Abstract

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that is characterized by social and communication impairments and restricted repetitive behaviors (American Psychiatric Association, 2013). While ASD is based exclusively on behavioral symptomology, many reports note immune dysregulation as a common underlying feature. These immune alterations are thought to begin early in fetal development and population-based studies suggest a link between ASD severity and maternal incidence of allergies and asthma. This project examined the effects of maternal immune activation, via a maternal allergic-asthma (MAA) model, in conjunction with offspring asthma on severity of ASD-like behaviors in mice. It was hypothesized that MAA offspring exposed to repeated asthma inductions throughout juvenile development would exhibit more severe ASD-like behaviors. To test this, female C57BL/6J (C57) mice were sensitized to the egg protein ovalbumin, OVA, or phosphate buffered saline, PBS, and pregnant females were exposed to either aerosolized OVA (i.e. MAA) or PBS- vehicle (i.e. Control), respectively, throughout gestation to elicit allergic-asthma episodes. Following parturition, mice from both MAA and Control dams were randomly assigned to undergo either subsequent OVA sensitizations and inductions or be exposed to PBS sensitizations and inductions. The resulting four groups, (Control-PBS, Control-OVA, MAA-PBS, and MAA-OVA) were then assessed for ASD-like behaviors following acute asthma inductions using a series of well-validated behavioral tasks. Results indicated that allergic-asthma exposure, either during gestation or in the juvenile period, elicited species atypical behaviors in social and anxiety-associated tasks.

Asthma-induced ASD-like Behaviors in Offspring of Maternal Allergic-Asthma Dams: A Model of the Double Hit Hypothesis

by

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A Thesis

Presented to the Faculty of Mount Holyoke College

in partial fulfillment of the requirements

for the Bachelor of Arts

with Honors

Departments of Neuroscience & Behaviors and Psychology & Education

Mount Holyoke College

South Hadley, Massachusetts

Acknowledgements

To *Jared Schwartzer, Ph.D.,* words cannot even begin to explain how thankful I am for all you have done for me over the past three years, but I am going to try. When I first entered your lab, I was shy and insecure, but thanks to your constant encouragement and support I have done something I never thought I could… written a thesis. Thank you for always going above and beyond to help me and all of your other advisees. You have shown me the qualities a true leader and good mentor has, and I will carry that with me for the rest of my life. From now on, when I look for advisors, they will all be in comparison to you. In sum, being a part of your lab has been the best and most rewarding part of my Mount Holyoke experience and I cannot (and do not) want to imagine my time here without it. Thank you for allowing me to be in your lab for three years, it has changed my life.

To *Katie McMenimen Ph.D.*, thank you for being on my thesis committee. Chemistry has always been a very challenging subject for me, so I was really nervous to take Biochemistry. However, you made the class fun and interesting and you were one of the best professors I have had at Mount Holyoke. Thank you for being one of my advisors.

To *Mara Benjamin, Ph.D.*, thank you for supporting me during this journey. Even though I only met you last semester, I felt like we connected instantly. You encouraged me to begin conversations that I normally shy away from and I appreciate your support during that time more than you will ever know. Thank you again for being on my thesis committee and serving as one of my last advisors at Mount Holyoke.

To *Jamie Church, Ph.D.*, thank you for always being in my corner. I am so happy that you joined the Schwartzer lab last year because I truly cannot imagine my senior year without you. From doing work together in your office to getting doughnuts over J-Term, your support and friendship means so much to me. I will always remember all of the laughs and good times we had together. And most importantly, thank you for showing me what it means to be a strong and confident woman in science, I admire you more than you know.

To *Felicity Emerson, Morgan Coburn, and Megan Johnson*, thank you for being the best lab mates a girl could ask for during the first two years of my time in the Schwartzer lab. Thank you for teaching me everything you knew about lab and being patient with me while I learned. You made lab fun and fostered an environment that made me even more excited about neuroscience. Even though we were not together during my last year of lab, I appreciate that you all checked in, asked how my thesis was going, and encouraged me along the way.

To *Fiona Chace-Donahue*, thank you for constantly supporting me and for always being someone I could talk to about lab things. Thank you for always being willing to come into lab and help me, even when it meant waking up early when you did not have to. Thank you for believing in me when I didn't believe in myself. And most importantly, thank you for your friendship.

To *Rebecca Goodier, Kerry Ouimette, Jordan Green, Laura Restrepo, and Aurora Aldama,* thank you for listening to my presentations in lab meetings and giving me feedback. Thank you for your intellectual questions that challenged me to think in different ways. Thank you for allowing me to teach you and being patient with me while I learned which teaching styles worked best for me. Lastly, thank you for your constant support during this year.

To *Suz Rose,* thank you for being the best thesis buddy around. I always looked forward to writing my thesis because it meant spending time with you. I am so proud of you for all that you have accomplished, and I am glad that we got to write our theses together. Thank you for listening to my presentation, answering my questions, and being an all-around wonderful person.

To *Cheryl, Janelle, and the Thesis 399 Class*, thank you for your support during this process. I enjoyed listening to your highs and lows and I appreciate you listening to mine. Thank you for all of your feedback and guidance.

To *Lenny Beliveau, Emily Bernstein, Irisa Lee, Lindsey McGinnis, Alice Richardson, and Deme Osei Tutu,* thank you for being my best friends at Mount Holyoke. You turned college into a second home and I could not have made it through school without all of you. Thank you for always encouraging me to pursue my dreams, sharing moments of success and failure, and always being down to get ice cream.

To *Mommy, Mama, and Michael*, thank you for everything you have done for me. I love you all more than anything in the world and I would not be where I am today without you. Thank you for pretending to understand my research and supporting me in all of my endeavors. Thank you for encouraging me to follow my dreams and never giving up on me when I chase them. Most importantly, thank you for always being there for me and loving me unconditionally.

To the *Harap Family*, thank you for your financial support. Your contributions helped to make this project a reality and I appreciate your generosity.

To the *Mice*, thank you for sacrificing your lives. I will never forget the sacrifices you made, and I promise to never take them for granted.

Table of Contents

List of Tables

List of Figures

Introduction

Autism Spectrum Disorders

Overview. Autism spectrum disorder (ASD) is a neurodevelopmental disorder that is characterized by social and communication impairments and restricted repetitive behaviors (American Psychiatric Association, 2013). ASD affects 1 in 59 children, making it the fastest growing developmental disorder in the United States (Center for Disease Control). ASD is 4.5 times more common in boys than girls and is reported to affect all racial, ethnic, and socioeconomic groups (Christensen, Baio, Braun, Bilder, Charles, & Constantino, et.al., 2016). Typically, symptoms of ASD begin by the age of six months, are established in two to three years, and persist throughout life (American Psychiatric Association, 2013). These individuals often require increased care in the form of both medical and behavioral interventions. It was estimated that the total cost per year for children with ASD in the United States was between 11.5 billion- 60.9 billion (US) dollars (Lavelle, Weinstein, Newhouse, Munir, Kuhlthau, & Prosser, 2014), which underscores the significant economic burden of ASD and the need to examine the underlying causes of ASD. While the specific etiology of ASD remains unknown, recent studies suggest that autism emerges from a confluence of genetic by environmental factors (Croen, Grether, Yoshida, Odouli, & Van de Water, 2005; Marchetto, Carromeu, Acab, Yu, Yeo, Mu, & Muotri, 2010; Sadin, Lichtenstein, Kuja-Halkola, Larsson, Hultman, & Reichenberg, 2014). Therefore, in order to understand the complexity of ASD, it is essential to understand both the genetic and environmental components of the disorder.

Genetic Factors Influencing Autism. Autism is one of the most heritably complex genetic disorders in psychiatry (Ma, Whitehead, Menold, Martin, Ashley-Koch, Mei, et.al., 2005). The majority of ASD cases are idiopathic, with only 20% of cases estimated to be attributed to a known genetic mutation or chromosomal copy number variations (Boddaert, Zilbovivius, Philipe, Robel, Bourgeois, Barthélemy, & Bahi-Buisson, 2009; Gillberg & Coleman, 1996; Pinto, Pagnamenta, Klei, Anney, Merico, Regan, & Almedia, 2010; Sebat, Lakshmi, Malhotra, Troge, Lese-Martin, Walsh, & Leotta, 2007). In order to identify autism-susceptibility genes studies have examined both direct mapping approaches, namely chromosomal methods and linkage, and indirect mapping approaches, such as the characterization of Rett syndrome (RTT) and fragile X syndrome which are less genetically complex (Ma, et.al., 2005).

In addition to ASD cases with known genetic variants (e.g. Fragile X syndrome and Rett syndrome), family history studies suggest that ASD might be caused by a confluence of genetic factors. Previous studies show that siblings of ASD individuals are at an approximately 25-fold higher risk of developing autism compared to the population, and twin studies show that there is a concordance of the autism phenotype in 20-30% of dizygotic twins and 60% of monozygotic twins (Geschwind, 2013; Hallmayer, Cleveland, Torres, Phillips, Cohen, & Torigoe, 2011). Another study examined more than 2 million families to evaluate the familial risk of ASD and estimated that genetic and non-genetic factors contributed equally to ASD susceptibility (Sandin, et.al., 2014). Using relative recurrence risk measures, Sandin et. al. 2014 estimated a 50% heritability for ASD, indicating that genetic factors explained only half the risk for autism. Considering that the monozygotic concordance is only 50-60% and not 100%, it is clear that genetics cannot be the only factor influencing the development of ASD. Together, these previous population-based studies underscore the important contribution of non-genomic factors that

contribute to the etiology of ASD. Therefore, research must expand beyond examining genetic factors and consider environmental influences as well.

Environmental Factors Influencing Autism. Environmental influences play a substantial role in the etiology of ASD highlighting the need to better characterize the myriad environmental factors implicated in neurodevelopmental disorders. Numerous studies point to the maternal environment, specifically the environment *in utero*, as a significant factor contributing to the incident of ASD (Dufour-Rainfray, Vourch'h, Tourlet, Guilloteau, Chalon, & Andres, 2011; Kinney, Munir, Crowley, & Miller, 2008; Newschaffer, Croen, Daniels, Giarelli, Grether, Levy, et.al., 2007). Epidemiological studies note that the risk of an ASD diagnosis in offspring is increased when mothers are exposed to air pollutants associated with freeways during gestation (Raz, Roberts, Lyall, Hart, Just, Laden, et.al., 2015; Volk, Hertz-Picciotto, Delwiche, Lurmann, McConnell, 2011). Moreover, exposure to environmental toxins (ET) have also been found to increase the risk of having a child diagnosed with ASD (Kim, Han, Lyoo, Min, Kim, & Renshaw, 2010). These toxins, both air pollutants and industry chemicals, are often teratogenic and may disrupt the normal function of cells and structures in the nervous, endocrine, and immune systems of the mother and developing fetus (Kim, et.al., 2010). In addition, environmental pollutants are hypothesized to impart epigenetic modifications that produce lasting changes in offspring development—a process known as developmental fetal programming (Kim, et.al., 2010). Air pollutants and environmental toxins activate the maternal immune system which can have neurodevelopmental consequences for the developing fetus. These associations between air pollution, chemical toxicants, and autism risk point to the maternal immune system as an underlying mechanism linking various environmental factors to

developmental deficits. As a result, research efforts are converging on the immune responses in search of signaling molecules that may impact offspring neurodevelopment.

Immune Activation

Maternal Immune Activation. Maternal immune activation (MIA) has been found to impact the neurodevelopment of the fetus as studies support that the activation of a mother's immune system during pregnancy may result in a higher risk of the offspring developing ASD (Malkova, Yu, Hsiao, Moore, & Patterson, 2012; Patel, Masi, Dale, Whitehouse, Pokorski, Alvares, et.al., 2017). In a nationwide study in Denmark, more than 20,000 mothers were hospitalized during pregnancy because of infection, and these infections were associated with a significantly increased probability of the child developing ASD (Atladóttir, Thorsen, østergaard, Schendel, Lemcke, Abdallah, et.al., 2010). These clinical findings highlight that the activation of the mother's immune system may influence the behavior of the offspring and give rise to the notion that biological mechanisms responsible for maternal immune inflammation may influence neurodevelopment in the fetal brain (Figure 1).

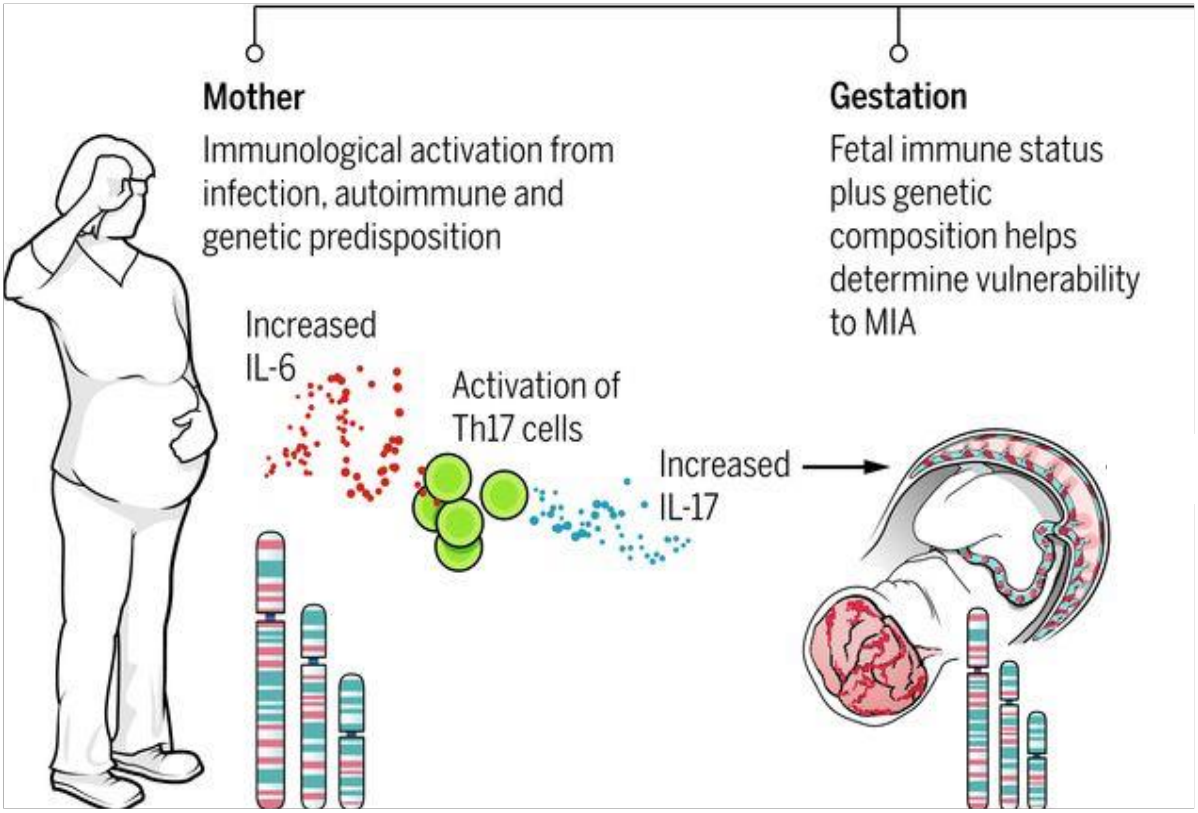


Figure 1: Maternal immune activation influencing fetal neurodevelopment. Adapted from Estes et.al., 2016.

Mothers who have allergies and asthma during pregnancy may also be at an increased risk for having a child diagnosed with ASD. A population-based study found that maternal asthma and allergies during pregnancy were associated with more severe autism-related symptoms, but autoimmune conditions had no effect on ASD severity (Patel, et.al., 2017). Though some environmental factors can be mitigated, for example neurotoxic chemicals, other influences (i.e. asthma, allergies, and illnesses) are on the rise and cannot be easily avoided. These correlational findings suggest a need to determine the connection between maternal immune activation during gestation and the increased risk of having a child later diagnosed with ASD.

Immune activation is characterized by the release of pro-inflammatory signals (i.e. cytokines), and recent research indicates that immune molecules are present in various tissue across the maternal-fetal axis, specifically the placenta, the amniotic fluid, and the fetal brain (Meyers, Feldon, & Dammann, 2011). Importantly, pre-clinical studies have demonstrated that in the absence of viral or bacterial infections, prenatal exposure to pro-inflammatory agents is sufficient to produce long lasting brain and behavioral dysfunction in offspring reminiscent of behavioral features associated with schizophrenia and ASD (Meyer, et.al., 2011). These studies suggest that the immune signaling molecules rather than the pathogen itself, are responsible for changes in fetal neurodevelopment. Taken together, these clinical and pre-clinical findings underscore the role prenatal cytokine exposure plays in meditating the effects of MIA on offspring.

Offspring Immune Dysregulation. In addition to the maternal immune environment, altered immune regulation in the offspring may also contribute to ASD severity given that many

children with ASD are reported to have dysregulated immune responses (Ashwood, Wills, & Van de Water, 2006; Ashwood, Krakowiak, Hertz-Picciottto, Hansen, Pessah, & Van de Water, 2011; Corcoran, Berry, & Hill, 2015). Several studies note immune-related co-morbidities associated with autism, including allergies, asthma, and skin disorders such as eczema and atopic dermatitis (Akintude, Rose, Krakowiak, Heuer, Ashwood, Hansen, et.al., 2015; Kotey, Etal, & Whitcomb, 2014). Further, multiple studies have shown that children with ASD have altered cytokine/chemokine profiles when compared with typically developing children (Ashwood, et.al., 2006, Careaga, Van de Water, & Ashwood, 2010; Onore, Careaga, & Ashwood, 2011), suggesting that the offspring immune system, in addition to the maternal immune profile, may be a factor contributing to the ASD phenotype. Importantly, asthma is characterized by imbalances in inflammatory processes and represents another branch of immune dysregulation. These similarities in immune-associated risks, in conjunction with the increases in prevalence of ASD and asthma, underscore a plausible common etiology (Becker & Schultz, 2010). To this end, a closer examination of the various immunological processes is warranted.

Immunology

Overview. The immune system has evolved to combat various pathogens and foreign invaders by developing complimentary branches of immunity that work together to protect and adapt to various environmental insults. These branches of immunity, namely the innate (natural) immune system and the adaptive (acquired) immune system, work together to defend the body against pathogenic invasion through a cascade of signaling molecules that recruit and activate immune-specific cell types (Reese & Campbell, 2011).

Innate Immunity. The innate immune system is active immediately upon encountering an infection and functions the same regardless of whether the pathogen has been encountered previously (Reese & Campbell, 2011). Although the body is protected by an outer covering (i.e. the skin) pathogens are still able to breech the barrier defense because the body needs to interact with the environment for gas exchange, nutrition, and reproduction. In a properly functioning immune system, the organism's immune system has the ability to recognize self from non-self so that immune cells can differentiate invaders and exclusively target foreign cells or viruses, a process known as molecular recognition.

Activation of the innate immune system by a foreign pathogen results in the release of a cascade of immune signaling molecules known as cytokines (Figure 2). These cytokines are crucial for recruiting additional immune cells to the site of infection where they work in concert to break down pathogens, initiate apoptosis in infected cells, and restore healthy tissue function. Importantly, while the innate system is working to kill the antigen, the adaptive immune system is also activated to educate the immune system to future encounters with the pathogen.

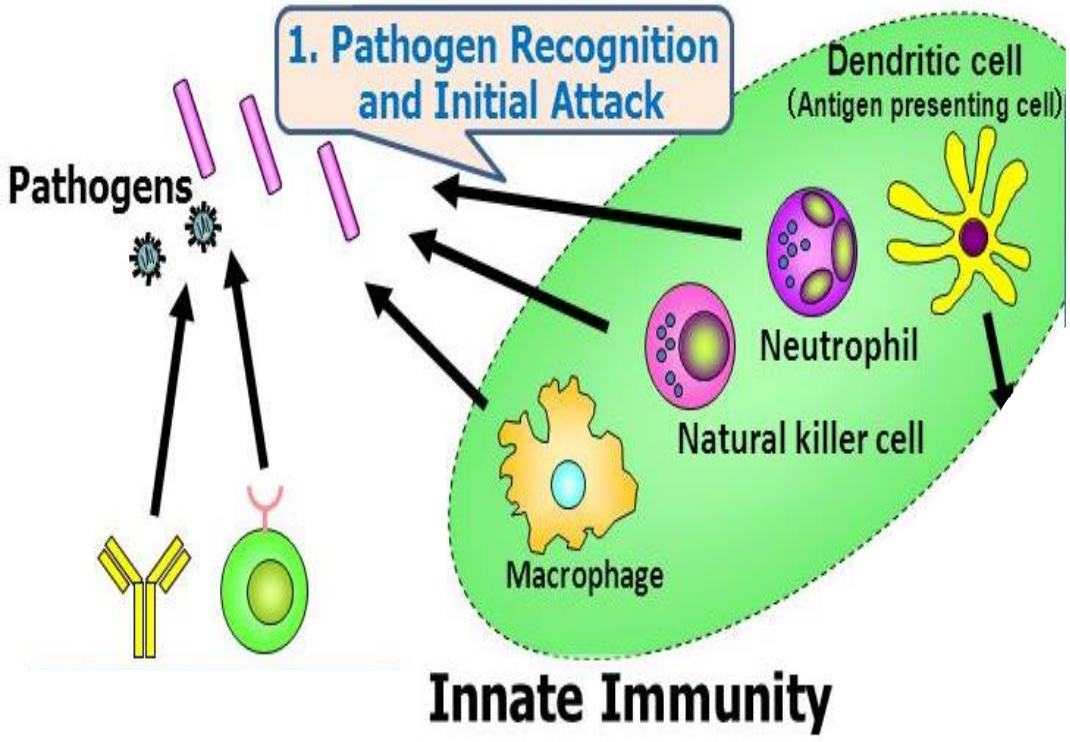


Figure 2: Innate Immunity. Adapted from Akira, et.al., 2011.

Adaptive Immunity. The adaptive immune system has a delayed activation and is another form of molecular recognition. While the innate immune system is working to kill the virus or disease, the adaptive immune system is creating a memory in order to enhance immune responses upon future encounters with the same pathogen. Many of the cytokines released during innate immune activation also recruit antigen presenting cells including CD4 positive T lymphocytes to the site of the infection where they serve to gather pathogen-specific antigens to present to memory forming B lymphocytes. Activation of these B cells results in cell replication and production of antibodies specific for the pathogen. By creating these memory cells, the immune system is preparing itself for another encounter with the pathogen. Specifically, the next time the antigen is present in the body, immune-mediated factors can immediately recognize the antigen and recruit the existing antibodies to destroy the pathogen (Figure 3). This rapid and immediate response inhibits the returning pathogen from infecting host cells and preventions. While adaptive immunity is vital for survival, the past century has seen an emergence of over activation of these adaptive immune responses that are producing deleterious health outcomes for many individuals, most notably through the rises in asthma and allergies. Allergic-asthma is characterized by the release of inflammatory factors from both innate and adaptive immune branches in response to innocuous stimuli. This immune hypersensitivity results in vascular permeability, smooth muscle contraction, and mucus production in the lungs as well as more global cytokine elevations in the peripheral immune system (Galli, Tsai, & Philiponsky, 2008).

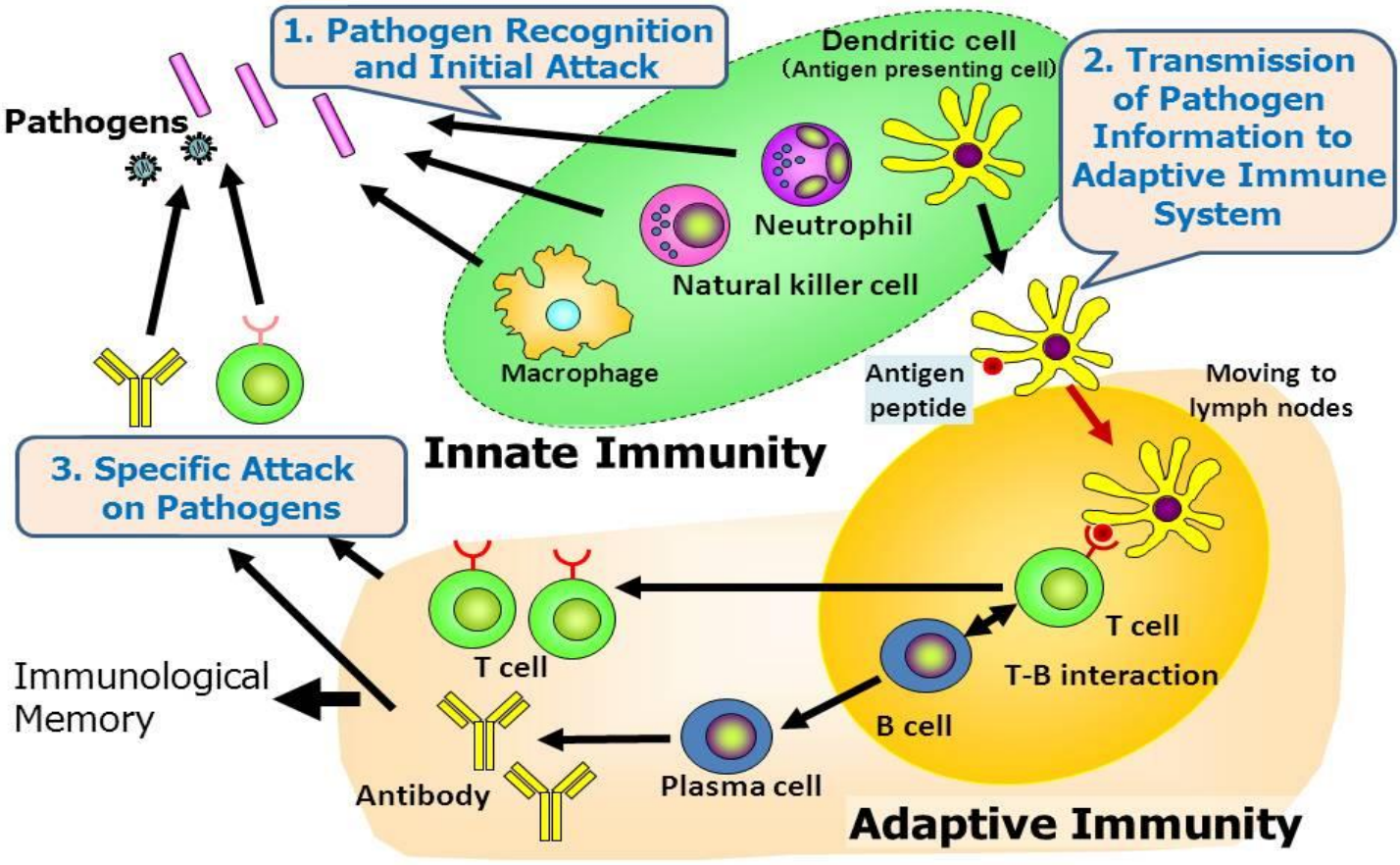


Figure 3: Innate and adaptive immunity. Adapted from Akira, et.al., 2011.

Asthma as an Immunological Factor Influencing Autism. Asthma, the second leading cause of child hospitalization in the United States (Kotey, et.al., 2014), is characterized by inflammation in the airways that results in wheezing, breathlessness, and a tight chest (Akintude, et.al., 2015). Asthma is more common in boys under the age of 18 when compared to girls of the same age range (Center for Disease Control), a trend that parallels the higher male to female ratio associated with the ASD diagnosis. This association raises questions around potential links between asthma and ASD severity. However, the relationship between ASD and asthma remains inconclusive. Akintude et.al. 2015 found that asthma is 35% more common in individuals with autism compared to typically developing individuals, whereas a similar epidemiological report concluded that food allergies and sensitivities are more common in ASD children compared to asthma (Lyall, Van De Water, Ashwood, & Hertz-Picciotto, 2015). Conversely, Zheng and colleagues found that there was no association between asthma and autism (Zheng, Zhang, Zhu, Huang, Qu, & Mu, 2016). These contradictory findings underscore the need to better understand the causal links between autism and asthma and suggest there may be additional factors that contribute to the etiology of ASD, specifically the combined influence of both maternal and offspring immune system dysregulation.

Second Hit Hypothesis

Overview. Given that the heritability of ASD is only 50-60% in monozygotic twins, the notion that environmental factors (i.e. air pollutants and toxins) influence immune signaling across the maternal-fetal axis, and the associations between both maternal and offspring immune activation via asthma and offspring neurodevelopment, it can be hypothesized that these seemingly unique

factors may work together to influence offspring neurodevelopment. It has been proposed that neurodevelopment is not influenced by a single mechanism but rather by multiple environmental and genetic factors that must co-occur to elicit neurodevelopmental deficits. Proposed in 1999 to explain the origins of schizophrenia, the second hit hypothesis attributes both genetic factors and non-genetic factors to the onset of schizophrenia (Bayer, Falkai, & Maier, 1999). Specifically, it is postulated that neuropsychiatric disorders emerge from an initial mutation to a gene that leads to the malfunction of a neural network (first hit) followed by a second hit, such as an environmental stimulus (i.e. infection) that provokes the mutant gene leading to the psychotic illness (Bayer, et.al., 1999) (Figure 4). More recently, the second hit hypothesis has expanded to include epigenetic modifications, in addition to de novo mutations, as an initial source of susceptibility. Therefore, the second hit hypothesis may also serve as a model for neuropsychiatric disorders and neurodevelopmental disorders broadly in that it accounts for a confluence of factors that interact to influence brain development and function.

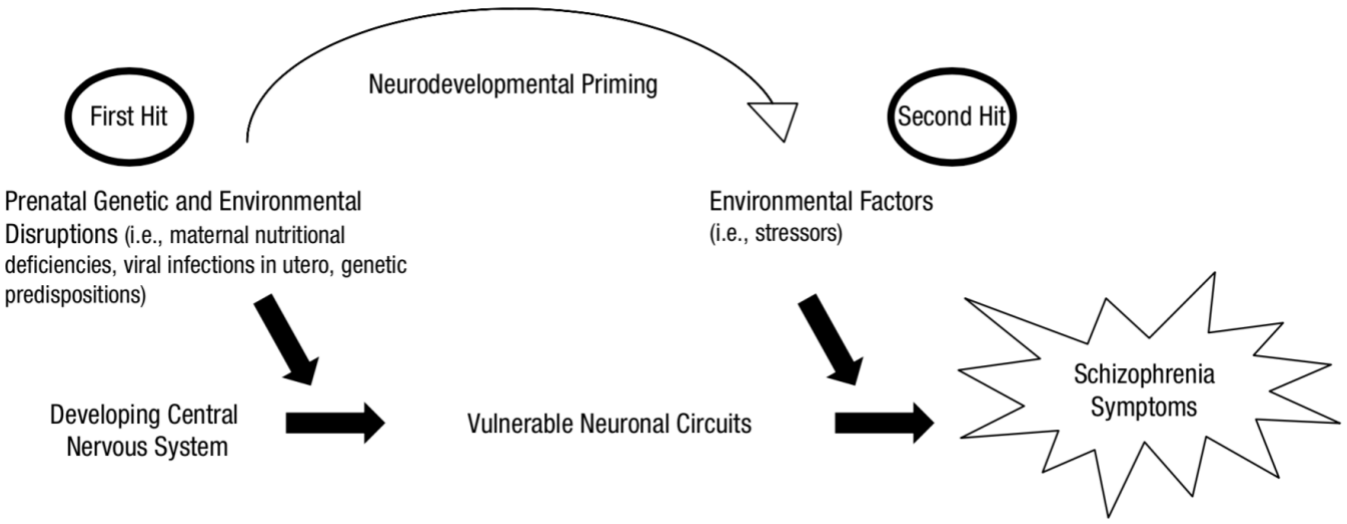


Figure 4: The second hit hypothesis as a model for schizophrenia. Adapted from Picci et.al., 2015.

Second Hit Hypothesis as a Model for ASD. The multiple-hit approach to studying the development of psychopathology is also applicable to the interacting mechanisms leading to abnormal neurodevelopment, namely MIA, ASD, and asthma. It is thought that a genetic or environmental "first hit" during critical periods of development make an individual more susceptible to a "second hit" later in life. These combinations of multiple factors are hypothesized to cause behavioral deficits as the body cannot sufficiently compensate and adapt to repeated environmental insults (Yee, Ribic, Coenen de Roo, & Fuchs, 2011). Specifically, individuals with autism experience two developmental insults that uniquely influence their neurodevelopment (Picci & Scherf, 2015). Support for this theory stems from a recent report that noted a decline in adaptive skills functions during adolescence in 30% of individuals with autism (Picci & Scherf, 2015). It is thought that these ASD individuals possessed an underlying neuronal system that is "built to fail" (first hit) such that upon encountering a second hit, specifically the combined effect of pubertal hormones, neural reorganization, and increasing social demands during adolescence, provide a "second hit" that disrupts the ability to transition into adult social roles and levels of adaptive functioning (Picci & Scherf, 2015) (Figure 5). Further, the second hit hypothesis would support the idea of an interaction between maternal immune activation and childhood asthma, namely that MIA exposes the fetus to the first hit causing atypical neurodevelopment and then a subsequent onset of asthma in the child (second hit) contributes to the emergence of autism-associated behavioral deficits.

While the double-hit hypothesis remains a plausible explanation for the underlying causes of neurodevelopmental disorders, association studies in humans are limited in causally testing the validity of the hypothesis. Therefore, researchers are looking to animal models to better corroborate the double-hit hypothesis.

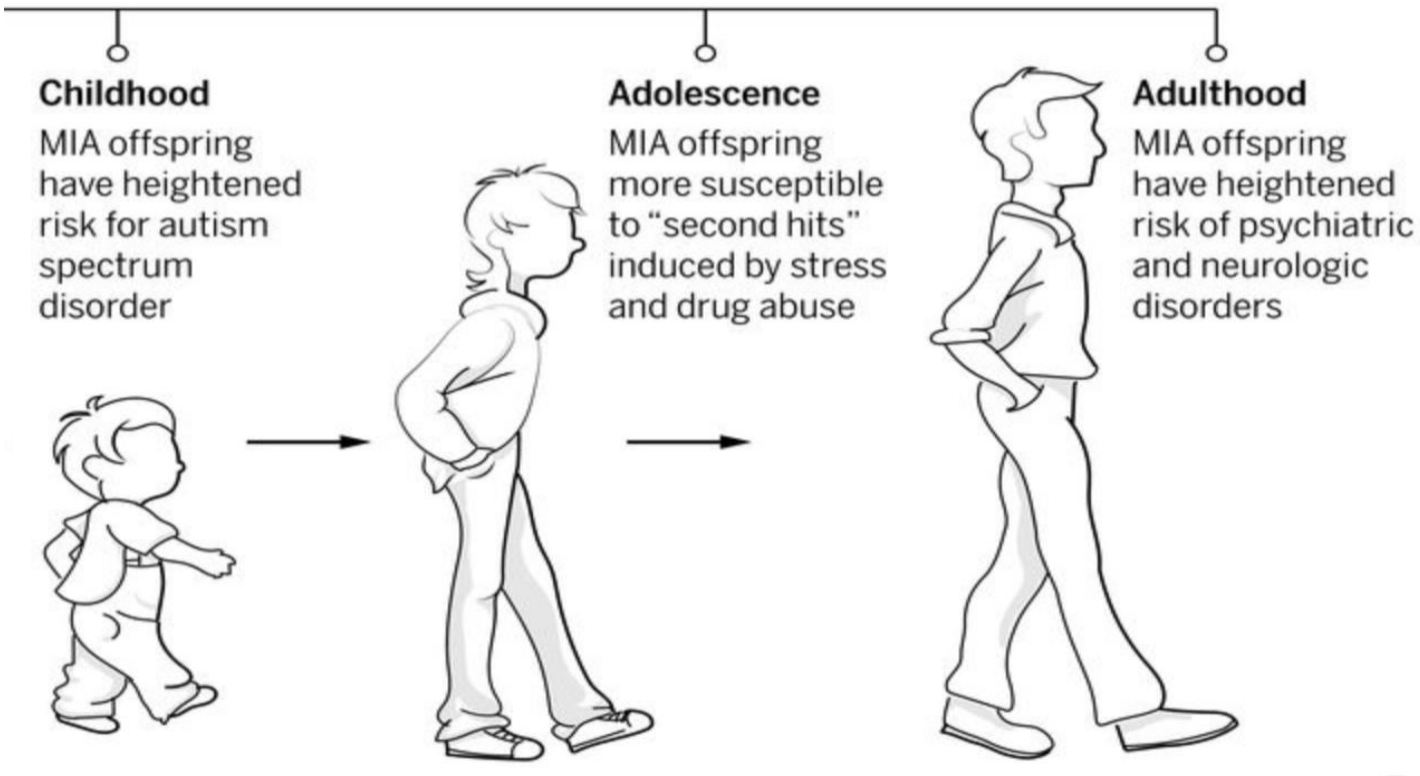


Figure 5:Offspring of MIA have an increased risk of getting ASD and they are more susceptible to a second hit. Adapted from Estes et.al., 2016.

Mouse Models

Overview. Previous population-based studies led to several common trends underlying the pathogenesis of ASD including that, 1) maternal immune activation may increase the risk of having a child diagnosed with ASD 2) individuals with ASD are more likely to have immune dysregulation (i.e. asthma and allergies) and 3) ASD severity may be increased as a result of the combination of multiple genetic and environmental insults. Several mouse models have been developed to direct causal links between environmental factors and offspring development through the use of behavioral tasks that measure changes in species-typical behaviors. Moreover, mouse behavioral tasks have been developed that have face validity for the hallmark characteristics to the ASD diagnosis, namely impairments in social interactions and restricted/repetitive behaviors (Crawley, 2007; Moy, Nadler, Young, Pereez, Holloway, Barbarom, et.al., 2007). These behavioral assays have strong translational value in testing theories on the etiology of ASD and other neurodevelopmental disorders.

Mouse Models of Immune Activation. Previous studies in rodents provide evidence for a causal link between MIA and the onset of ASD-like behaviors in the offspring. One study in mice found that when dams were injected with the double stranded RNA viral mimic polyinosinic:polycytidylic acid (Poly (I:C)) during pregnancy, a strong activator of the innate immune system, their offspring displayed three hallmark behaviors of autism, namely less ultrasonic vocalizations, decreased sociability, and increased repetitive/ restricted behaviors (Malkova, et.al., 2012).

More recently, Williamson et.al. 2011 used a rat model of MIA to test the plausibility of the second hit hypothesis. Specifically, rat pups were injected with a systemic infection on

postnatal day 4 and then injected with a bacterial membrane protein mimic, lipopolysaccharide (LPS), in adulthood and run through a memory task (Williamson, Scholar, Mistry, Smith, & Biblo, 2011). It was found that the rats exposed to a subsequent immune challenge had memory impairments that were only present when inflammation was present during both early development and later in life. These findings support the idea that an immune challenge in early life can affect neurodevelopment only when unmasked by a second immune challenge (Williamson et.al., 2011).

Together, the work of Williamson and Malkova provides a framework for an experimental paradigm that can test the validity of the second hit model. However, both studies are limited given that their immunological manipulations only activate the innate immune system. This is significant given that adaptive immune activation triggers distinct cascades of inflammatory factors that have been associated with autism risk (Goines & Van de Water, 2011). Therefore, a controlled experiment that explores the combination of maternal and offspring-mediated responses using physiologically-relevant insults is necessary to validate the double hit hypothesis in an animal model.

Limitations in the Current Animal Models of Immune Activation. To date, most research in the MIA field has focused on the effects of viral and bacterial infections of the mother on offspring development. In order to study immune dysfunction in mothers these models use Poly(I:C) or LPS. While models of maternal immune activation produce behavioral deficits in offspring, their translational power is limited because they fail to model the entirety of the immune response seen in infected mothers. Viruses and bacteria activate both the innate and the adaptive immune systems in humans, whereas Poly(I:C) and LPS activate only the innate branch. Specifically,

Poly(I:C) and LPS initiate an immune response by activating specific pattern recognition receptors that stimulate innate immune activation. However, these small molecules are not large enough to be presented as antigens to initiate B-cell activation or adaptive immune system signaling factors. As a result, there are few studies to date that are able to model the complete immune response associated with inflammation in humans. This is particularly concerning given that recent reports highlight adaptive-mediated maternal inflammation as the strongest contributor of autism risk, namely allergies and asthma (Patel et.al, 2017).

Maternal Allergic-Asthma Mouse Model. Epidemiological studies revealed that mothers with allergies or asthma during pregnancy were at an increased risk of having a child with ASD (Croen et.al., 2005; Lynall et.al., 2014). Therefore, allergies and asthma represent a novel source of MIA in mothers that is recently uncovered as a major-contributor to ASD severity (Patel et.al. 2017).

The maternal allergic-asthma (MAA) model is a novel approach that not only activates both the innate and adaptive immune systems, but also mimics the physiological response to allergies and asthma seen in humans (Schwartzer, Careaga, Coburn, Rose, Hughes, & Ashwood, 2016). Therefore, this model has the potential to allow researchers to investigate the relationship between maternal immune activation by activating both innate and adaptive branches of the immune system (Schwartzer et.al., 2016). In the MAA model, female mice are sensitized to the egg protein ovalbumin (OVA) and then an asthma response is elicited by inducing airway hypersensitivity from subsequent OVA exposures throughout gestation (MAA group) (Schwartzer, et.al., 2016) (Figure 6).

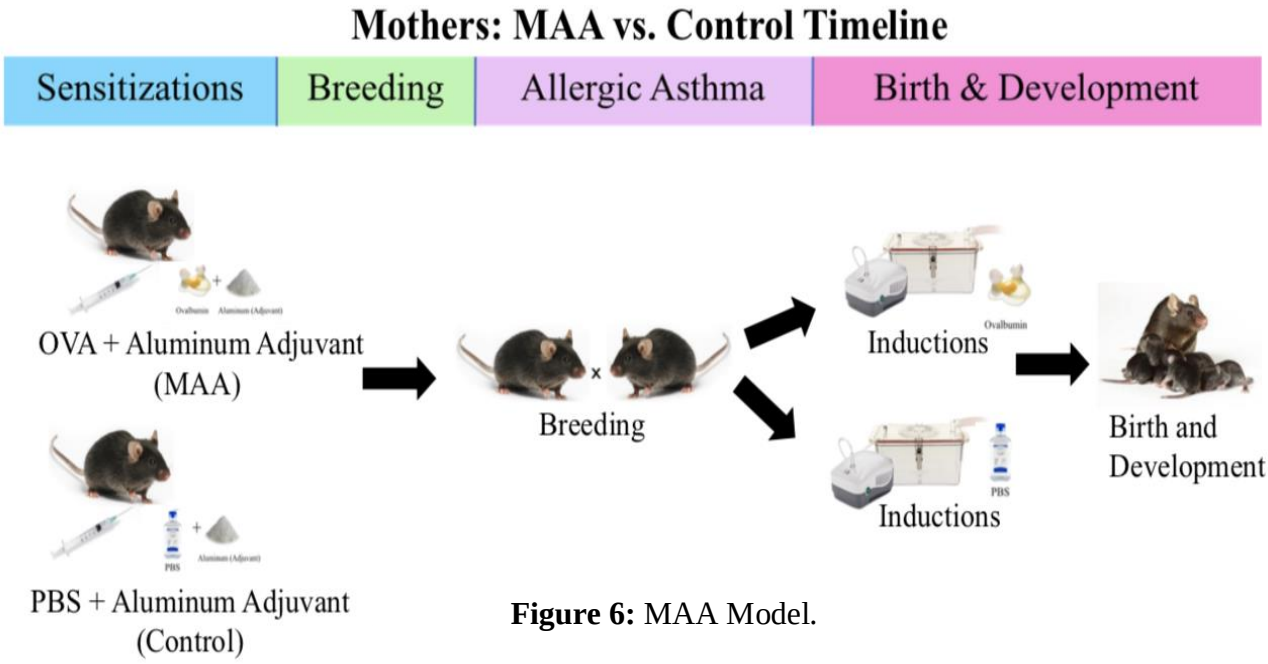


Figure 6: MAA Model.

Using this model, it was found that MAA offspring showed ASD-like behavioral deficits characterized by spending less time socializing when compared to control mice (Schwartzer, et al., 2016). Moreover, MAA offspring spent significantly more time engaged in a repetitive digging behavior analogous to the restricted/repetitive behavioral associated with ASD (Schwartzer, et.al., 2016).

The MAA model parallels other MIA models in that it demonstrates a role for maternal inflammation in eliciting ASD-like behaviors in the offspring. However, it is unknown how the MAA model would affect the behavior of MAA offspring who are later exposed to subsequent allergic-asthma episodes in young adulthood. This is important considering that using the MAA model allows researchers to examine the combined influence of innate and adaptive immunity on behavioral deficits, an experimental advantage not offered by traditional Poly(I:C) and LPS models. No studies have been conducted using the MAA model to examine the second hit hypothesis. As the second hit hypothesis has only been demonstrated through viral/bacterial mimics, these studies are limited because of their failure to activate both branches of immunity, therefore they are not completely replicating the immune response seen in humans. Taking together the relationship between MIA and ASD, asthma and ASD, the second hit hypothesis, and the limitations in the current models of MIA it remains unknown if offspring born to MAA mothers would express more severe ASD-like behaviors in response to subsequent allergic-asthma exposures. The recent development of the MAA model provides a powerful tool to address whether the second hit hypothesis, through transgenerational allergic-asthma, plays a role in the onset and severity of ASD-like behaviors. Thus, the current study attempts to use the MAA model to examine the second hit hypothesis as a model of ASD.

Research Objective. The purpose of this study was to examine if MAA offspring exhibit more serve ASD-like behaviors in response to subsequent immune challenges when compared to controls. Pregnant damns were exposed to asthma inductions during gestation to activate the immune system and their offspring encountered subsequent immune challenges via the same asthma inductions. The resulting groups were then measured for deficits in social behaviors and restricted/repetitive behaviors, two-core feature of ASD. It was hypothesized that MAA offspring exposed to subsequent asthma inductions during the juvenile period would exhibit more severe ASD-like deficits when compared to all other conditions. That is, mice that experienced multiple immune challenges (i.e. *in utero* and postnatally) were predicted to display the greatest behavioral deficits, supporting the second hit hypothesis as a model for autism.

Methods

Animals

Male and female C57Bl/67 (C57) (Jackson Laboratory, Bar Harbor, Maine USA) mice were bred and maintained at Mount Holyoke College at ambient room temperature on a 12 h light/dark cycle (lights on at 0800h). Mice were housed in individually ventilated cages (IVC) with same-sex littermates. Food and water were provided *ad libitum*. Mice were grouped 2-5 per cage and pregnant mice were single housed following the start of pregnancy. All mice were provided nestles for enrichment. Additional mice were bred and maintained at Mount Holyoke College, under the same conditions, to serve as stimulus mice for social behavioral tasks. Behavioral procedures were performed during the light cycle and all procedures were approved by the Mount Holyoke College Institutional Animal Care and Use Committee in accordance with the guidelines provided by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Maternal Allergy/Asthma Induction

Sexually naïve female C57 mice were randomly assigned to either the allergic-asthma group (MAA) or the control group (Control). Female mice were sensitized with 10µg ovalbumin (MAA) (OVA, Sigma, St. Louis, MO, USA) in 1mg $(Al)OH_3$ (InvivoGEN, San Diego, CA, USA) dissolved in 200µl phosphate-buffered saline (PBS) or vehicle alone (Control) via intraperitoneal injection (IP) and again 1 week later. One week following the second sensitization period, male and female mice were mated overnight, and females were checked daily for the presence of seminal plugs. Upon discovery of a seminal plug, noted as gestational day 0.5 (G0.5), female mice were removed from their littermates and single-housed. Throughout

gestation, pregnant mice were exposed to either an aerosolized solution of 1% (wt/vl) OVA in PBS (MAA) or PBS alone (Control) for seven consecutive days via a 45-minute induction session. These induction sessions occurred either at G2.5-9.5 or G10.5-17.5, which correspond to early-middle gestation or middle-late gestation in humans (Clancy, Darlington, & Finlay, 2011; Malkova, et.al., 2012; Meyer, Nyffeler, Yee, Knuesel, & Feldon, 2008; Schwartzer, Careaga, Chang, Onore, & Ashwood, 2015). Following the final induction session, mice were left single-housed and undisturbed until the birth of their litters (for reference see figure 6). Pups remained with their mother until weaning on P21 after which offspring were group housed with 2-5 of their same-sex littermates.

Offspring Allergy/Asthma Induction

Offspring of both MAA and Control mothers were randomly assigned to either the allergic-asthma (OVA) group or control (PBS) group. Male and female mice were sensitized with 10µg ovalbumin (OVA, Sigma, St. Louis, MO, USA) in 1mg $(Al)OH_3$ (InvivoGEN, San Diego, CA, USA) dissolved in 200µl PBS (OVA) or vehicle alone (PBS) via IP injection on P28 and again one week later on P35. One week following second sensitization, pups were exposed to either an aerosolized solution of 1% (wt/vl) OVA in PBS or PBS alone for 45 minutes on P42 and P49 to initiate allergic-asthma responses in the offspring. The resulting groups were classified into four experimental conditions (Control-PBS, Control-OVA, MAA-PBS, MAA-OVA) (Figure 7). Offspring from each group were then exposed to subsequent OVA or PBS inductions once per week followed by behavioral assessments four hours post induction (Figure 8).

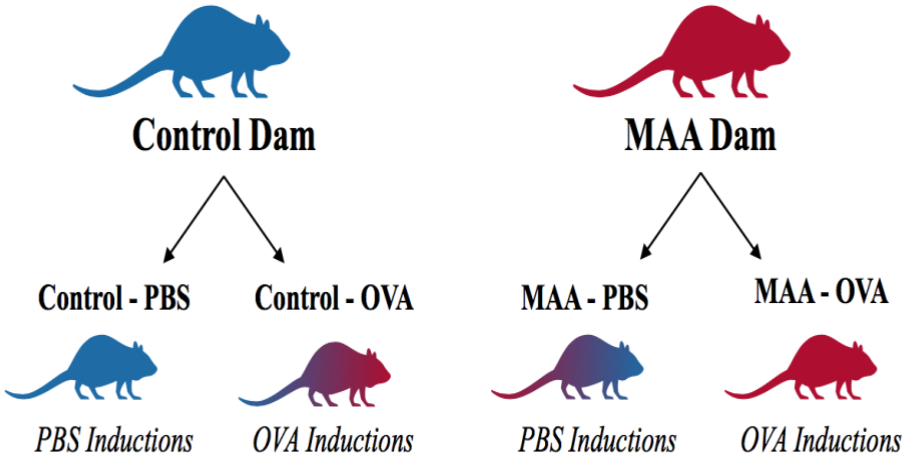


Figure 7: Four groups in the study.

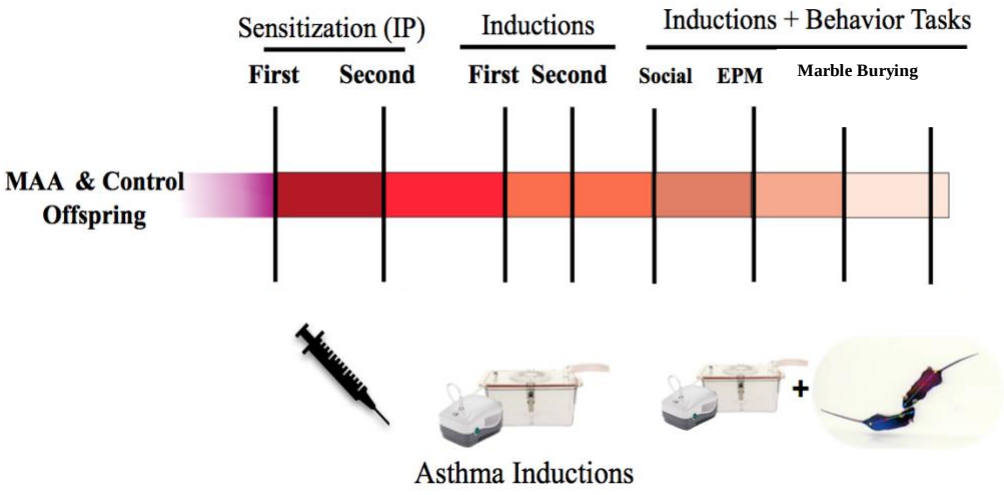


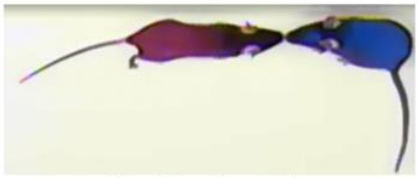
Figure 8: Timeline for MAA and Control Offspring.

Behavioral Assessments: Experimental Design

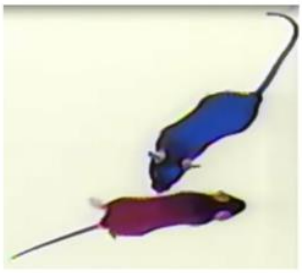
Between P56 and P71, MAA and Control offspring were given three more inductions, each occurring seven days apart, and evaluated for sociability, anxiety, and repetitive motor behaviors four hours after an allergic-asthma exposure.

1. Reciprocal Social Interaction (RSI)

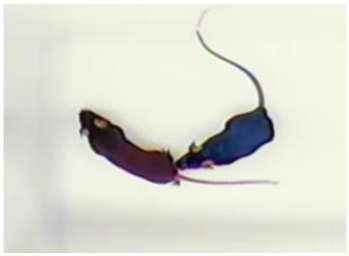
Experimental offspring were placed into a clean arena (40x50x20cm) and marked with either blue hair chalk or pink hair chalk for computer-guided detection. Paris of mice from the same mother-offspring treatment condition were placed in opposite corners of the arena and the mice were allowed to interact freely for 30 minutes while being video recorded. All pairings were with non-littermates to ensure social interactions were with novel stimuli. Mice were analyzed for locomotion and social interactions using Ethovision XT 11.5. Horizontal locomotor activity was measured for total distance travelled and average velocity during the 30-minute social interaction. Further, social interactions were detected using Ethovision's three-point body module to assess nose-nose, nose-body sniff, and nose-anogenital sniffs (Figure 9), as well as total time engaged in social interactions.



Nose-Nose Interaction



Nose-Body Interaction



Nose-Anogenital Interaction

Figure 9: The three interactions assessed in mice during RSI.

Figure 10: Elevated Plus Maze.

Figure 11: Set up of marble burying task.

2. Elevated Plus Maze

Experimental offspring were tested for changes in anxiety using an elevated plus maze as described in Schwartzer, et.al., 2016 (Figure 10). Mice were placed in the center of the elevated plus maze and allowed to freely explore for 5 minutes under a spotlight. The elevated plus maze was cleaned with 70% ethanol between each trial. Behaviors were video recorded and analyzed using EthoVision XT 11.5 for number of entries into each arm, risk taking behaviors, and percentage of time spent in open arms. Percent of time spent in open arm was calculated as the total amount of time sent in open arms divided by the time spent in both open and closed arms. Decreased percent time exploring the open arms were interpreted as increased anxiety.

3. Marble Burying

Repetitive marble burying behavior was assessed using procedures modified from Deacon, 2006. Mice were habituated for 10 minutes to a clean plastic cage (37 x 14x 12.5 cm) filled with a 4-centimeter-thick layer of fresh corncob bedding. Following habituation, animals were returned to their home cage and 15 clean glass marbles were laid out in rows of 3x5 placed equidistantly apart (Figure 11). Mice were then returned to their cages and left undisturbed to explore for 10 minutes. At the end of the 10-minute period, mice were carefully removed from the arena and a photo was taken of the cage for later analysis. The marbles were wiped down with 70% ethanol between trials and a new cage with fresh corncob bedding was provided for each mouse. Marble burying was defined as 75% of the marble covered by bedding and assessed by two raters blind to treatment condition.

Statistical Analysis

Data were analyzed with SPSS v. 24 using a three-way factorial ANOVA. Maternal treatment, offspring treatment, and sex were set as fixed factors (Table 1). For the RSI task, distance was added in as a covariate. For significant interactions, simple main effects analyses were conducted to determine the nature of the interaction.

Exclusion Criteria

Data points that were greater than 1.5 quartiles from the mean were excluded from only the behavioral task in which they were an outlier.

Table 1: Sample size of maternal treatment, offspring treatment and sex

Maternal Treatment	**Offspring Treatment**	**Sex**	**Number of Mice**
MAA	OVA	Male	6
MAA	OVA	Female	7
MAA	PBS	Male	6
MAA	PBS	Female	6
Control	OVA	Male	8
Control	OVA	Female	6
Control	PBS	Male	6
Control	PBS	Female	8

Results

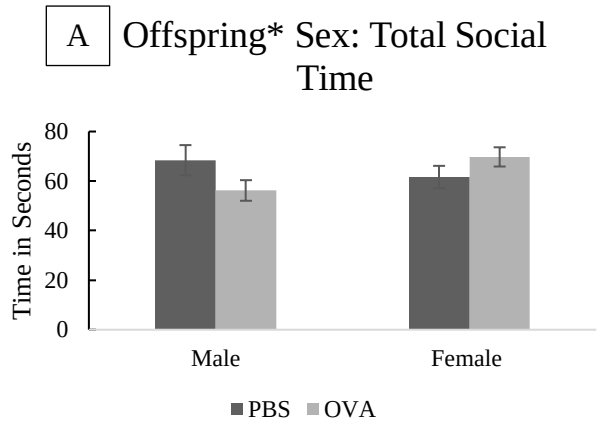
Reciprocal Social Interaction

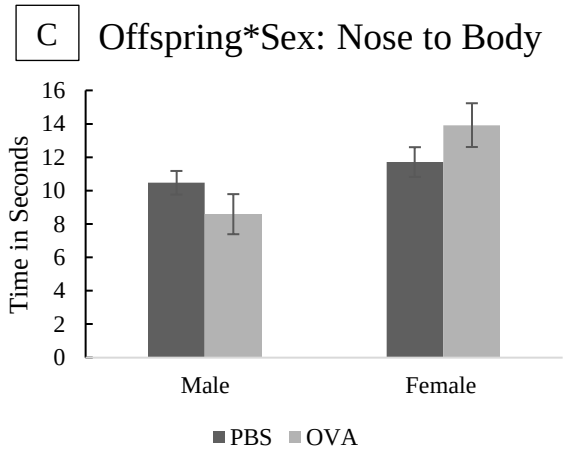
In order to determine the behavioral consequences of maternal and offspring allergic-asthma exposure, mice were assessed for changes in sociability in the reciprocal social interaction task. A three-way analysis of variance assessing the total time mice engaged in social interactions, with distance as a covariate, revealed no significant main effects for maternal treatment, $F(1, 51) = 0.001$, $p = 0.970$, offspring treatment, $F(1, 51) = 0.247$, $p = 0.622$, or sex, $F(1, 51) = 0.512$, $p = 0.478$. In addition, there was no significant maternal treatment by offspring treatment interaction for total time engaged in all social behaviors, $F(1, 51) = 1.50$, $p = 0.227$ and no significant interaction between maternal treatment and sex, $F(1, 51) = 0.072$, $p = 0.789$. However, there was a trend found for an interaction between offspring and sex, $F(1, 51) = 4.87$, $p = 0.071$. A simple main effects analysis revealed a trend towards a significant reduction in social interaction from OVA males ($M = 56.19$, $SD = 16.81$) when compared to PBS males ($M = 64.86$, $SD = 17.80$), $p = 0.060$. This interaction was not present in female mice, $p = 0.229$ (Figure 12A).

Individual social behavior postures were also assessed to determine if treatment conditions altered specific nose to nose postures, body sniffing, or anogenital sniffing behaviors. A three-way analysis of variance was conducted, with distance travelled as a covariate, to determine the amount of time mice spent in a nose to nose interaction, calculated by assessing the total amount of time the nose of one mouse was within 1.5 centimeters of the nose of its partner. There were no significant main effects for maternal treatment, $F(1, 51) = 0.539$, $p = 0.467$, offspring treatment, $F(1, 51) = 0.004$, $p = 0.950$, or sex, $F(1, 51) = 0.208$, $p = 0.651$. Moreover, there were no significant maternal treatment by offspring treatment interactions found for total time spent in a nose to nose behavior, $F(1, 51) = 0.034$, $p = 0.856$. However, there was a

trend present for the interaction between offspring treatment and sex $F(1, 51) = 3.84$, $p = 0.057$ (Figure 12B). However, after a simple main effects analysis was conducted these findings did not remain significant, $p > 0.05$ for all values. Lastly, when maternal treatment was factored into the sex by offspring treatment interaction, no significant three-way interaction was detected, $F(1, 51) = 0.712$, $p = 0.403$.

Next, mice were assessed for total time spent in a nose to body interaction in which the nose of one mouse was sniffing the body of its partner. Using a three way factorial ANOVA, with distance as a covariate, it was found that there was no main effect for maternal treatment, $F(1, 51) = 0.738$, $p = 0.395$, or offspring treatment, $F(1, 51) = 0.003$, $p = 0.957$. However, there was a significant main effect for sex on nose to body interactions, $F(1, 51) = 9.44$, $p < 0.05$, with males (M =9.46, SD = 3.07) spending significantly less time in a nose to body interaction when compared to females (M =12.73, SD = 4.56). Interestingly, there were no interactions found for maternal treatment by offspring treatment, $F(1, 51) = 1.87$, $p = 0.179$, maternal treatment by sex, $F(1, 51) = 0.123$, $p = 0.728$, or maternal treatment by offspring treatment by sex, $F(1, 51) = 0.108$, $p = 0.744$. However, there was a trend for an interaction between offspring treatment and sex, $F(1, 51) = 3.25$, $p = 0.068$ (Figure 12C). When a simple main effects analysis was run, it was revealed that this interaction did not remain significant, $p > 0.05$ for all comparisons.





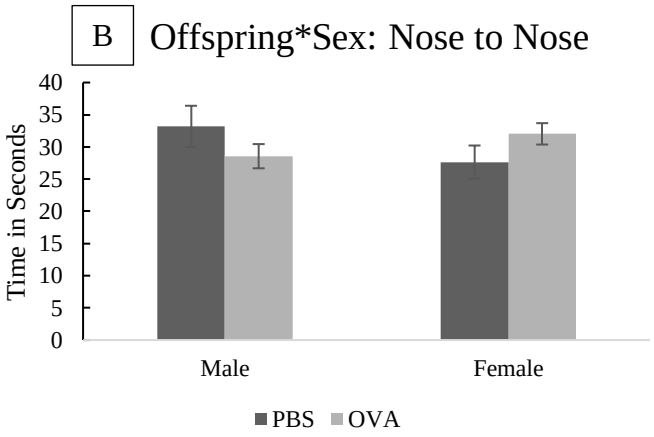


Figure 12: *Reciprocal social interaction trends*. (**A**) There was a trend showing that OVA males (n = 14) spent less time in a social behavior when compared to PBS-males (n = 12), p= 0.060. PBS-females (n = 14) spent an equal amount of time in a social behavior when compared to OVA-females (n = 12), p = 0.229. (**B**) There was a trend showing that PBS male mice spent more time in a nose to nose interaction when compared to PBS females and OVA female spent more time in a nose to nose interaction when compared to OVA males. (**C**) There was a trend showing that OVA females spent more time in a nose to body interaction when compared to OVA males and this trend was not present in PBS offspring.

Lastly, mice were assessed for time spent in a nose to anogenital sniff behavior, defined as the nose of one mouse sniffing the anogenital region of its partner. There were no significant main effects found for maternal treatment, $F(1, 51) = 0.199$, $p = 0.657$, offspring treatment, $F(1, 51) = 0.949$, $p = 0.335$, and sex, $F(1, 51) = 0.271$, $p = 0.605$. Further, no significant interactions were detected between maternal treatment and sex, $F(1, 51) = 0.200$, $p = 0.657$, offspring treatment and sex, $F(1, 51) = 2.58$, $p = 0.166$, or the three way interaction between maternal treatment, offspring treatment, and sex, $F(1, 51) = 0.023$ $p = 0.880$. However, there was a significant interaction between maternal treatment and offspring treatment, $F(1, 51) = 4.36$ $p = 0.045$. A simple main effects analysis revealed that MAA-PBS mice ($M = 26.70$, $SD = 12.73$) spent more time in a nose to tail interaction when compared to MAA-OVA mice ($M = 19.45$, $SD = 4.86$), $p = 0.042$ (Figure 13). This interaction was not present in Control-OVA or Control-PBS mice, $p = 0.422$. Together, these results suggest that adult social behaviors are altered by both maternal and offspring allergic-asthma exposure.

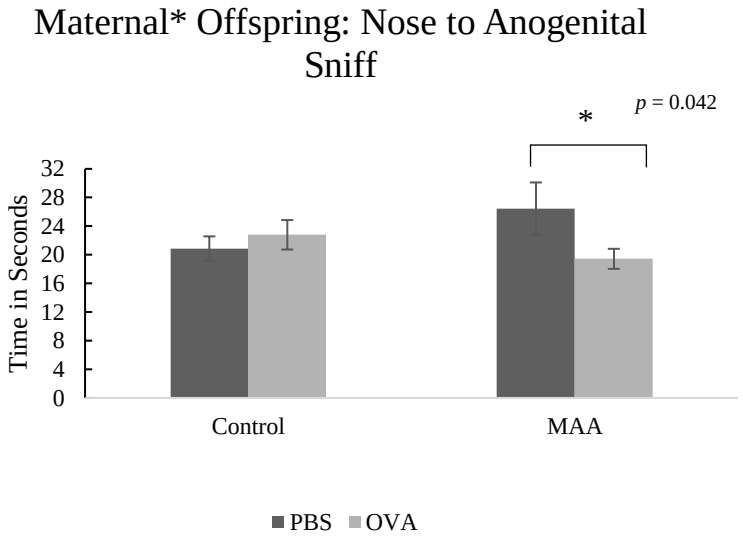


Figure 13: *Double hit hypothesis reduces social interaction*. MAA-PBS mice (n = 12) spent more time in a nose to anogenital behavior when compare to MAA-OVA mice (n =13), p = 0.042. This was not seen in Control-PBS or Control-OVA mice, p = 0.422.

Elevated Plus Maze

Mice were assessed for changes in anxiety-like behaviors using the elevated plus maze. A three-way factorial ANOVA revealed a significant main effect for maternal treatment on the percent time spent in the open arm of the arena, $F(1, 53) = 8.7$, $p < 0.05$. Specifically, MAA offspring ($M = 48.02\%$, $SD = 0.139$) spent significantly more time in the open arm of the elevated plus maze when compared to Control offspring ($M = 38.16\%$, $SD = 0.118$) (Figure 14A). There was also a significant main effect found for sex, $F(1, 53) = 5.62$, $p < 0.05$, with female mice ($M = 46.61\%$, $SD = 0.117$) spending more time on the open arms of the maze compared to males ($M = 38.87\%$, $SD = 0.145$) (Figure 14B). However, no significant interactions were found for maternal treatment by offspring treatment, $F(1, 53) = 0.026$, $p = 0.873$, or maternal treatment by offspring treatment by sex, $F(1, 53) = 2.66$, $p = 0.110$.

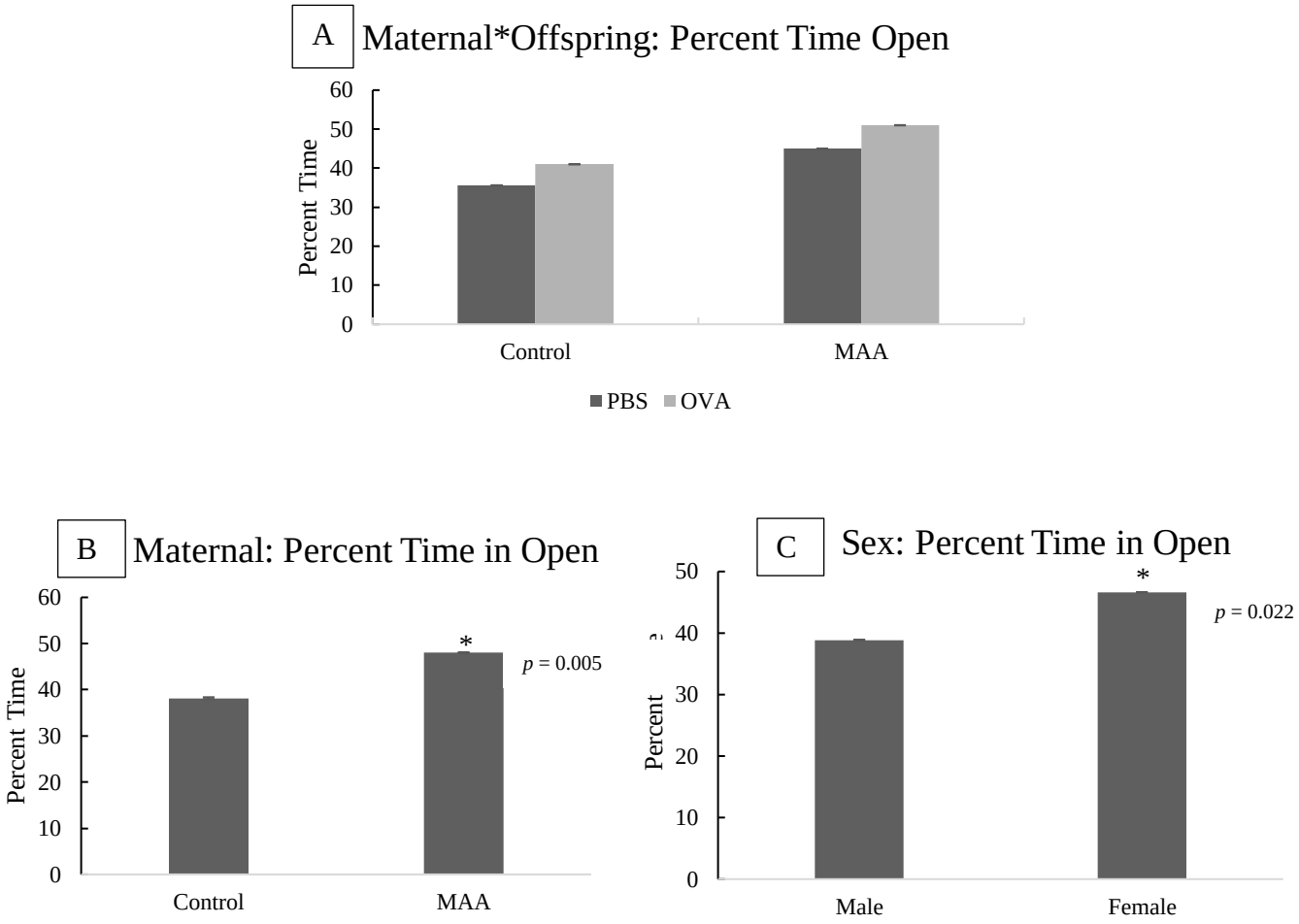


Figure 14: *Elevated plus maze.* (**A**) There was no significant difference found between MAA-OVA and MAA-PBS mice or Control-OVA and Control-PBS mice. (**B**) MAA offspring (n = 25) spent significantly more time in the open arm of the elevated plus maze when compared to Control offspring (n = 28), p = 0.005. (**C**) Furthermore, males (n = 26) spent less time in the open arm of the maze when compared to females (n = 27), p = 0.022.

Mice were assessed for peeking behavior, defined as the time when the mouse's body was in the center of the maze and its head was in the open arm or hanging over the side of the maze. In addition, edge exploration was defined when the mouse's body was in the open arm with its head hanging over the side of the maze. For peeking behavior, there was no significant main effect for maternal treatment, $F(1, 52) = 2.99$, $p = 0.091$, offspring treatment, $F(1, 52) = 0.991$, $p = 0.325$, or sex, $F(1, 52) = 0.020$, $p = 0.888$. In addition, there were no significant interactions for maternal treatment by offspring treatment, $F(1, 52) = 1.04$, $p = 0.314$, offspring treatment by sex, $F(1, 52) = 0.018$, $p = 0.893$, or the three-way interaction of maternal treatment by offspring treatment by sex, $F(1, 52) = 0.113$, $p = 0.738$. Interestingly, there was a trend for an interaction between maternal treatment and sex, $F(1, 52) = 3.27$, $p = 0.077$. A simple main effects analysis revealed that MAA female mice ($M = 31.82$, $SD = 6.33$) spent more time in a peeking behavior when compared to MAA male mice ($M = 27.64$, $SD = 7.68$), $p = 0.031$. This interaction was not present in PBS mice, $p = 0.956$ (Figure 15).

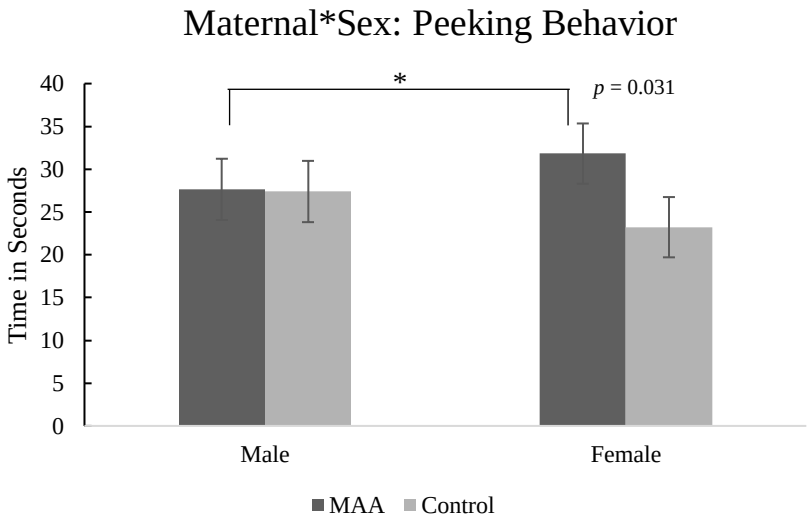


Figure 15: *Peeking behavior*. MAA female mice (n = 13) spent more time in a peeking behavior when compared to MAA male mice (n = 12), p = 0.031. This trend was not seen in Control mice.

For edge exploration behavior there was a significant main effect for sex found, $F(1, 52) = 5.44$, $p = 0.024$, indicating that male and female mice spent unequal times in an edge exploration behavior. Specifically, males ($M = 33.29$, $SD = 14.46$) spent more time in an edge exploration behavior when compared to females ($M = 24.79$, $SD = 11.01$). Additionally, there was a trend towards a significant main effect for offspring treatment on edge exploration behavior, $F(1, 52) = 3.40$, $p = 0.072$, suggesting that OVA mice ($M = 32.37$, $SD = 14.66$) spend more time in an edge exploration behavior when compared to PBS mice ($M = 25.41$, $SD = 12.42$). No significant interactions were found between maternal treatment and offspring treatment, $F(1, 52) = 0.455$ $p = 0.504$, and maternal treatment and sex, $F(1, 52) = 0.878$ $p = 0.354$. However, there was a significant interaction between offspring treatment and sex, $F(1, 52) = 7.85$, $p < 0.05$. A simple main effect analysis revealed that PBS males ($M = 24.58$, $SD = 14.40$) spent less time in an edge exploration behavior when compared to OVA males ($M = 37.36$, $SD = 9.96$) $p = 0.015$. This interaction was not seen in females, $p = 0.498$ (Figure 16). There was also a significant three way interaction between maternal treatment, offspring treatment and sex, $F(1, 52) = 4.74$, $p = 0.035$. A simple main effect analysis revealed that Control PBS males ($M = 20.27$, $SD = 13.86$) spent less time engaged in an edge behavior when compared to Control OVA males ($M = 45.50$, $SD = 14.27$), $p = 0.000$. This effect was not seen in MAA PBS or OVA males and was also not seen in females, $p > 0.05$ for all comparisons.

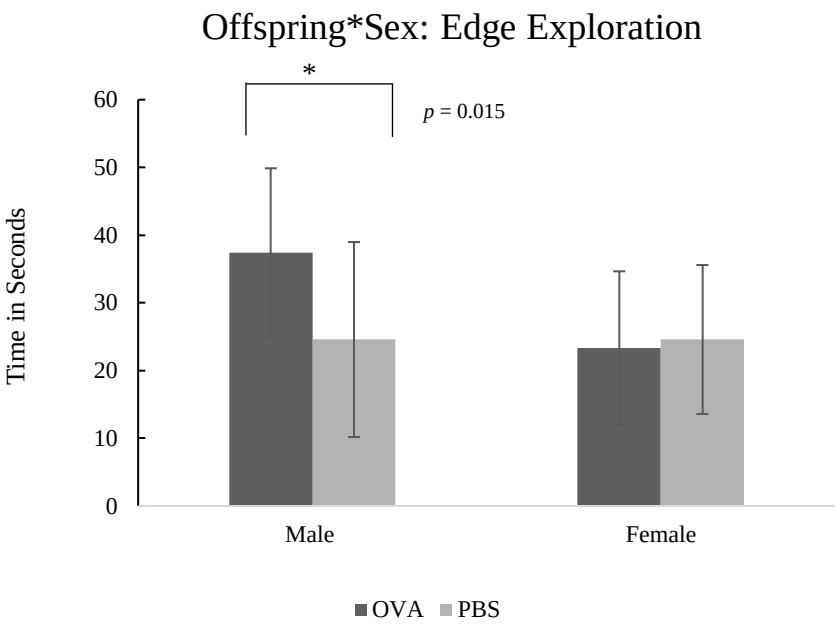


Figure 16: *Edge Exploration*. OVA males (n = 14) spent more time in an edge exploration behavior when compared to PBS males (n=12), p = 0.015. Conversely, OVA females (n = 12) and PBS females (n = 14) spent similar amounts of time in an edge exploration behavior, p = 0.498.

Marble Burying

To assess for the presence of restricted/repetitive behaviors, mice were run through the marble burying task. There were no significant main effects found for maternal treatment, $F(1, 53) = 0.863$, $p = 0.358$, offspring treatment, $F(1, 53) = 1.397$, $p = 0.243$, or sex, $F(1, 53) = 0.712$, $p = 0.243$. However, there was a significant interaction between offspring treatment and sex, $F(1, 52) = 6.48$, $p < .05$. A simple main effects analysis showed that PBS female mice buried more marbles ($M = 5.23$ $SD = 8.34$) when compared to OVA females ($M = .000$, $SD = .000$), $p = 0.013$ (Figure 17). This interaction was not present in males, $p = 0.380$. Lastly, there was no significant interaction found for maternal treatment by offspring treatment, $F(1, 53) = .080$, $p = 0.779$ or maternal treatment by offspring treatment by sex, $F(1, 53) = 1.60$, $p = 0.212$.

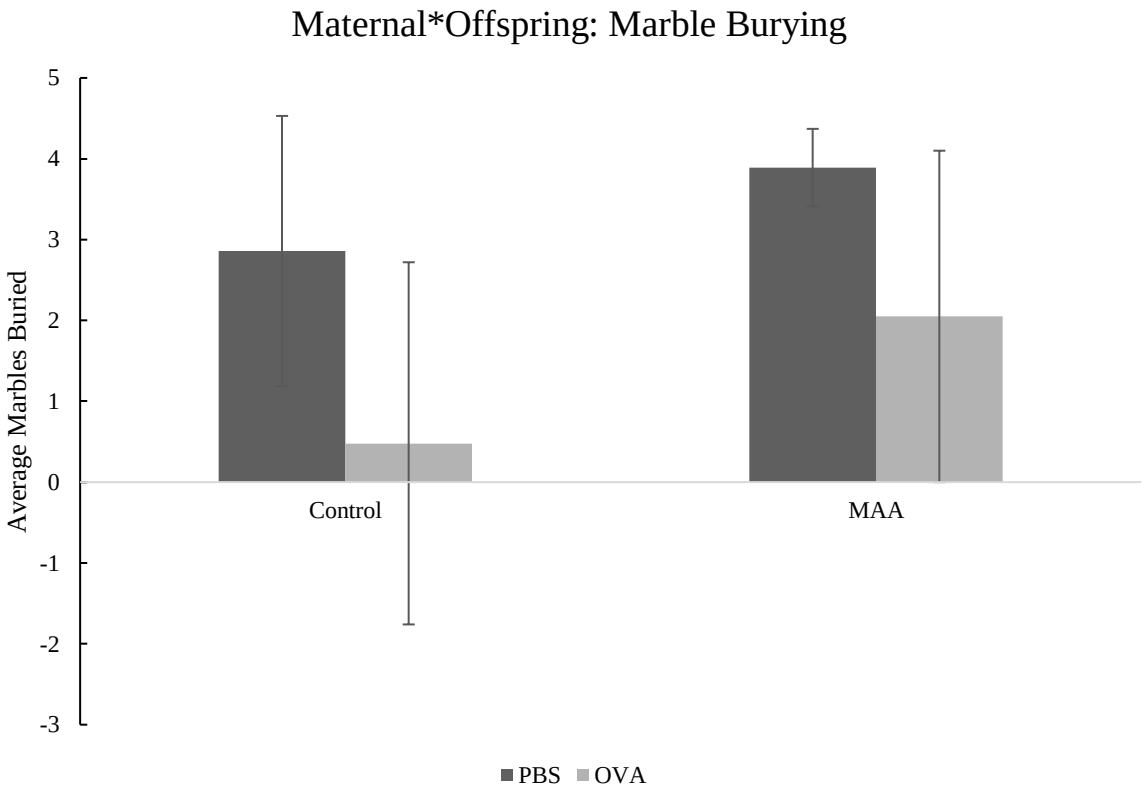


Figure 17: *Marble burying is not affected by the Double Hit Hypothesis.* There were no differences between Control-PBS and Control-OVA mice on number of marbles buried. Furthermore, there were no differences between MAA-PBS and MAA-OVA mice on marbles buried.

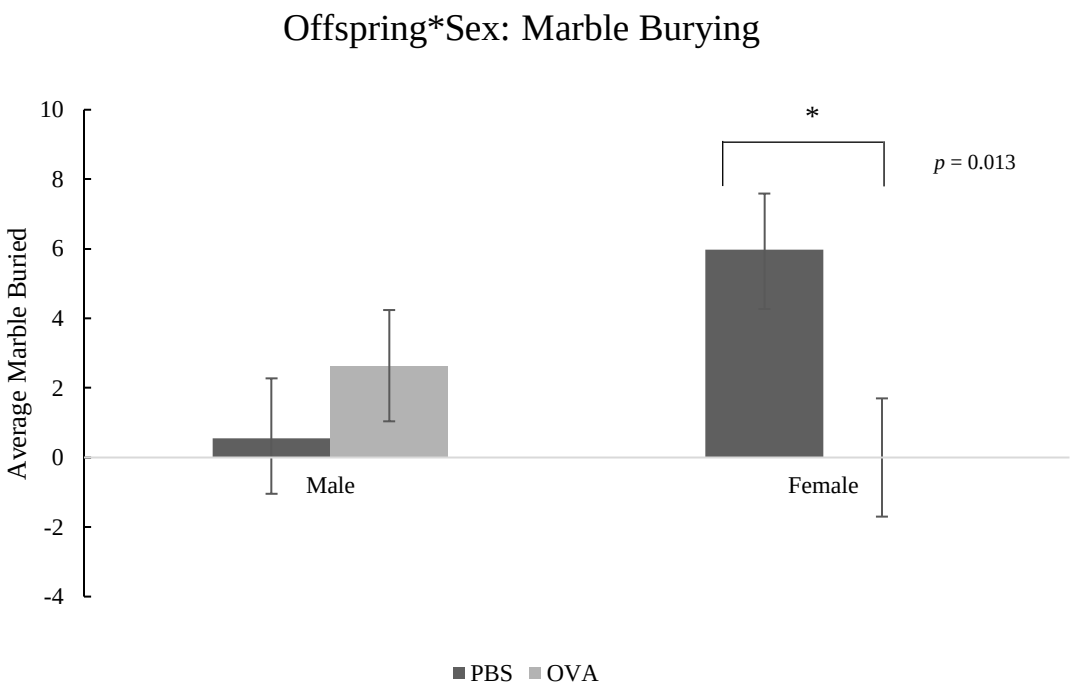


Figure 18: *Marble burying.* PBS females (n = 14) buried significantly more marbles when compared to OVA females (n = 12), p = 0.013. However, this interaction was not present in males, p = 0.380.

Discussion

Overview. Maternal immune activation during pregnancy is a well-established risk factor for neurodevelopmental disorders (Knuesel, Chicha, Britschgi, Schobel, Bodmer, Hellings, et.al., 2014). Previous studies have determined that it is the inflammatory signals, not the specific pathogen, released during immune activation in the mother that contributes to atypical neurodevelopment in the offspring (Penner & Brown, 2007). Furthermore, a wealth of studies linking immune system alterations to autism have led to the notion that ASD, in some patients, may represent an immune-mediated or autoimmune disorder (Ashwood & Van de Water, 2004). Moreover, Picci & Scherf, 2015 postulated that a two-hit hypothesis could be used as a model of autism in humans. Despite these compelling correlations in clinical studies, researchers are unable to effectively demonstrate a causal role for the immune system due to ethical limitations. However, animal models can be utilized to test the effects of MIA on the generation of autism-like behavioral deficits in an attempt to model the human condition. Current animal models of immune activation utilize the viral and bacterial mimics, Poly (I:C) or LPS, to demonstrate the link between immune activation and ASD-like behaviors (Ashwood & de Water, 2004). But, these studies are limited because they fail to consider other immunological agents, namely allergies and asthma, that can equally affect neurodevelopment. In humans, infections, allergies, and asthma activate both the innate and adaptive branches of immunity, but the current animal models of immune activation only activate the innate branch, thus the translational power of these current models is lacking. Importantly, the maternal allergic-asthma (MAA) model developed by Schwartzer, et. al. activates both the innate and adaptive branches of immunity in rodents which allows researchers to explore the combined effects of innate and adaptive immunity on neurodevelopment (Schwartzer, et.al., 2015).

The current study marks the first attempt at activating both the innate and adaptive branches of the immune system in both the mother and the offspring to test the feasibility of utilizing the second hit hypothesis as a model of neurodevelopmental disorders, specifically autism. Using the MAA animal model of immune activation, pregnant dams were exposed to repeated OVA-inductions; then, their offspring were exposed to subsequent OVA allergic-asthma challenges and assessed for ASD-like behavioral deficits using a series of well-validated behavioral tasks. The findings of this study demonstrate that concomitant exposures to allergic-asthma during fetal development and during the juvenile period alter social and anxiety-like behaviors but do not disrupt repetitive marble burying behavior. These data suggest that social-emotional processing may be more sensitive to allergic-asthma compared to repetitive motor behaviors. Taken together, the findings support the notion that exposure to allergic-asthma can elicit species atypical social and anxiety-related behaviors in a MAA mouse model.

Social Interaction. In order to determine the relationship between maternal allergic asthma, offspring allergic asthma, and ASD-like social behaviors, offspring completed the reciprocal social interaction task. In this task, mice are allowed to freely interact for 30 minutes and their behaviors are recorded and analyzed for time spent in a social behavior, with decreased amount of time in a social behavior being analogous to social impairments seen in humans with autism. This task encourages more complex and intricate social behaviors which allows for a better understanding of social interactions (Schwartzer, et.al., 2016). Results indicate that allergic-asthma exposure elicits behavioral deficits in a social interaction task and these behavioral changes are more apparent in male mice. Specifically, the RSI data showed that MAA-OVA mice spent less time engaging in an anogenital sniffing behavior when compared to Control-PBS

mice. Additionally, OVA mice spent significantly less time in social interactions when compared to PBS mice and decreases in social behaviors were observed in male, but not female, mice. There was also an interaction between sex and offspring treatment with OVA males spending less time in a social behavior when compared to OVA females.

The combined effects of maternal and offspring asthma led to more severe decreases in specific social behavioral postures. Specifically, MAA-OVA mice spent less time in a nose-to-anogenital sniff behavior when compared to Control-PBS mice. This interaction between maternal and offspring treatment supports the double hit hypothesis that prenatal priming can be further exacerbated by a second environmental insult during the juvenile period. However, these results are limited by the fact that MAA-PBS treatment did not have a significant effect on social behaviors. It has previously been shown that MAA offspring show decreased social ability in the juvenile reciprocal social interaction (JRSI) task (Schwartzer, et.al., 2016). This is in contrast to the current findings that did not report a main effect of maternal treatment on social behavior. Importantly, behavioral assessments in the previous Schwartzer, et.al. 2016 study examined changes in social interactions during the juvenile period. However, no study to date has presented on changes in adult social interaction using the reciprocal social interaction task. Given that the present study assessed social interactions in adulthood, it is possible that the lack of decrease in total social time in MAA offspring may reflect a compensatory improvement in social behaviors that occurs between juvenile and adult development. In fact, studies examining social behavior deficits in the BTBR mouse model of autism have shown that repeated social interactions with highly social cage-mates can rescue the social behavior deficits that are characteristic of this mouse strain (Yang, Perry, Weber, Katz, & Crawley, 2010). That is, it remains unknown whether the social deficits observed during the juvenile period following

MAA may diminish in adulthood and only become unmasked after a second environmental hit in later in life.

The differences in sociability seen in the offspring treatment groups are reminiscent of other immune-mediated behaviors often associated with sickness behavior. Sickness behavior occurs when a sick animal exhibits several of the following well-characterized behavioral changes: reduction in food and water intake, low activity and exploration, increased sleep, and reduced social and sexual interactions (Bilbo & Schwarz, 2012). Therefore, the finding that OVA-exposed mice exhibit less social interactions is consistent with the idea that sickness due to immune activation elicits abnormal behaviors in the offspring. Specifically, pro-inflammatory cytokines are released during immune activation and these cytokines are shown to be the central mediators of sickness behavior (Dantzer & Kelley, 2007). Importantly, studies have found that peripheral immune activation and subsequent cytokine release can trigger the production and release of pro-inflammatory cytokines in the brain, and these central nervous system cytokines influence neural circuits, plasticity, and connectivity in brain regions that are associated with ASD (Allan & Rothwell, 2001; Levin & Godukhin, 2016). Taken together, the findings that OVA mice engage in less social behaviors when compared to PBS mice supports the idea that maternal immune activation can alter offspring neurodevelopment and furthermore that acute OVA-inductions initiate the release of pro-inflammatory cytokines which disrupt brain function and lead to sickness behaviors. Due to time constraints, the current study was unable to corroborate this hypothesis by analyzing the brains of offspring for the presence of pro-inflammatory cytokines. But additional studies are underway to determine whether MAA-OVA mice would express more pro-inflammatory cytokines in their brains compared to other groups.

The sex-based differences found in this study, specifically greater social interaction observed in female mice, support previous results that have found that female C57 mice are more social than C57 male mice (An, Zou, Wu, Yang, Tai, Zeng, et.al., 2011). These results are interesting because they suggest that males are naturally less social when compared to females and are in line with the theory that autism is 4.5 times more common in boys than girls (Werling & Geschwind, 2014). It is suggested that this disparity between male and female autism prevalence is due to males being genetically predisposed to engage in less social behavior (Baron-Cohen Baron-Cohen, Lombardo, Auyeung, Ashwin, Chakrabarti, & Knickmeyer, 2011). In other words, if females have a natural drive to be more social than males, it would take a more severe deficit in social behavior to see the change. Whereas males already engage in fewer social behaviors, changes in their social brain networks following immune activation would be more easily observed compared to females. Despite this notion that sex differences in autism are due to innate differences in sociability, few research reports in humans provide compelling evidence to support this claim. Therefore, other genetic sensitivities, particularly x-linked gene variants, may also contribute to sex differences in the behavioral response to allergic asthma.

The interaction found between offspring treatment and sex supports the biological sex-based differences seen in human asthma. Female OVA mice spent more time in a nose-to-body interaction when compared to male OVA mice which suggests that females may not be as sensitive to juvenile asthma when compared to males. Epidemiologic studies carried out in humans have found that asthma usually begins in infancy or early childhood with males being twice as likely to develop asthma when compared to females. However, in adulthood asthma occurs more frequently among women (Postma, 2007). The findings in this study corroborate the epidemiological findings given that male mice showed greater sensitivity to the behavioral

consequences of acute asthma. These sex differences in behavioral responses could be attributed to an exacerbated immune response in males in early development that was not seen in females. That is, male sensitivity to acute asthma are hypothesized to contribute to the more severe behavioral deficits observed in male offspring. Speculation aside, the social interaction results demonstrate the contribution of both maternal and offspring immune alterations in shaping the ASD-phenotype and these deficits are more pronounced in male offspring. These findings parallel the trends seen in humans, namely that asthma affects women more than men and boys more than girls, and that autism is 4.5 times more common in boys than girls and provides evidence in support of the double hit hypothesis as a plausible model for autism.

Anxiety Related Behaviors. In addition to social behavior deficits, maternal and offspring immune activation may play a role in altering anxiety-like behaviors. Although unexpected, this study found that OVA mice spent more time in the open arm of the elevated plus maze when compared to PBS mice; these findings suggest that OVA mice experience less anxiety when compared to PBS mice. These results are not consistent with previous findings that MAA mice spend equal amounts of time in the open arm of the elevated plus maze when compared to PBS mice (Schwartzer, et.al., 2016). However, the fact that MAA mice are less anxious does not negatively affect the decreased social interactions seen in MAA mice. In fact, these results are interesting given that OVA mice engaged in less social behavior compared to PBS mice, despite showing decreased anxiety-like behavior in the elevated plus maze. This raises the question of how anxiety and arousal states may be contributing to changes in risk-taking behavior in MAA offspring. Specifically, anxiety disorders are often characterized by hyperarousal (i.e. heighten baseline of arousal) which supports a state of hypervigilance (scanning the environment for

threatening stimuli) (Green & Ben-Sasson, 2010). Thus, when OVA mice were placed on the elevated plus maze they may have been under aroused which resulted in less anxiety-like behaviors and more exploratory behaviors. Alternatively, OVA mice may be hyper-responsive to social-specific stimuli but not respond to other forms anxiety. Specifically, in the RSI task OVA mice may have been over-aroused and hypervigilant when faced with a social situation and thus exhibited less social behaviors but this anxiogenic behavior may not generalize to other non-social activities. In other words, OVA mice do not show generalized anxiety when compared to PBS mice but may experience more social anxiety when given a social-specific stimulus. Furthermore, these results, in the context of observed social behaviors, strengthen the notion that our model induced ASD-specific behavior deficits. Specifically, a decrease in social behaviors in the absence of increased anxiety-like behavior suggests that the deficits observed in anogenital sniffing and total social time cannot be attributed to increases in generalized anxiety. More likely, decreases in total social time are driven by decreased interest in social interactions. Use of a social motivation task, such as the three-chamber social approach task (Yang, Silverman, & Crawley, 2011), may be useful in specifically identifying the role of social motivation compared to motivation for other non-social exploration in the observed MAA-OVA behavioral deficits.

Unexpectedly, MAA females and OVA males spent more time in risk taking behaviors when compared to Control females and PBS males, respectively. Although this is surprising given that OVA mice are believed to be exhibiting sickness-like behaviors, and thus decreased arousal, following acute allergic asthma inductions, it is known that fear motivated behavior takes over sickness behavior (Dantzer, 2004). In a study done by Aubert and colleagues (1997) it was discovered that lactating mice injected with LPS and exposed to cold ambient temperature expressed pup-retrieving and nest-building behaviors indicating that behavioral expression of

LPS-induced sickness behavior can be lessened when the priority of another behavior is greater (Aubert, Goodall, Dantzer, & Gheusi, 1997). This same logic can be applied to explain the findings in the current study that allergic-asthma mice engaged in more risk-taking behaviors. While MAA females and OVA males may have been exhibiting sickness behaviors, these mice may have perceived the elevated plus maze as more of a life-threatening danger, when compared to controls, thus overcoming their sickness behavior temporarily in order to try and escape the maze by engaging in peeking and edge exploration behaviors. In the future, it would be important to address this by using the allergic-asthma model to challenge the immune system and then place mice in a more aversive behavioral task, such as the forced swim task, to determine if the mice can overcome their sickness behavior when placed in a more life-threatening situation.

Despite the finding that OVA-inductions decreased anxiety-like behavior, there are several limitations that need to be considered. First, the maze was left in the same orientation for each trial and not rotated. This is significant given that different environmental cues in the room (e.g. shadows and other stimuli) may contribute to open or closed arm preferences. However, the unexpected results are not likely attributed to the lack of rotation of the maze because secondary analysis of movements (data not shown) showed that mice travelled to both arms and did not favor the same arm. Although these data found that mice exposed to allergic-asthma, either during gestation or during the juvenile period, exhibited less anxiety-like behaviors, it remains unclear if these behaviors were a result of a lack of sufficient arousal in the experimental mice. For example, perhaps the aversive light was not bright enough to elicit a response, or the mice were already accustomed to being exposed to the testing environments and were not fearful of the maze. Taken together with the social approach findings, the elevated plus maze results suggest that the deficits in social behavior following allergic asthma exposure cannot be

attributed to an increase in generalized anxiety, but rather are due to changes in social-specific brain networks.

Restricted/Repetitive Behaviors. The marble burying task is a measurement of restricted/ repetitive behaviors thought to model the motor stereotypies associated with ASD. Results from the present study indicated that PBS females exhibited more restricted/repetitive behaviors when compared to OVA females. These results are not consistent with previous studies which indicated that MAA offspring buried more marbles when compared to PBS offspring (Schwartzer, et.al., 2016) and that a second hit could unmask behavioral deficits (Williamson et.al., 2011). Although these results are unexpected, they raise the question of the relationship between anxiety and restricted/repetitive behaviors given that MAA offspring were less anxious than PBS mice and MAA mice buried fewer marbles when compared to PBS mice. Importantly, marble burying has been shown to be independent of anxiety-like behavior (Thomas, Burant, Bui, Graham, Yuva-Paylor, & Paylor, 2009) and the current study's marble burying results support this distinction given the disparate findings between repetitive marble burying and elevated plus maze behaviors.

Importantly, the control mice for this study did not exhibit species-typical levels of marble burying behavior. Specifically, it has been found that naïve C57 males bury upwards of 50% of the marbles (Malkova et.al., 2012). However, the data in this study do not suggest this given that the Control-PBS mice in both sexes only buried approximately 15% of the marbles. The lack of appropriate control outcomes brings into question the validity of the marble burying data. Given that few animals in all conditions buried any marbles, external factors, for example lighting or odor cues, may have confounded the experimental task. Furthermore, locomotion was

not video recorded for this task thus these abnormal findings could be attributed to reduction in overall locomotor activity, possibly as a result of habituation to the testing area and environment from prior social and anxiety behavioral tasks. Due to the abnormal behavior of mice in this task, it remains inconclusive whether mice exposed to allergic-asthma *in utero* and in young adulthood exhibit more restricted/repetitive behaviors.

Limitations. While this study successfully generated preliminary behavioral data, it represents only one of several planned cohorts needed to reach an adequate sample size estimated from previous power analyses. Only 54 mice completed the study and of those 54, there were only 6-8 mice per condition when including sex as a factor. Furthermore, of the 6-8 mice in each group, the dams were split into two different exposure windows (i.e. early or late exposure to OVA during gestation). In the Schwartzer lab there is an ongoing project examining developmental windows for the timeframe that maternal immune activation influences offspring neurodevelopment, namely early-middle and middle-late gestation. These offspring were born from mothers there were part of an ongoing research project aimed at examining critical development windows. Thus, the findings from this thesis are highly underpowered given that only half of the mice in each of the 4 groups received the same MAA induction schedule. For example, in the MAA-OVA group half of the offspring were born to mothers who exhibited allergic-asthma in the early window and the other half were born to mother who were given inductions in the late window. To increase statistical power, mice were collapsed across timing conditions and this factor was not analyzed in the present analysis. However, it remains unknown whether timing, specifically exposure during early verses late maternal asthma inductions, differentially influences subsequent immune challenges on ASD-like behaviors.

Additionally, litter effects were not taken into account. Litter effects account for the fact that the offspring from rodents are essentially dizygotic twins and thus share similar prenatal and early postnatal environments, which leads to animals from the same litter having highly correlated results (Lazic & Essioux, 2013). Importantly, failing to account for litter effects can lead to false positive and false negative results based on variations in maternal care rather than the independently modified variable. Although this study controlled for litter effects to some extent in that the offspring of each dam were split equally into the PBS and OVA groups, there are still potential litter effects influencing the main effects for maternal treatment as these effects were not statistically accounted for in this study. Given that there were only two moms per treatment group and her pups were further divided into two groups it is hypothesized that liter effects may play a large role in the results of the current study and the potential confounds should be addressed when additional cohorts are available to increases statistical power.

Conclusion. Maternal immune activation has emerged as a potential risk factor for having a child with ASD. However, individuals with ASD also have been shown to have dysregulated immune systems. Thus, together these two pieces of evidence lead to the conclusion that these two factors may work together to produce ASD behavioral deficits. This study is the first attempt to use the double-hit hypothesis as a model for autism utilizing the novel maternal allergic-asthma model. The results suggest that allergic-asthma exposure, either during gestation or in the juvenile period, elicits species atypical behaviors in both social interaction task and in an anxiety-associated task. Overall, these findings support the notion that exposure to allergic-asthma, either during gestation or in the juvenile period, influences brain and behavior development to elicit abnormal social and anxiety-related behaviors.

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