5p | 3.43 | 2.31 | -2.17 | 0.0141 |
| mmu-miR-5108 | 0.8 | 0.58 | -1.16 | 0.0143 |
| mmu-miR-6546-5p | 0.84 | 0.63 | -1.16 | 0.0144 |
| mmu-miR-7070-3p | 0.94 | 0.67 | -1.2 | 0.0145 |
| mmu-miR-3101-3p | 0.76 | 1.13 | 1.29 | 0.0152 |
| mmu-mir-7652 | 0.7 | 0.99 | 1.22 | 0.0152 |
| mmu-miR-3964 | 0.92 | 0.84 | -1.06 | 0.0152 |
| mmu-mir-7115 | 0.67 | 0.95 | 1.21 | 0.0154 |
| gi:555853 | 6.67 | 6.27 | -1.32 | 0.0166 |
| mmu-miR-7050-5p | 1.92 | 1.19 | -1.66 | 0.0166 |
| mmu-miR-669b-5p | 1.4 | 0.98 | -1.34 | 0.0167 |
| mmu-miR-7679-5p | 0.64 | 0.85 | 1.16 | 0.0171 |

| | | | | |
|-----------------|------|------|-------|--------|
| mmu-miR-1298-3p | 0.79 | 0.7 | -1.07 | 0.0173 |
| mmu-mir-339 | 0.81 | 0.51 | -1.23 | 0.0174 |
| mmu-miR-7688-3p | 0.57 | 0.9 | 1.26 | 0.0176 |
| gi:555853 | 6.53 | 5.97 | -1.48 | 0.0182 |
| mmu-miR-664-3p | 0.63 | 0.91 | 1.22 | 0.0187 |
| mmu-miR-154-3p | 0.89 | 1.08 | 1.15 | 0.0189 |
| mmu-miR-6963-3p | 0.68 | 1.06 | 1.3 | 0.0194 |
| gi:555853 | 6.47 | 6.12 | -1.28 | 0.0194 |
| mmu-mir-669h | 1.1 | 0.85 | -1.19 | 0.0195 |
| mmu-miR-7049-3p | 0.99 | 0.86 | -1.1 | 0.0198 |
| mmu-mir-761 | 1.1 | 0.74 | -1.28 | 0.02 |
| mmu-mir-7006 | 0.66 | 0.81 | 1.11 | 0.0204 |
| mmu-mir-3091 | 1.08 | 0.82 | -1.2 | 0.0212 |
| mmu-miR-873b | 0.89 | 0.7 | -1.14 | 0.0213 |
| mmu-miR-7008-5p | 0.76 | 0.98 | 1.17 | 0.0218 |
| mmu-miR-3057-5p | 0.87 | 0.58 | -1.22 | 0.0222 |
| mmu-mir-5132 | 0.74 | 0.92 | 1.13 | 0.0224 |
| mmu-let-7i | 0.96 | 0.7 | -1.2 | 0.0224 |
| mmu-mir-6981 | 0.73 | 0.97 | 1.18 | 0.0225 |
| mmu-mir-6918 | 0.85 | 0.53 | -1.25 | 0.0225 |
| mmu-miR-290b-3p | 0.8 | 1.04 | 1.18 | 0.0228 |
| mmu-mir-15b | 0.98 | 0.84 | -1.1 | 0.0229 |

| | | | | |
|------------------|------|------|-------|--------|
| mmu-miR-3473d | 0.96 | 0.64 | -1.25 | 0.0237 |
| gi:555853 | 6.3 | 5.8 | -1.42 | 0.0237 |
| mmu-miR-7210-3p | 0.88 | 0.68 | -1.15 | 0.0238 |
| mmu-mir-7668 | 1.27 | 0.98 | -1.23 | 0.024 |
| mmu-mir-137 | 0.87 | 0.73 | -1.11 | 0.0244 |
| mmu-miR-5130 | 3.39 | 2.77 | -1.54 | 0.0249 |
| mmu-miR-6956-5p | 1.01 | 1.2 | 1.14 | 0.0251 |
| mmu-mir-551b | 1.12 | 0.81 | -1.24 | 0.0251 |
| mmu-miR-181a-5p | 0.78 | 1.01 | 1.18 | 0.0252 |
| mmu-mir-1906-1 | 1.08 | 0.83 | -1.19 | 0.0253 |
| mmu-mir-1906-2 | 1.08 | 0.83 | -1.19 | 0.0253 |
| mmu-mir-704 | 0.69 | 0.83 | 1.1 | 0.0264 |
| mmu-mir-6917 | 0.67 | 1 | 1.25 | 0.0264 |
| mmu-miR-449a-5p | 0.68 | 0.89 | 1.15 | 0.027 |
| mmu-miR-219b-5p | 0.62 | 0.9 | 1.22 | 0.0272 |
| mmu-miR-3070a-5p | 0.93 | 1.24 | 1.24 | 0.0278 |
| mmu-miR-3070b-5p | 0.93 | 1.24 | 1.24 | 0.0278 |
| mmu-miR-134-5p | 0.66 | 0.95 | 1.22 | 0.0278 |
| mmu-miR-32-3p | 0.94 | 1.35 | 1.33 | 0.0284 |
| mmu-miR-6392-5p | 0.67 | 0.97 | 1.24 | 0.0286 |
| mmu-mir-7076 | 1 | 0.84 | -1.11 | 0.0288 |
| mmu-miR-124-5p | 0.94 | 0.81 | -1.09 | 0.0295 |

| | | | | |
|-----------------|------|------|-------|--------|
| mmu-mir-450a-1 | 0.77 | 1.05 | 1.22 | 0.0302 |
| mmu-miR-1931 | 3.82 | 4.36 | 1.45 | 0.0306 |
| mmu-mir-451a | 0.88 | 0.68 | -1.15 | 0.0309 |
| mmu-miR-378a-5p | 1.04 | 0.87 | -1.12 | 0.031 |
| mmu-miR-29c-3p | 0.65 | 0.83 | 1.13 | 0.031 |
| mmu-mir-6516 | 0.94 | 1.26 | 1.25 | 0.0312 |
| mmu-miR-467e-5p | 0.79 | 0.99 | 1.15 | 0.0314 |
| mmu-miR-6338 | 0.87 | 0.55 | -1.25 | 0.0318 |
| mmu-miR-1947-3p | 0.8 | 0.84 | 1.02 | 0.0322 |
| mmu-mir-7055 | 0.94 | 0.71 | -1.18 | 0.0328 |
| mmu-miR-433-5p | 0.77 | 1.06 | 1.22 | 0.0328 |
| mmu-miR-138-5p | 0.99 | 0.8 | -1.14 | 0.0338 |
| mmu-miR-6392-3p | 0.67 | 1.06 | 1.31 | 0.0342 |
| mmu-miR-669h-3p | 0.85 | 1.14 | 1.22 | 0.0344 |
| mmu-miR-1953 | 0.75 | 1.01 | 1.19 | 0.0345 |
| mmu-miR-7080-5p | 1.35 | 1.02 | -1.25 | 0.0345 |
| mmu-miR-493-3p | 1.08 | 0.77 | -1.24 | 0.0348 |
| gi:555853 | 6.39 | 5.9 | -1.41 | 0.0349 |
| mmu-mir-497 | 0.89 | 1.09 | 1.15 | 0.035 |
| mmu-mir-7648 | 0.73 | 0.9 | 1.13 | 0.0351 |
| mmu-miR-21b | 0.72 | 1.05 | 1.25 | 0.0354 |
| gi:555853 | 6.25 | 5.86 | -1.32 | 0.0356 |

| | | | | |
|-----------------|------|------|-------|--------|
| gi:555853 | 6.44 | 6.01 | -1.35 | 0.0358 |
| mmu-miR-7678-5p | 0.85 | 1.29 | 1.36 | 0.0361 |
| mmu-miR-1946a | 0.83 | 1.23 | 1.32 | 0.0361 |
| mmu-mir-493 | 0.87 | 0.84 | -1.02 | 0.0375 |
| mmu-miR-374c-5p | 0.6 | 0.9 | 1.23 | 0.0375 |
| mmu-miR-1949 | 0.67 | 0.96 | 1.23 | 0.038 |
| mmu-miR-8107 | 1.44 | 1.85 | 1.32 | 0.0381 |
| mmu-miR-7076-5p | 0.83 | 1.07 | 1.18 | 0.0383 |
| mmu-mir-7682 | 1.03 | 0.69 | -1.27 | 0.0388 |
| mmu-mir-302b | 0.72 | 1.02 | 1.23 | 0.039 |
| mmu-miR-377-5p | 0.75 | 1.02 | 1.21 | 0.0391 |
| mmu-mir-297a-2 | 0.95 | 0.74 | -1.16 | 0.0393 |
| mmu-miR-7040-3p | 0.73 | 1.07 | 1.26 | 0.0395 |
| mmu-miR-20a-3p | 0.88 | 0.67 | -1.16 | 0.0398 |
| mmu-miR-6997-5p | 0.88 | 0.84 | -1.03 | 0.04 |
| mmu-mir-466m | 1.06 | 0.83 | -1.17 | 0.0401 |
| mmu-miR-6995-3p | 0.73 | 0.98 | 1.19 | 0.0403 |
| mmu-mir-6971 | 0.75 | 1.08 | 1.26 | 0.0404 |
| mmu-mir-7223 | 0.65 | 0.8 | 1.12 | 0.0406 |
| mmu-mir-139 | 0.68 | 0.8 | 1.09 | 0.0406 |
| mmu-miR-302b-5p | 0.67 | 0.91 | 1.19 | 0.0408 |
| mmu-mir-1264 | 0.67 | 0.9 | 1.17 | 0.0411 |

| | | | | |
|-----------------|------|------|-------|--------|
| mmu-miR-1982-3p | 0.71 | 0.81 | 1.07 | 0.0413 |
| mmu-miR-6906-3p | 1.12 | 0.87 | -1.19 | 0.0414 |
| mmu-mir-697 | 1.01 | 0.81 | -1.15 | 0.0417 |
| mmu-miR-3470b | 1.02 | 0.91 | -1.08 | 0.042 |
| mmu-miR-1950 | 0.75 | 0.98 | 1.17 | 0.0421 |
| mmu-miR-141-3p | 0.9 | 1.17 | 1.21 | 0.0424 |
| mmu-miR-6930-3p | 0.89 | 0.68 | -1.16 | 0.0425 |
| mmu-mir-6372 | 0.69 | 0.97 | 1.21 | 0.0427 |
| mmu-miR-148a-3p | 0.73 | 0.52 | -1.16 | 0.043 |
| mmu-miR-3088-5p | 0.9 | 0.69 | -1.16 | 0.0438 |
| mmu-miR-574-3p | 0.75 | 0.95 | 1.14 | 0.0442 |
| mmu-miR-6966-3p | 0.84 | 0.59 | -1.19 | 0.0444 |
| mmu-miR-7212-3p | 0.83 | 1.1 | 1.2 | 0.0447 |
| mmu-mir-6354 | 0.85 | 0.57 | -1.21 | 0.0453 |
| mmu-mir-3085 | 0.86 | 1.2 | 1.26 | 0.0454 |
| gi:555853 | 6.5 | 6.15 | -1.27 | 0.0454 |
| mmu-mir-6546 | 1.09 | 0.82 | -1.2 | 0.0459 |
| mmu-mir-758 | 0.82 | 0.95 | 1.1 | 0.0459 |
| mmu-mir-590 | 0.62 | 0.95 | 1.26 | 0.0463 |
| mmu-miR-290a-3p | 0.81 | 0.95 | 1.1 | 0.0463 |
| mmu-miR-351-5p | 0.68 | 0.88 | 1.14 | 0.0465 |

| | | | | |
|-----------------|------|------|-------|--------|
| mmu-miR-7241-5p | 1.07 | 0.91 | -1.11 | 0.0466 |
| mmu-miR-6236 | 0.67 | 0.77 | 1.07 | 0.0469 |
| mmu-miR-292b-5p | 0.83 | 0.99 | 1.12 | 0.0469 |
| mmu-mir-129-1 | 1.09 | 0.74 | -1.27 | 0.0477 |
| mmu-miR-344b-5p | 1 | 0.72 | -1.22 | 0.048 |
| mmu-miR-344i | 1.21 | 1 | -1.16 | 0.0486 |
| mmu-miR-6537-3p | 0.93 | 0.68 | -1.19 | 0.0488 |
| mmu-mir-6339 | 0.85 | 1.16 | 1.24 | 0.0491 |
| mmu-miR-6992-5p | 0.84 | 0.96 | 1.09 | 0.0496 |
| mmu-miR-744-3p | 0.57 | 0.8 | 1.18 | 0.0496 |
| mmu-miR-7671-3p | 1.47 | 1.89 | 1.33 | 0.0498 |
| mmu-miR-6385 | 0.77 | 1.28 | 1.42 | 0.0499 |
| mmu-miR-6937-3p | 1.11 | 0.9 | -1.15 | 0.05 |

Table 3: List of significant differentially expressed microRNAs in wild type non-stressed and wild type stressed dams at embryonic day 21 (E21). MicroRNAs of interest are indicated in bold.

| MicroRN A | Wild Type Non-Stressed | Wild Type Stressed | Wild Type Stressed vs Wild Type Non-Stressed Fold Change | Wild Type Stressed vs Wild Type Non-Stressed P Value |
|------------------|-------------------------------|---------------------------|---|---|
| mmu-mir-690 | 0.81 | 1.19 | 1.3 | 0.0005 |
| mmu-miR-7028-3p | 1.04 | 0.66 | -1.3 | 0.0009 |
| mmu-miR-297a-5p | 0.9 | 1.77 | 1.83 | 0.0011 |
| mmu-mir-1942 | 1.14 | 0.79 | -1.27 | 0.0022 |
| mmu-miR-590-5p | 0.98 | 0.64 | -1.26 | 0.0022 |
| mmu-miR-129-1-3p | 1.16 | 0.7 | -1.38 | 0.0022 |
| mmu-miR-7219-5p | 1.04 | 0.6 | -1.35 | 0.0026 |
| mmu-miR-5108 | 0.53 | 0.88 | 1.27 | 0.0026 |
| mmu-miR-149-5p | 0.94 | 0.66 | -1.22 | 0.0027 |
| mmu-mir-7027 | 0.95 | 0.69 | -1.19 | 0.0031 |
| mmu-miR-878-5p | 0.64 | 0.94 | 1.23 | 0.0035 |
| mmu-miR-5128 | 8.14 | 7.29 | -1.8 | 0.0037 |
| mmu-mir-21a | 0.6 | 0.96 | 1.28 | 0.0044 |
| mmu-miR-7051-5p | 0.7 | 1 | 1.23 | 0.0049 |
| mmu-mir-7022 | 1.03 | 0.66 | -1.29 | 0.0052 |
| mmu-miR-99b-3p | 1.11 | 0.67 | -1.36 | 0.0053 |
| mmu-mir-344g | 0.59 | 0.89 | 1.23 | 0.0054 |
| mmu-mir-467a-2 | 0.68 | 1.01 | 1.26 | 0.0055 |
| mmu-mir-467a-4 | 0.68 | 1.01 | 1.26 | 0.0055 |
| mmu-mir-467a-5 | 0.68 | 1.01 | 1.26 | 0.0055 |

| | | | | |
|------------------|------|------|-------|--------|
| mmu-mir-467a-7 | 0.68 | 1.01 | 1.26 | 0.0055 |
| mmu-mir-467a-8 | 0.68 | 1.01 | 1.26 | 0.0055 |
| mmu-mir-467a-9 | 0.68 | 1.01 | 1.26 | 0.0055 |
| mmu-miR-1943-3p | 1.02 | 0.74 | -1.21 | 0.0057 |
| mmu-mir-1247 | 0.74 | 1.15 | 1.33 | 0.0057 |
| mmu-mir-6396 | 0.75 | 1.03 | 1.21 | 0.0067 |
| mmu-miR-448-5p | 1.03 | 0.67 | -1.29 | 0.0071 |
| mmu-miR-6941-5p | 0.69 | 1.21 | 1.43 | 0.0071 |
| mmu-let-7c-2 | 1.08 | 0.81 | -1.2 | 0.0072 |
| mmu-miR-6929-3p | 1.03 | 0.62 | -1.32 | 0.0074 |
| mmu-miR-6973b-5p | 0.78 | 1.06 | 1.22 | 0.0075 |
| mmu-mir-7037 | 1 | 0.81 | -1.14 | 0.0076 |
| mmu-mir-486 | 1.08 | 0.76 | -1.25 | 0.0086 |
| mmu-mir-485 | 0.72 | 0.89 | 1.12 | 0.0086 |
| mmu-miR-7210-3p | 0.79 | 1.17 | 1.3 | 0.0087 |
| mmu-miR-7028-5p | 0.81 | 1.25 | 1.36 | 0.0088 |
| mmu-miR-669b-5p | 1.07 | 1.44 | 1.29 | 0.01 |
| mmu-miR-342-5p | 0.98 | 0.73 | -1.19 | 0.0103 |
| mmu-mir-2136 | 1 | 0.71 | -1.22 | 0.0103 |
| mmu-miR-496b | 1.13 | 0.8 | -1.26 | 0.0108 |
| mmu-mir-3063 | 0.98 | 0.71 | -1.2 | 0.0108 |
| mmu-miR-6390 | 0.64 | 0.89 | 1.19 | 0.0109 |

| | | | | |
|-----------------|------|------|-------|--------|
| mmu-miR-1950 | 0.76 | 1.02 | 1.19 | 0.0111 |
| mmu-miR-8116 | 1.34 | 0.72 | -1.54 | 0.0112 |
| mmu-mir-7067 | 0.52 | 0.85 | 1.25 | 0.0115 |
| mmu-mir-5121 | 0.56 | 1.06 | 1.41 | 0.0116 |
| mmu-mir-182 | 0.9 | 0.67 | -1.17 | 0.0118 |
| mmu-miR-532-3p | 1.23 | 0.65 | -1.49 | 0.0123 |
| mmu-miR-328-5p | 1.75 | 2.81 | 2.09 | 0.0124 |
| mmu-miR-363-3p | 0.46 | 0.7 | 1.18 | 0.0128 |
| mmu-miR-7064-5p | 1.12 | 0.8 | -1.26 | 0.0129 |
| mmu-miR-6998-5p | 0.8 | 1.17 | 1.29 | 0.0132 |
| mmu-miR-7077-3p | 0.83 | 0.63 | -1.15 | 0.0132 |
| mmu-mir-7578 | 0.42 | 0.79 | 1.29 | 0.0136 |
| mmu-miR-152-3p | 0.85 | 0.61 | -1.18 | 0.0139 |
| mmu-miR-2137 | 3.43 | 4.65 | 2.33 | 0.014 |
| mmu-miR-7651-3p | 1.21 | 0.88 | -1.26 | 0.014 |
| mmu-mir-6978 | 1.05 | 0.77 | -1.22 | 0.0141 |
| mmu-miR-345-5p | 0.76 | 1.19 | 1.34 | 0.0143 |
| mmu-mir-713 | 0.93 | 0.65 | -1.22 | 0.0144 |
| mmu-mir-3086 | 1.1 | 0.81 | -1.22 | 0.0154 |
| gi:555853 | 5.93 | 6.44 | 1.43 | 0.0155 |
| mmu-miR-25-3p | 0.92 | 3.9 | 7.86 | 0.0157 |
| mmu-mir-6546 | 0.65 | 0.9 | 1.19 | 0.0159 |

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|-----------------|------|------|-------|--------|
| mmu-mir-487b | 1.08 | 0.65 | -1.34 | 0.0165 |
| mmu-miR-3069-3p | 0.67 | 0.79 | 1.09 | 0.0166 |
| mmu-miR-615-3p | 0.71 | 0.99 | 1.21 | 0.0168 |
| mmu-mir-6516 | 1 | 1.25 | 1.19 | 0.017 |
| mmu-miR-501-5p | 0.65 | 0.99 | 1.26 | 0.0173 |
| mmu-mir-7654 | 1.3 | 0.95 | -1.28 | 0.0174 |
| mmu-miR-7214-5p | 0.96 | 0.71 | -1.19 | 0.018 |
| mmu-mir-6951 | 1.25 | 0.86 | -1.31 | 0.0188 |
| mmu-miR-7a-5p | 1.18 | 0.79 | -1.31 | 0.0189 |
| mmu-miR-8101 | 2.69 | 4.54 | 3.59 | 0.0191 |
| mmu-miR-876-5p | 0.49 | 0.87 | 1.3 | 0.0192 |
| mmu-miR-190a-5p | 0.54 | 0.81 | 1.2 | 0.0203 |
| mmu-mir-100 | 0.87 | 0.79 | -1.06 | 0.0206 |
| mmu-miR-7045-3p | 1.45 | 0.87 | -1.5 | 0.0207 |
| mmu-miR-6538 | 6.1 | 8.33 | 4.69 | 0.0207 |
| mmu-miR-449b | 1.13 | 0.8 | -1.26 | 0.0209 |
| mmu-miR-6380 | 1.03 | 0.72 | -1.24 | 0.0209 |
| mmu-miR-6934-3p | 0.94 | 0.77 | -1.13 | 0.0214 |
| mmu-miR-6980-5p | 0.96 | 1.31 | 1.28 | 0.0217 |
| mmu-miR-7015-5p | 1.15 | 0.95 | -1.15 | 0.0224 |
| mmu-mir-499 | 0.61 | 0.86 | 1.19 | 0.0226 |
| mmu-miR-717 | 0.61 | 0.71 | 1.08 | 0.0226 |

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|-----------------|------|------|-------|--------|
| mmu-let-7k | 1.12 | 0.85 | -1.21 | 0.0228 |
| mmu-miR-5622-5p | 1.22 | 0.96 | -1.2 | 0.0228 |
| mmu-miR-466f-5p | 0.65 | 1.28 | 1.55 | 0.0229 |
| mmu-miR-338-3p | 0.98 | 0.63 | -1.28 | 0.0231 |
| mmu-miR-505-5p | 1.08 | 0.63 | -1.37 | 0.0232 |
| mmu-miR-6997-3p | 0.85 | 1.03 | 1.13 | 0.0245 |
| mmu-mir-365-1 | 1.07 | 0.86 | -1.16 | 0.0251 |
| mmu-miR-3057-3p | 0.63 | 1.1 | 1.38 | 0.0251 |
| mmu-miR-6951-3p | 0.71 | 0.8 | 1.07 | 0.0258 |
| mmu-mir-1932 | 1.05 | 0.66 | -1.32 | 0.0262 |
| mmu-mir-5108 | 0.9 | 0.64 | -1.2 | 0.0262 |
| mmu-miR-6541 | 0.76 | 0.86 | 1.07 | 0.0264 |
| mmu-mir-5128 | 1.09 | 0.78 | -1.24 | 0.0266 |
| mmu-miR-539-3p | 1.26 | 0.77 | -1.4 | 0.0268 |
| mmu-miR-1306-3p | 0.8 | 1.21 | 1.33 | 0.0271 |
| mmu-mir-453 | 0.65 | 0.9 | 1.19 | 0.0276 |
| mmu-miR-3077-5p | 3.49 | 3.95 | 1.38 | 0.0276 |
| mmu-miR-3082-3p | 0.7 | 1.03 | 1.26 | 0.0276 |
| mmu-mir-361 | 0.79 | 1.03 | 1.18 | 0.0279 |
| mmu-miR-126a-3p | 0.68 | 1 | 1.25 | 0.0281 |
| mmu-miR-149-3p | 4.83 | 6.2 | 2.57 | 0.0282 |

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|-----------------|------|------|-------|--------|
| mmu-miR-30e-3p | 0.8 | 0.97 | 1.13 | 0.0284 |
| mmu-miR-193a-5p | 1.04 | 0.71 | -1.26 | 0.0285 |
| mmu-miR-190a-3p | 3.65 | 1.6 | -4.14 | 0.0288 |
| mmu-mir-219c | 1.21 | 0.93 | -1.21 | 0.0291 |
| mmu-mir-6407 | 0.6 | 0.96 | 1.28 | 0.0291 |
| mmu-miR-7035-5p | 0.78 | 1.06 | 1.22 | 0.0297 |
| mmu-miR-291a-5p | 0.94 | 0.73 | -1.16 | 0.0299 |
| mmu-mir-7684 | 1.34 | 1.69 | 1.27 | 0.0302 |
| mmu-miR-7000-3p | 1.26 | 0.77 | -1.4 | 0.0304 |
| mmu-miR-6378 | 1.07 | 0.64 | -1.34 | 0.0311 |
| mmu-mir-7658 | 1.08 | 0.81 | -1.2 | 0.0316 |
| mmu-miR-7671-3p | 1.56 | 1.17 | -1.31 | 0.0316 |
| mmu-miR-375-3p | 0.87 | 0.53 | -1.27 | 0.032 |
| mmu-miR-7021-3p | 0.74 | 0.64 | -1.08 | 0.0326 |
| mmu-miR-3106-3p | 0.7 | 1 | 1.23 | 0.0326 |
| mmu-mir-669h | 0.66 | 0.91 | 1.19 | 0.0337 |
| mmu-miR-106a-5p | 0.79 | 1.38 | 1.51 | 0.0339 |
| mmu-miR-376a-5p | 0.76 | 0.58 | -1.13 | 0.034 |
| mmu-miR-7056-3p | 0.98 | 0.68 | -1.23 | 0.034 |
| mmu-miR-379-3p | 0.55 | 0.83 | 1.21 | 0.0342 |
| mmu-miR-665-5p | 0.84 | 1.04 | 1.15 | 0.0344 |

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|-------------------|------|------|-------|--------|
| mmu-miR-664-3p | 1.01 | 0.77 | -1.18 | 0.0346 |
| mmu-miR-92a-2-5p | 1.22 | 0.96 | -1.2 | 0.0346 |
| mmu-mir-1900 | 0.59 | 0.97 | 1.3 | 0.0356 |
| mmu-miR-103-1-5p | 0.96 | 0.74 | -1.16 | 0.0357 |
| mmu-mir-669c | 1.01 | 0.71 | -1.23 | 0.0359 |
| mmu-miR-1963 | 0.9 | 0.63 | -1.21 | 0.036 |
| mmu-miR-2861 | 6 | 6.61 | 1.52 | 0.0361 |
| mmu-miR-7649-3p | 0.78 | 0.59 | -1.14 | 0.0367 |
| mmu-miR-6339 | 0.93 | 0.8 | -1.1 | 0.037 |
| mmu-miR-682 | 0.52 | 0.74 | 1.16 | 0.0373 |
| mmu-miR-1258-5p | 1.04 | 0.65 | -1.31 | 0.0373 |
| mmu-miR-6383 | 0.67 | 0.87 | 1.15 | 0.0381 |
| mmu-mir-5621 | 0.66 | 0.95 | 1.22 | 0.0383 |
| mmu-miR-350-3p | 0.85 | 0.75 | -1.07 | 0.0383 |
| mmu-miR-5626-5p | 0.82 | 0.69 | -1.1 | 0.0388 |
| mmu-mir-191 | 1.01 | 0.71 | -1.23 | 0.0391 |
| mmu-mir-668 | 1.15 | 0.74 | -1.33 | 0.0395 |
| mmu-miR-669d-2-3p | 1.17 | 0.87 | -1.23 | 0.0403 |
| mmu-mir-489 | 0.73 | 0.96 | 1.17 | 0.0403 |
| mmu-miR-7045-5p | 3.79 | 5.28 | 2.81 | 0.0404 |
| mmu-mir-3100 | 0.93 | 0.8 | -1.1 | 0.0406 |
| mmu-mir-195a | 0.77 | 1.1 | 1.26 | 0.0408 |

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|-----------------|------|------|-------|--------|
| mmu-mir-3070a | 0.72 | 1.01 | 1.22 | 0.0408 |
| mmu-miR-3473g | 1.22 | 2.47 | 2.37 | 0.0409 |
| mmu-miR-6915-3p | 0.82 | 0.62 | -1.15 | 0.041 |
| mmu-mir-370 | 0.56 | 0.82 | 1.2 | 0.0414 |
| mmu-miR-7658-5p | 1.33 | 1.59 | 1.19 | 0.0418 |
| mmu-miR-6931-5p | 1.81 | 3.68 | 3.65 | 0.0419 |
| mmu-miR-6972-5p | 0.83 | 1.2 | 1.29 | 0.0422 |
| mmu-mir-21c | 1.09 | 0.8 | -1.23 | 0.0423 |
| mmu-mir-7676-1 | 0.98 | 0.84 | -1.1 | 0.0426 |
| mmu-mir-7676-2 | 0.98 | 0.84 | -1.1 | 0.0426 |
| mmu-mir-6901 | 0.71 | 0.94 | 1.18 | 0.043 |
| mmu-miR-7015-3p | 0.69 | 0.91 | 1.16 | 0.0431 |
| mmu-miR-669i | 1.14 | 0.72 | -1.34 | 0.0432 |
| mmu-miR-702-3p | 0.88 | 1.05 | 1.12 | 0.044 |
| mmu-mir-6910 | 0.64 | 0.79 | 1.11 | 0.0441 |
| mmu-mir-684-1 | 0.77 | 0.98 | 1.16 | 0.0442 |
| mmu-mir-684-2 | 0.77 | 0.98 | 1.16 | 0.0442 |
| mmu-miR-764-5p | 0.88 | 0.59 | -1.22 | 0.0443 |
| mmu-mir-8116 | 0.92 | 0.72 | -1.15 | 0.0443 |
| mmu-mir-24-1 | 0.62 | 0.85 | 1.17 | 0.0448 |
| mmu-miR-1983 | 0.96 | 0.65 | -1.23 | 0.0451 |
| mmu-miR-1955-3p | 0.7 | 1.21 | 1.43 | 0.0452 |

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|-----------------|------|------|-------|--------|
| mmu-mir-7650 | 0.58 | 0.93 | 1.27 | 0.0454 |
| mmu-miR-599 | 0.8 | 1.02 | 1.17 | 0.0459 |
| mmu-miR-3103-3p | 0.96 | 0.81 | -1.11 | 0.0473 |
| mmu-mir-467b | 0.58 | 0.87 | 1.22 | 0.0477 |
| gi:555853 | 5.79 | 6.29 | 1.41 | 0.0484 |
| mmu-mir-5129 | 1.12 | 0.77 | -1.27 | 0.0485 |
| mmu-miR-93-5p | 1.41 | 4.79 | 10.44 | 0.0485 |
| gi:555853 | 5.78 | 6.4 | 1.54 | 0.0485 |
| mmu-mir-7117 | 0.87 | 1.05 | 1.14 | 0.0486 |
| mmu-miR-29a-5p | 1.21 | 0.87 | -1.26 | 0.0489 |
| mmu-miR-484 | 0.75 | 1.05 | 1.23 | 0.0496 |

Table 4: List of significant differentially expressed microRNAs in heterozygous SERT, (SERT (+/-)) non-stressed and wild type non-stressed dams at embryonic day 21 (E21). MicroRNAs of interest are indicated in bold.

| MicroRNA | SERT (+/-) Non-Stressed | Wild Type Non-Stressed | SERT (+/-) Non-Stressed vs Wild Type Non-Stressed Fold Change | SERT (+/-) Non-Stressed vs Wild Type Non-Stressed P Value |
|-----------------|--------------------------------|-------------------------------|--|--|
| mmu-let-7c-2 | 0.67 | 1.08 | -1.33 | 0.0004 |
| mmu-mir-7029 | 1.06 | 0.69 | 1.29 | 0.0005 |
| mmu-mir-669h | 1.1 | 0.66 | 1.37 | 0.0008 |
| mmu-mir-6951 | 0.62 | 1.25 | -1.55 | 0.0009 |
| mmu-miR-5128 | 7.21 | 8.14 | -1.91 | 0.0009 |
| mmu-mir-5120 | 1.03 | 0.63 | 1.32 | 0.0011 |
| mmu-mir-182 | 0.59 | 0.9 | -1.23 | 0.0012 |
| mmu-mir-3100 | 0.67 | 0.93 | -1.2 | 0.0022 |
| mmu-miR-378a-5p | 1.04 | 0.67 | 1.29 | 0.0023 |
| gi:555853 | 6.67 | 5.81 | 1.81 | 0.0023 |
| mmu-miR-7234-3p | 1.08 | 0.87 | 1.15 | 0.0024 |
| mmu-mir-15b | 0.98 | 0.62 | 1.28 | 0.0024 |
| mmu-mir-6546 | 1.09 | 0.65 | 1.36 | 0.0025 |
| mmu-miR-8093 | 1.33 | 0.96 | 1.29 | 0.0025 |
| mmu-miR-7221-5p | 0.83 | 0.5 | 1.26 | 0.003 |
| mmu-mir-2136 | 0.63 | 1 | -1.29 | 0.0031 |
| mmu-miR-6906-3p | 1.12 | 0.7 | 1.35 | 0.0032 |
| mmu-mir-7668 | 1.27 | 0.8 | 1.39 | 0.0037 |
| mmu-miR-664-3p | 0.63 | 1.01 | -1.31 | 0.0038 |
| mmu-miR-190a-3p | 1.2 | 3.65 | -5.46 | 0.004 |
| mmu-miR-3091-3p | 1.03 | 0.73 | 1.23 | 0.0041 |

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|-----------------|------|------|-------|--------|
| mmu-miR-3066-5p | 0.68 | 1 | -1.25 | 0.0051 |
| mmu-mir-7027 | 0.73 | 0.95 | -1.16 | 0.0053 |
| mmu-let-7c-2 | 0.68 | 0.93 | -1.19 | 0.0054 |
| mmu-miR-6386 | 0.92 | 0.66 | 1.19 | 0.0054 |
| mmu-miR-379-3p | 0.96 | 0.55 | 1.33 | 0.0057 |
| mmu-miR-7034-5p | 1.13 | 0.73 | 1.32 | 0.0058 |
| mmu-miR-7077-3p | 0.59 | 0.83 | -1.18 | 0.006 |
| mmu-mir-7658 | 0.62 | 1.08 | -1.38 | 0.006 |
| mmu-miR-6546-5p | 0.84 | 0.63 | 1.16 | 0.0061 |
| mmu-miR-496b | 0.78 | 1.13 | -1.27 | 0.0068 |
| mmu-miR-6937-3p | 1.11 | 0.73 | 1.3 | 0.0071 |
| mmu-miR-7050-5p | 1.92 | 1.09 | 1.77 | 0.0071 |
| mmu-mir-6962 | 0.62 | 0.97 | -1.28 | 0.0074 |
| gi:555853 | 6.39 | 5.79 | 1.52 | 0.0079 |
| mmu-mir-6407 | 0.95 | 0.6 | 1.27 | 0.0081 |
| mmu-miR-7028-3p | 0.72 | 1.04 | -1.25 | 0.0082 |
| mmu-mir-7578 | 0.76 | 0.42 | 1.26 | 0.0087 |
| mmu-miR-6998-5p | 1.11 | 0.8 | 1.24 | 0.0092 |
| mmu-mir-7241 | 0.68 | 1.11 | -1.34 | 0.0092 |
| mmu-miR-292b-5p | 0.83 | 1.12 | -1.22 | 0.0093 |
| mmu-miR-297a-5p | 1.53 | 0.9 | 1.55 | 0.0094 |
| mmu-miR-484 | 1.04 | 0.75 | 1.22 | 0.0096 |
| mmu-miR-328-5p | 2.89 | 1.75 | 2.21 | 0.0097 |
| mmu-mir-21c | 0.62 | 1.09 | -1.39 | 0.0099 |

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|-----------------|------|------|-------|--------|
| mmu-mir-7037 | 0.85 | 1 | -1.11 | 0.0101 |
| mmu-miR-188-5p | 1.42 | 0.88 | 1.45 | 0.0102 |
| mmu-miR-6418-3p | 0.79 | 1.09 | -1.23 | 0.0104 |
| mmu-miR-6419 | 0.9 | 0.56 | 1.27 | 0.0108 |
| mmu-let-7k | 0.81 | 1.12 | -1.23 | 0.011 |
| mmu-miR-23a-5p | 0.68 | 1.04 | -1.28 | 0.0113 |
| mmu-mir-1942 | 0.78 | 1.14 | -1.28 | 0.0115 |
| mmu-miR-106a-5p | 1.5 | 0.79 | 1.64 | 0.0118 |
| mmu-miR-7217-3p | 0.78 | 1.01 | -1.17 | 0.0133 |
| mmu-miR-1982-3p | 0.71 | 0.92 | -1.16 | 0.0139 |
| mmu-miR-7679-5p | 0.64 | 0.85 | -1.16 | 0.0139 |
| mmu-miR-3547-5p | 3.29 | 2.18 | 2.15 | 0.0142 |
| mmu-miR-6951-3p | 0.85 | 0.71 | 1.11 | 0.0147 |
| mmu-mir-434 | 1.03 | 0.71 | 1.25 | 0.0148 |
| mmu-miR-1983 | 0.61 | 0.96 | -1.27 | 0.0162 |
| mmu-miR-5108 | 0.8 | 0.53 | 1.2 | 0.0163 |
| mmu-miR-344e-3p | 1.11 | 0.81 | 1.23 | 0.0167 |
| mmu-mir-8120 | 0.95 | 0.66 | 1.23 | 0.0168 |
| mmu-miR-149-3p | 6.04 | 4.83 | 2.32 | 0.0171 |
| mmu-miR-7055-3p | 0.77 | 0.99 | -1.16 | 0.0171 |
| mmu-miR-6948-3p | 0.76 | 0.92 | -1.11 | 0.0174 |
| mmu-mir-6978 | 0.69 | 1.05 | -1.28 | 0.0174 |
| mmu-miR-2137 | 4.76 | 3.43 | 2.52 | 0.0175 |
| mmu-miR-3103-3p | 0.67 | 0.96 | -1.23 | 0.0177 |

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|-----------------|------|------|-------|--------|
| mmu-mir-15a | 0.92 | 0.65 | 1.2 | 0.0178 |
| mmu-mir-344g | 0.84 | 0.59 | 1.19 | 0.018 |
| mmu-miR-7086-5p | 0.69 | 1.04 | -1.28 | 0.0186 |
| mmu-miR-882 | 1.11 | 0.6 | 1.43 | 0.02 |
| mmu-miR-344i | 1.21 | 0.85 | 1.28 | 0.0201 |
| mmu-mir-467b | 0.99 | 0.58 | 1.33 | 0.0202 |
| mmu-miR-7680-3p | 0.65 | 0.86 | -1.16 | 0.0202 |
| mmu-mir-200b | 0.76 | 0.85 | -1.06 | 0.0206 |
| mmu-mir-32 | 0.94 | 0.67 | 1.21 | 0.0207 |
| mmu-miR-300-5p | 0.71 | 1.06 | -1.27 | 0.0208 |
| mmu-mir-7073 | 0.71 | 0.94 | -1.17 | 0.0211 |
| mmu-miR-667-5p | 2.15 | 1.44 | 1.63 | 0.0213 |
| mmu-miR-467d-3p | 0.74 | 1.07 | -1.25 | 0.0227 |
| mmu-miR-299a-5p | 1.08 | 0.89 | 1.14 | 0.0227 |
| mmu-miR-26b-5p | 0.61 | 0.93 | -1.25 | 0.0229 |
| mmu-mir-6410 | 0.88 | 0.78 | 1.07 | 0.023 |
| mmu-mir-6939 | 0.67 | 0.6 | 1.05 | 0.0233 |
| mmu-miR-669e-5p | 1.12 | 0.79 | 1.26 | 0.0236 |
| mmu-miR-6946-3p | 0.68 | 1.01 | -1.26 | 0.0243 |
| mmu-miR-590-5p | 0.75 | 0.98 | -1.17 | 0.0247 |
| mmu-mir-6355 | 0.91 | 0.72 | 1.14 | 0.0251 |
| gi:555853 | 6.3 | 5.78 | 1.43 | 0.0253 |
| mmu-miR-6970-5p | 1.98 | 1.43 | 1.46 | 0.0257 |
| mmu-mir-10a | 0.65 | 0.83 | -1.13 | 0.0258 |

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|-----------------|------|------|-------|--------|
| mmu-mir-5113 | 0.69 | 0.98 | -1.22 | 0.027 |
| mmu-mir-3091 | 1.08 | 0.82 | 1.2 | 0.0271 |
| mmu-miR-3065-5p | 0.89 | 0.7 | 1.14 | 0.0275 |
| mmu-miR-27b-3p | 0.73 | 1.06 | -1.26 | 0.0277 |
| mmu-miR-149-5p | 0.75 | 0.94 | -1.14 | 0.0286 |
| mmu-mir-297a-2 | 0.95 | 0.77 | 1.13 | 0.0287 |
| mmu-miR-3065-3p | 0.84 | 0.66 | 1.13 | 0.0289 |
| mmu-miR-3544-3p | 0.94 | 0.71 | 1.17 | 0.0293 |
| mmu-miR-5627-3p | 0.82 | 1.16 | -1.26 | 0.0293 |
| mmu-miR-5130 | 3.39 | 2.78 | 1.53 | 0.0293 |
| mmu-miR-7230-3p | 0.99 | 0.73 | 1.2 | 0.0295 |
| mmu-miR-7688-3p | 0.57 | 0.92 | -1.27 | 0.0298 |
| mmu-miR-6538 | 8.12 | 6.1 | 4.07 | 0.03 |
| mmu-miR-1839-5p | 1.07 | 0.66 | 1.32 | 0.0303 |
| mmu-mir-7080 | 0.74 | 0.93 | -1.14 | 0.0304 |
| mmu-miR-673-5p | 0.54 | 0.88 | -1.27 | 0.0305 |
| mmu-miR-1298-3p | 0.79 | 0.73 | 1.05 | 0.0308 |
| mmu-mir-1194 | 1.09 | 0.73 | 1.28 | 0.0317 |
| mmu-mir-1264 | 0.67 | 0.86 | -1.14 | 0.0318 |
| mmu-miR-7017-5p | 0.99 | 0.79 | 1.15 | 0.0323 |
| mmu-miR-665-5p | 1.13 | 0.84 | 1.22 | 0.0326 |
| mmu-miR-7214-5p | 0.75 | 0.96 | -1.16 | 0.0328 |
| mmu-miR-466i-3p | 0.86 | 1.06 | -1.14 | 0.0328 |
| mmu-miR-6989-5p | 1.28 | 0.88 | 1.32 | 0.0333 |

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|------------------|------|------|-------|--------|
| mmu-miR-6394 | 0.93 | 0.72 | 1.16 | 0.0336 |
| mmu-mir-3090 | 0.65 | 0.91 | -1.19 | 0.0337 |
| mmu-miR-216b-5p | 0.83 | 1.01 | -1.13 | 0.0341 |
| mmu-mir-1895 | 0.85 | 1.11 | -1.19 | 0.0344 |
| mmu-mir-7654 | 1 | 1.3 | -1.23 | 0.0345 |
| mmu-mir-7067 | 0.79 | 0.52 | 1.2 | 0.0349 |
| mmu-miR-7660-3p | 0.82 | 0.98 | -1.11 | 0.0349 |
| mmu-miR-574-5p | 1.47 | 0.99 | 1.4 | 0.0349 |
| mmu-miR-6936-3p | 0.73 | 0.6 | 1.1 | 0.0357 |
| mmu-miR-6339 | 0.83 | 0.93 | -1.07 | 0.0359 |
| mmu-miR-1895 | 3.21 | 2.24 | 1.95 | 0.0359 |
| mmu-miR-7045-5p | 5.13 | 3.79 | 2.53 | 0.0361 |
| mmu-mir-8090 | 1.04 | 0.81 | 1.17 | 0.0364 |
| mmu-mir-26a-2 | 0.95 | 0.79 | 1.12 | 0.0364 |
| mmu-miR-363-3p | 0.66 | 0.46 | 1.15 | 0.0366 |
| mmu-miR-199a-3p | 0.78 | 0.56 | 1.16 | 0.0366 |
| mmu-miR-199b-3p | 0.78 | 0.56 | 1.16 | 0.0366 |
| mmu-miR-3073b-5p | 0.7 | 0.86 | -1.12 | 0.037 |
| mmu-mir-551b | 1.12 | 0.9 | 1.17 | 0.037 |
| mmu-miR-214-5p | 0.73 | 0.89 | -1.11 | 0.0373 |
| mmu-mir-494 | 1.22 | 0.88 | 1.26 | 0.0374 |
| mmu-miR-1943-3p | 0.7 | 1.02 | -1.25 | 0.0379 |
| mmu-miR-5116 | 0.84 | 1.18 | -1.27 | 0.0379 |
| mmu-miR-7047-5p | 2.77 | 2.12 | 1.57 | 0.0379 |

| | | | | |
|-----------------|------|------|-------|--------|
| mmu-miR-290b-3p | 0.8 | 1.02 | -1.16 | 0.0389 |
| mmu-mir-491 | 0.91 | 0.68 | 1.18 | 0.0395 |
| mmu-miR-190a-5p | 0.75 | 0.54 | 1.15 | 0.0401 |
| gi:555853 | 6.48 | 6 | 1.4 | 0.0401 |
| mmu-mir-7066 | 0.91 | 0.52 | 1.32 | 0.0402 |
| mmu-miR-7070-3p | 0.94 | 0.72 | 1.16 | 0.0407 |
| mmu-miR-449b | 0.82 | 1.13 | -1.25 | 0.0409 |
| gi:555853 | 6.27 | 5.93 | 1.27 | 0.0413 |
| mmu-mir-184 | 0.82 | 0.78 | 1.03 | 0.0414 |
| mmu-miR-6923-5p | 1.12 | 0.81 | 1.24 | 0.0417 |
| mmu-miR-7052-5p | 1.41 | 1.07 | 1.27 | 0.0424 |
| mmu-miR-1934-5p | 0.7 | 1.07 | -1.29 | 0.0426 |
| mmu-miR-7062-3p | 0.48 | 0.81 | -1.26 | 0.0427 |
| mmu-mir-3475 | 0.7 | 0.91 | -1.15 | 0.0431 |
| mmu-miR-8119 | 1.1 | 0.92 | 1.14 | 0.0432 |
| mmu-miR-326-5p | 1.07 | 0.57 | 1.41 | 0.0433 |
| mmu-mir-680-1 | 0.68 | 1 | -1.25 | 0.0434 |
| mmu-mir-6353 | 0.66 | 0.96 | -1.23 | 0.0435 |
| mmu-miR-5113 | 1.12 | 0.82 | 1.23 | 0.0436 |
| mmu-mir-6237 | 1.02 | 0.75 | 1.2 | 0.0437 |
| mmu-mir-98 | 1.06 | 0.63 | 1.35 | 0.0439 |
| mmu-miR-29a-5p | 0.82 | 1.21 | -1.31 | 0.045 |
| mmu-miR-1971 | 2.97 | 1.24 | 3.32 | 0.045 |
| mmu-miR-1967 | 0.86 | 0.73 | 1.1 | 0.0452 |

| | | | | |
|-----------------|------|------|-------|--------|
| mmu-miR-494-3p | 1.56 | 1.13 | 1.35 | 0.0461 |
| gi:555853 | 6.47 | 6.01 | 1.38 | 0.0461 |
| mmu-miR-344c-3p | 0.68 | 1.02 | -1.27 | 0.0464 |
| mmu-miR-1190 | 0.76 | 0.95 | -1.14 | 0.0466 |
| mmu-mir-3473g | 0.89 | 1.15 | -1.2 | 0.0476 |
| mmu-miR-19b-3p | 1.2 | 0.81 | 1.31 | 0.0482 |
| mmu-mir-219c | 0.92 | 1.21 | -1.23 | 0.0485 |
| mmu-mir-6922 | 0.68 | 0.56 | 1.08 | 0.0485 |
| mmu-miR-345-5p | 1.13 | 0.76 | 1.28 | 0.049 |
| mmu-miR-133a-5p | 0.64 | 0.87 | -1.17 | 0.0491 |
| mmu-miR-434-3p | 1.08 | 0.72 | 1.29 | 0.0491 |
| mmu-miR-7a-5p | 0.83 | 1.18 | -1.27 | 0.0492 |
| mmu-mir-486 | 0.85 | 1.08 | -1.18 | 0.0493 |
| mmu-miR-34a-3p | 1.07 | 0.82 | 1.19 | 0.0498 |
| mmu-miR-6971-3p | 0.75 | 0.91 | -1.12 | 0.0498 |
| mmu-miR-532-5p | 0.83 | 0.54 | 1.23 | 0.0499 |

Table 5: List of significant differentially expressed microRNAs in heterozygous SERT, (SERT (+/-)) stressed and wild type stressed dams at embryonic day 21 (E21). MicroRNAs of interest are indicated in bold.

| MicroRNA | SERT (+/-) Stressed | Wild Type Stressed | SERT (+/-) Stressed vs Wild Type Stressed Fold Change | SERT (+/-) Stressed vs Wild Type Stressed P Value |
|-----------------|----------------------------|---------------------------|--|--|
| mmu-miR-7210-3p | 0.68 | 1.17 | -1.4 | 0.0002 |
| mmu-miR-467d-3p | 0.71 | 1.31 | -1.51 | 0.0008 |
| mmu-mir-451a | 0.69 | 0.93 | -1.19 | 0.0013 |
| mmu-miR-133c | 0.62 | 1.04 | -1.34 | 0.0015 |
| mmu-miR-344e-5p | 0.64 | 1.03 | -1.31 | 0.0016 |
| mmu-miR-344h-5p | 0.64 | 1.03 | -1.31 | 0.0016 |
| mmu-miR-1947-3p | 0.84 | 0.65 | 1.14 | 0.0019 |
| mmu-mir-362 | 0.66 | 0.88 | -1.17 | 0.002 |
| mmu-miR-5108 | 0.58 | 0.88 | -1.23 | 0.0021 |
| mmu-miR-669b-5p | 0.98 | 1.44 | -1.37 | 0.0026 |
| mmu-miR-9-3p | 0.72 | 1.05 | -1.26 | 0.0026 |
| mmu-miR-878-5p | 0.61 | 0.94 | -1.26 | 0.0032 |
| mmu-miR-7684-3p | 1.57 | 1.06 | 1.42 | 0.0038 |
| mmu-miR-140-5p | 0.61 | 0.95 | -1.27 | 0.0049 |
| mmu-miR-6966-3p | 0.59 | 0.79 | -1.14 | 0.0052 |
| mmu-miR-376a-5p | 0.81 | 0.58 | 1.17 | 0.0058 |
| mmu-mir-6373 | 1 | 0.7 | 1.23 | 0.0059 |
| mmu-mir-3544 | 0.68 | 1.04 | -1.29 | 0.0059 |
| mmu-mir-873b | 0.85 | 0.68 | 1.13 | 0.006 |
| mmu-mir-6931 | 0.98 | 0.66 | 1.25 | 0.006 |
| mmu-miR-15a-3p | 0.62 | 0.82 | -1.15 | 0.0064 |

| | | | | |
|----------------------|------|------|-------|--------|
| mmu-mir-451a | 0.68 | 0.97 | -1.22 | 0.0066 |
| mmu-mir-5621 | 0.67 | 0.95 | -1.21 | 0.0069 |
| mmu-miR-367-3p | 0.84 | 0.67 | 1.13 | 0.0071 |
| mmu-mir-6918 | 0.53 | 0.95 | -1.33 | 0.0071 |
| mmu-mir-1970 | 0.75 | 0.96 | -1.16 | 0.0076 |
| mmu-mir-2136 | 1.03 | 0.71 | 1.25 | 0.0078 |
| mmu-miR-7051-5p | 0.76 | 1 | -1.18 | 0.0081 |
| mmu-miR-7671-3p | 1.89 | 1.17 | 1.65 | 0.0084 |
| mmu-miR-377-5p | 1.02 | 0.75 | 1.21 | 0.0091 |
| mmu-miR-7680-5p | 0.93 | 0.75 | 1.13 | 0.0098 |
| mmu-mir-7220 | 0.63 | 0.91 | -1.21 | 0.0099 |
| mmu-mir-6372 | 0.97 | 0.7 | 1.21 | 0.0102 |
| mmu-mir-8105 | 0.88 | 1.45 | -1.48 | 0.0102 |
| mmu-miR-290a-3p | 0.95 | 0.75 | 1.15 | 0.0107 |
| mmu-miR-6967-3p | 0.89 | 0.76 | 1.09 | 0.0111 |
| mmu-mir-152 | 0.91 | 0.66 | 1.19 | 0.012 |
| mmu-mir-6396 | 0.76 | 1.03 | -1.2 | 0.0122 |
| mmu-miR-2139 | 0.79 | 0.6 | 1.14 | 0.0127 |
| mmu-mir-875 | 0.68 | 0.86 | -1.13 | 0.0129 |
| mmu-miR-3102-3p.2-3p | 0.81 | 1.12 | -1.24 | 0.0133 |
| mmu-miR-3074-1-3p | 1.04 | 0.66 | 1.3 | 0.0134 |
| mmu-miR-5098 | 0.65 | 0.86 | -1.16 | 0.0139 |
| mmu-miR-6347 | 3.75 | 3.05 | 1.62 | 0.0146 |
| mmu-mir-6348 | 0.96 | 0.75 | 1.16 | 0.0147 |

| | | | | |
|-----------------|------|------|-------|--------|
| mmu-mir-6905 | 0.66 | 0.83 | -1.13 | 0.0154 |
| mmu-miR-6541 | 0.68 | 0.86 | -1.13 | 0.0159 |
| mmu-miR-6358 | 0.86 | 0.67 | 1.14 | 0.016 |
| mmu-miR-7657-3p | 0.62 | 0.67 | -1.04 | 0.0164 |
| mmu-miR-7066-3p | 0.74 | 0.95 | -1.16 | 0.0165 |
| mmu-mir-3070a | 0.69 | 1.01 | -1.25 | 0.0166 |
| mmu-miR-7221-5p | 0.9 | 0.64 | 1.2 | 0.0167 |
| mmu-mir-7660 | 0.97 | 0.71 | 1.2 | 0.0177 |
| mmu-miR-5622-3p | 1.65 | 1.14 | 1.43 | 0.0177 |
| mmu-mir-7117 | 0.89 | 1.05 | -1.12 | 0.018 |
| mmu-miR-7056-3p | 0.83 | 0.68 | 1.11 | 0.0183 |
| mmu-miR-6370 | 0.74 | 1.02 | -1.21 | 0.0185 |
| gi:555853 | 5.98 | 6.44 | -1.38 | 0.0185 |
| mmu-miR-7676-5p | 0.62 | 1.21 | -1.51 | 0.0188 |
| mmu-miR-6904-5p | 0.48 | 1.03 | -1.46 | 0.0188 |
| mmu-miR-344c-5p | 1.25 | 0.95 | 1.24 | 0.0189 |
| mmu-mir-690 | 0.93 | 1.19 | -1.2 | 0.0189 |
| mmu-miR-211-3p | 0.84 | 1.5 | -1.58 | 0.0189 |
| mmu-miR-5625-3p | 1.05 | 0.61 | 1.35 | 0.0193 |
| mmu-miR-6995-3p | 0.98 | 0.75 | 1.17 | 0.0194 |
| mmu-miR-6992-5p | 0.96 | 0.8 | 1.12 | 0.0194 |
| mmu-miR-6934-3p | 1.06 | 0.77 | 1.23 | 0.0199 |
| mmu-miR-1931 | 4.36 | 3.75 | 1.53 | 0.0201 |
| mmu-miR-7044-5p | 2.01 | 3.06 | -2.07 | 0.0211 |

| | | | | |
|------------------|------|------|-------|--------|
| mmu-mir-758 | 0.95 | 0.7 | 1.19 | 0.0211 |
| mmu-mir-8113 | 1.13 | 0.88 | 1.19 | 0.0214 |
| mmu-miR-683 | 0.98 | 0.71 | 1.21 | 0.0228 |
| mmu-mir-28b | 1.33 | 0.95 | 1.29 | 0.0228 |
| mmu-miR-29c-3p | 0.83 | 0.62 | 1.16 | 0.0229 |
| mmu-mir-3063 | 0.89 | 0.71 | 1.13 | 0.0231 |
| mmu-miR-493-3p | 0.77 | 1.03 | -1.2 | 0.0233 |
| mmu-miR-568 | 0.99 | 0.87 | 1.09 | 0.0235 |
| mmu-miR-129-1-3p | 1.06 | 0.7 | 1.29 | 0.0236 |
| mmu-miR-6920-5p | 1.12 | 0.73 | 1.31 | 0.0238 |
| mmu-mir-3095 | 0.95 | 0.79 | 1.11 | 0.0244 |
| mmu-mir-3109 | 0.69 | 0.95 | -1.2 | 0.0247 |
| mmu-mir-3085 | 1.2 | 0.77 | 1.35 | 0.0247 |
| mmu-mir-1931 | 0.92 | 0.72 | 1.15 | 0.0247 |
| mmu-mir-1247 | 0.86 | 1.15 | -1.23 | 0.0254 |
| mmu-mir-23a | 0.89 | 1.08 | -1.15 | 0.0266 |
| mmu-mir-590 | 0.95 | 0.68 | 1.21 | 0.0277 |
| mmu-miR-8097 | 1.16 | 0.89 | 1.21 | 0.0278 |
| mmu-miR-6380 | 0.99 | 0.72 | 1.2 | 0.0282 |
| mmu-mir-7052 | 0.78 | 1.01 | -1.17 | 0.0288 |
| mmu-miR-361-5p | 0.65 | 1.08 | -1.34 | 0.0289 |
| mmu-mir-6901 | 0.7 | 0.94 | -1.18 | 0.029 |
| mmu-miR-5128 | 7.63 | 7.29 | 1.26 | 0.0295 |
| mmu-miR-8107 | 1.85 | 1.24 | 1.53 | 0.0298 |

| | | | | |
|------------------|------|------|-------|--------|
| mmu-miR-344-5p | 0.63 | 0.98 | -1.27 | 0.0301 |
| mmu-miR-381-5p | 0.96 | 0.71 | 1.19 | 0.0302 |
| mmu-mir-5135 | 1.16 | 0.84 | 1.25 | 0.0303 |
| mmu-miR-3110-3p | 0.61 | 0.95 | -1.27 | 0.0306 |
| mmu-miR-195a-5p | 0.66 | 0.99 | -1.26 | 0.0307 |
| mmu-mir-497 | 1.09 | 0.87 | 1.16 | 0.0308 |
| mmu-miR-669f-5p | 1.04 | 1.42 | -1.3 | 0.0309 |
| mmu-miR-3070a-5p | 1.24 | 1 | 1.19 | 0.032 |
| mmu-miR-3070b-5p | 1.24 | 1 | 1.19 | 0.032 |
| mmu-miR-7210-5p | 0.98 | 0.82 | 1.12 | 0.033 |
| mmu-mir-7056 | 0.82 | 1.01 | -1.14 | 0.0338 |
| mmu-miR-6933-5p | 1.05 | 0.66 | 1.31 | 0.034 |
| mmu-miR-130b-3p | 0.74 | 1.13 | -1.3 | 0.0342 |
| mmu-let-7i-3p | 0.65 | 1.01 | -1.29 | 0.0344 |
| mmu-miR-7028-5p | 0.86 | 1.25 | -1.31 | 0.0347 |
| mmu-mir-7688 | 0.8 | 0.7 | 1.07 | 0.0352 |
| mmu-mir-876 | 0.71 | 0.99 | -1.22 | 0.0356 |
| mmu-mir-378d | 0.66 | 0.94 | -1.21 | 0.0356 |
| mmu-miR-3095-3p | 0.91 | 0.71 | 1.15 | 0.0364 |
| mmu-miR-767 | 0.63 | 0.91 | -1.22 | 0.0366 |
| mmu-miR-105 | 0.65 | 0.92 | -1.2 | 0.0369 |
| mmu-mir-222 | 0.6 | 0.8 | -1.15 | 0.0369 |
| mmu-miR-5623-3p | 0.7 | 0.63 | 1.05 | 0.0371 |
| mmu-miR-7035-3p | 0.99 | 0.7 | 1.23 | 0.0375 |

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|---------------------|-------|-------|-------|--------|
| mmu-miR-6420 | 1.25 | 0.81 | 1.35 | 0.0386 |
| mmu-miR-7685-5p | 0.75 | 1.23 | -1.4 | 0.0388 |
| mmu-miR-21b | 1.05 | 0.79 | 1.2 | 0.0389 |
| mmu-miR-1a-1-5p | 0.96 | 0.73 | 1.17 | 0.0391 |
| mmu-miR-6900-3p | 1.22 | 0.71 | 1.42 | 0.0393 |
| mmu-miR-467e-5p | 0.99 | 0.78 | 1.16 | 0.0393 |
| spike_in-control-36 | 13.45 | 14.28 | -1.77 | 0.0394 |
| mmu-miR-1943-5p | 0.67 | 0.85 | -1.13 | 0.0395 |
| mmu-miR-98-3p | 0.7 | 0.85 | -1.11 | 0.0396 |
| mmu-miR-106a-5p | 0.79 | 1.38 | -1.51 | 0.0399 |
| mmu-let-7j | 0.74 | 0.84 | -1.07 | 0.0408 |
| mmu-mir-7053 | 0.85 | 1.09 | -1.18 | 0.0419 |
| mmu-miR-7660-5p | 0.89 | 0.66 | 1.17 | 0.0421 |
| mmu-miR-28c | 0.59 | 0.78 | -1.14 | 0.0422 |
| mmu-mir-135b | 0.89 | 0.82 | 1.05 | 0.0429 |
| mmu-mir-8110 | 0.71 | 0.94 | -1.18 | 0.043 |
| mmu-mir-3074-1 | 0.79 | 0.91 | -1.09 | 0.043 |
| mmu-miR-7059-3p | 0.55 | 0.79 | -1.18 | 0.0432 |
| mmu-mir-1950 | 0.74 | 0.98 | -1.18 | 0.0433 |
| mmu-miR-695 | 1.04 | 0.64 | 1.32 | 0.0438 |
| mmu-mir-489 | 0.76 | 0.96 | -1.15 | 0.044 |
| mmu-mir-29b-2 | 1.06 | 0.67 | 1.31 | 0.0442 |
| mmu-mir-465c-1 | 2.26 | 3.12 | -1.81 | 0.0446 |
| mmu-mir-465c-2 | 2.26 | 3.12 | -1.81 | 0.0446 |

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|-----------------|------|------|-------|--------|
| mmu-miR-7015-5p | 1.12 | 0.95 | 1.13 | 0.0446 |
| mmu-mir-1906-1 | 0.83 | 0.9 | -1.05 | 0.0448 |
| mmu-mir-1906-2 | 0.83 | 0.9 | -1.05 | 0.0448 |
| mmu-mir-6936 | 0.84 | 0.72 | 1.08 | 0.0448 |
| mmu-miR-688 | 0.99 | 0.58 | 1.33 | 0.0452 |
| mmu-miR-7014-5p | 0.85 | 1.2 | -1.27 | 0.0459 |
| mmu-miR-6941-5p | 0.87 | 1.21 | -1.27 | 0.0463 |
| mmu-miR-449a-5p | 0.89 | 0.75 | 1.1 | 0.0464 |
| mmu-mir-3100 | 0.87 | 0.8 | 1.05 | 0.047 |
| mmu-miR-92b-3p | 0.75 | 1.06 | -1.24 | 0.0472 |
| gi:555853 | 5.8 | 6.4 | -1.52 | 0.0475 |
| mmu-miR-181a-5p | 1.01 | 0.81 | 1.16 | 0.0479 |
| mmu-mir-92a-2 | 0.76 | 1.09 | -1.26 | 0.0493 |
| mmu-miR-6952-3p | 0.88 | 0.69 | 1.14 | 0.0493 |

VITA

Candice King is the only child of Gloria and Godfrey King, born in the island republic of Trinidad and Tobago, where her parents supported her love of science and biology from an early age.

Candice emigrated to the United States and settled in Brooklyn, New York where she graduated from Prospect Heights High School. She later attended and earned her associate and bachelor's degrees in biology from Kingsborough Community College and Medgar Evers College, respectively. Her undergraduate research focused upon establishing the American oyster, *Crassostrea virginica*, as a model organism for Parkinson's Disease and demonstrating the efficacy of p-Aminosalicylic acid on manganese neurotoxicity, dopamine and serotonin levels in *C. virginica*. After which, she attended the University of Missouri-Columbia and earned her Doctor of Philosophy in Biological Sciences in the lab of Dr. David Q. Beversdorf. During her studies, she investigated the effects of the combination of maternal genetic stress susceptibility and prenatal stress exposure on maternal epigenetic profiles and offspring behavior.