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Sex Differences in the Anatomy of MAM E17 Treated Rats: A Developmental Model of
Schizophrenia

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Submitted in Partial Completion of the
Requirements for Departmental Honors in Psychology

Bridgewater State University

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Date: 05/09/2022

Abstract

Enlarged ventricles and reduced cortical volume are neuroanatomical abnormalities correlated with schizophrenia and typically more severe in males. The MAM model of schizophrenia is a developmental disruption model that involves exposing animals to a teratogen, methylazoxymethanol acetate (MAM), to reflect the neuroanatomical traits of schizophrenia. Rodents exposed to MAM on embryonic day 17 (E17) experience a reduction of cortical volume and increased ventricular volume. Measuring brain weight and ventricular volume can be used to inversely measure the severity of cortical reduction. The circling method was used to measure the lateral ventricles of a sample of 27 rodents; 8 MAM-females, 7 MAM-males, 5 control females, and 7 control males. The body weights of all subjects were recorded for 12 weeks. There were significant effects of sex, time, and treatment on body weight as well as significant time by treatment and time by sex interactions. Rodents exposed to MAM had lower body weight throughout the 12-week period and, after puberty, the females of both the control group and MAM group had lower body weight than male counterparts. Significant effects of sex and treatment were also found on brain weight. The rodents exposed to MAM had lower average brain weight than the control groups. Finally, there was a significant interaction of sex and treatment on ventricular volume. Male rodents exposed to MAM had greater ventricular volume than all other groups, respectively. These results reflect a similar pattern of ventricular enlargement and cortical reduction to that seen in humans diagnosed with schizophrenia.

Sex Differences in the Anatomy of MAM E17 Treated Rats: A Developmental Model of Schizophrenia

This study uses the Methylazoxymethanol acetate (MAM) developmental disruption model of schizophrenia to examine sex differences in the effects of exposing rodents to a neurotoxin (MAM) on gestational day 17. Rodents who experience prenatal exposure to MAM develop behavioral and anatomical abnormalities that reflect the pathology of schizophrenia. To gain insight on the possible pathological origin of sex differences observed in schizophrenia, this study uses the MAM model to analyze ventricular volume, brain weight, and body weight in a sample of female and male rodents.

Pathology of Schizophrenia

Schizophrenia is a complex mental disorder that is characterized by a combination of behavioral, cognitive, and anatomical traits. The likelihood of developing schizophrenia in one's lifetime is approximately 4.0/1,000 (Bhugra, 2005). According to a systematic review on the epidemiology of schizophrenia, the proportion of individuals diagnosed with schizophrenia within a population may vary depending on sex, migration status, and geographic location (McGrath et al., 2008). The cause of this disorder is still unclear, but research suggests it is the product of complex genetic and environmental interactions (Stilo & Murray, 2019).

The behavioral characteristics associated with schizophrenia are typically referred to as positive and negative symptoms. Data from the Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) research showed that roughly 19% of patients diagnosed with schizophrenia display prominent negative symptoms, 20% display prominent positive symptoms, and 21% display both negative and positive symptoms (Rabinowitz et al., 2013). Positive

symptoms, otherwise referred to as psychotic symptoms, are symptoms that are added to typical behavior such as hallucinations and delusions. Negative symptoms are behavioral and emotional deficits that fall into two domains: reduced expression of emotions and reduced pleasure and motivation (Blanchard & Cohen, 2005). According to the Brief Negative Symptom Scale (BNSS), these symptoms include flat affect (emotionless face), alogia (limited speech), asociality, anhedonia (lack of pleasure), and avolition (lack of motivation) (Kirkpatrick et al., 2010). These symptoms are considered negative because they are the result of typical behaviors being subtracted or taken away.

Cognitive symptoms of schizophrenia are similar to negative symptoms, as they are both deficits, but these symptoms concern mental processes such as memory and executive functioning rather than behavior. Cognitive impairment is one of the core traits of schizophrenia, affecting approximately 73% of those diagnosed (Palmer et al., 1997). Studies using the Wisconsin Card Sorting Test (WCST) have shown that patients with schizophrenia also struggle significantly with cognitive flexibility, which is the ability to adapt to rules that change (Everett et al., 2001). In addition, individuals with schizophrenia are observed to have impaired memory consolidation during sleep (Wamsley et al., 2012). The Measurement and Treatment Research to Improve Cognition in Schizophrenia evaluation, established by the National Institute on Mental Health (NIMH-MATRICES), identifies seven major areas of cognitive impairment associated with schizophrenia: working memory, attention/vigilance, verbal learning and memory, visual learning and memory, reasoning and problem solving, processing speed, and social cognition (Green et al., 2004). Behavioral and cognitive symptoms are linked to the frontal lobe abnormalities associated with schizophrenia (Mubarik & Tohid, 2016).

Anatomical differences in individuals affected by schizophrenia can be seen before and after behavioral symptoms appear. Lane and Albee (1966) found that lower weight at birth is associated with increased risk of developing schizophrenia. Later research supported this finding, showing that individuals who developed schizophrenia were more likely to be underweight at birth, throughout childhood, and after puberty (Sugawara et al., 2018; Wahlbeck et al., 2001). Differences in body weight seen in schizophrenia are theorized to be due to disruptions during development (Wahlbeck et al., 2001).

Significant physical abnormalities that are linked to schizophrenia can also be seen in specific structures of the brain. Reduced brain volume is a common feature of schizophrenia (Hajima et al., 2012; Vita et al., 2006). The reduction of brain volume is concentrated in regions of the cerebral cortex, the wrinkled outer layer of the brain, that play a major role in the cognitive functions impaired by schizophrenia. Research using MRI brain imaging suggests that the frontal lobe and medial temporal lobe structures, such as the hippocampus and basal ganglia, tend to be significantly reduced in volume in individuals diagnosed with schizophrenia (Wright et al., 2000). These areas of the brain are important for memory and executive functioning. Another common feature of schizophrenia is ventricular enlargement, which is the increase in volume of the lateral ventricles (Johnstone et al., 1976; Vita et al., 2006; Wright et al., 2000). The lateral ventricles are part of the brain's ventricular system. The ventricular system is made up of spaces within the brain filled with cerebrospinal fluid, which act as cushions for the brain and carry nutrients. More recent meta-analytic evidence shows that ventricular enlargement increases over time (Kempton et al., 2010). Since there is limited space inside the cranium, the ventricles likely increase in volume to compensate for the cortex decreasing in volume over time. Therefore,

evidence of progressive ventricular enlargement is indirect evidence of progressive cortical reduction.

Anatomical features of schizophrenia, such as ventricular enlargement and cortical reduction, are associated with the severity of cognitive impairment (Johnstone et al., 1976). Progressive cortical reduction, the decrease in volume of the cerebral cortex over time, in the brains of individuals diagnosed with schizophrenia is associated with increasing severity of cognitive impairment over time; as the cerebral cortex shrinks, the cognitive symptoms increase in severity (Kubota et al., 2015; Johnstone et al., 1976). Severity of cognitive impairment and negative behavioral symptoms is also positively correlated with increases in ventricular volume (Cahn et al., 2006; Haijma et al., 2012; Johnstone et al., 1976). Ventricular volume is negatively associated with cortical volume, meaning that those who experience a reduction of cortical volume are also found to have significantly larger ventricles (Arango et al., 2008). These findings show that volumetric measurement of the lateral ventricles can be used to predict both the extent of cortical reduction as well as the severity of cognitive impairments in those affected by schizophrenia. This study uses the measurement of the lateral ventricles, along with brain weight, as a way to indirectly measure cortical reduction.

Sex Differences in Schizophrenia

Research on sex differences in schizophrenia has been extensive but does not produce much consensus. Ultimately, the interaction between sex and pathology of schizophrenia is still ambiguous due to inconsistencies in methodologies and sample sizes across studies (Goldstein, 1993). Many studies include fewer female patients, which may lead researchers to erroneously conclude that there is no significant difference in the way schizophrenia manifests in females and males. Moreover, many studies involving animal models purposefully exclude female subjects

because of the confounding effects of sex hormones such as estrogen. Still, there is some consistent evidence of behavioral and anatomical sex differences in schizophrenia.

The most frequently studied sex differences concern behavioral symptoms. Females tend to experience later onset of symptoms compared to males, but the overall prevalence of schizophrenia is not significantly different for females and males (Jablensky et al., 1992; Kao et al., 2013). Females diagnosed with schizophrenia tend to experience more positive symptoms (auditory hallucinations and delusions of persecution) and are more responsive to antipsychotic medication (Leung & Chue, 2000). Males tend to experience more severe negative symptoms, such as deficits in social functioning, before and after being diagnosed with schizophrenia (Canuso & Pandina, 2007; Rietschel et al., 2015). Interestingly, poor cognitive functioning in males diagnosed with schizophrenia is related to the severity of anatomical abnormalities, but not in females diagnosed with schizophrenia (Gur et al., 2000).

Neuroanatomical differences between males and females diagnosed with schizophrenia are not as consistently replicated as behavioral sex differences. Several studies suggest that there are not significant sex differences in anatomical traits of schizophrenia (Flaum et al., 1995; Wright et al., 2000). However, others have argued that males have more severe neuroanatomical abnormalities (Gur et al., 2000; Leung & Chue, 2000; Nopoulos et al., 1997). One difference that is consistently supported by empirical research is that males diagnosed with schizophrenia typically have significantly greater ventricular volume than females diagnosed with schizophrenia (Arango et al., 2008; Nopoulos et al., 1997). The current study analyzes sex differences in ventricular volume using a developmental animal model of schizophrenia in order to better understand sex differences observed in humans with schizophrenia.

Etiology of Schizophrenia

Schizophrenia is not caused by one single factor. This complicated disorder is theorized to be the result of many genetic and environmental factors (Stilo & Murray, 2019). There is evidence of a strong genetic component in developing schizophrenia; several genes have been identified and shown to increase the risk (Prata et al., 2019; Schizophrenia Working Group of the Psychiatric Genomic Consortium, 2014). In addition, certain environmental factors that can increase the risk of developing schizophrenia have also been identified. Factors that can increase the probability of an individual developing schizophrenia, especially in combination with genetic factors, include childhood trauma, low socioeconomic status at birth, immigration, living in urban areas, and complications during pregnancy and birth (Bourque et al., 2010; Cannon et al., 2002; Harrison et al., 2001; Varese et al., 2012; Vassos et al., 2012). Complications during pregnancy can disrupt typical brain development, which may lead to abnormalities in the brain during adulthood. For example, prenatal maternal infection has been found to increase the risk of developing schizophrenia as an adult (Buka et al., 2001). Even more, the neurons of people diagnosed with schizophrenia tend to show a pattern of disorganization that can only be the result of a disruption in the neuromigration that occurs during the second trimester of pregnancy (Scheibel & Kovelman, 1981). Findings on the correlation between prenatal injury and the development of schizophrenia serve as the basis for many neurodevelopmental theories of schizophrenia.

Due to ethical and practical reasons, neurodevelopmental theories of schizophrenia are often studied through the lens of animal models. According to the ethical principles of psychologists established by the American Psychological Association (n.d.), researchers must make an effort to minimize the harm done to human participants. Abiding by these standards, humans cannot be intentionally exposed to a chemical that disrupts the development of the

nervous system. Further, there are too many confounding variables to investigate a causal link between accidental exposure to such chemicals and the development of schizophrenia. It is for these reasons that controlled laboratory experiments using animal models are used to investigate the role that prenatal brain injury plays in the development of brain abnormalities similar to those seen in schizophrenia.

There are a multitude of theories and animal models used to study the potential causes and the development of schizophrenia. Animal models are generally divided into four categories: drug-induced, genetic, lesion, and developmental (Jones et al., 2011). Drug-induced models typically involve the administration of drugs that produce schizophrenia-like behaviors in animals. For example, repeated administration of stimulants, such as amphetamines, or dissociative anesthetics, such as PCP, impairs cognitive functioning, learning, and social behavior in ways that mimic schizophrenia (Mouri et al., 2007). Genetic models involve identifying and altering genes in a way that increases the likelihood of developing traits comparable to schizophrenia. For example, the *Disc1* genetic model is commonly used to study schizophrenia using mice and rats. *Disc1* is a gene that is suggested to play an important role in neuromigration during prenatal brain development, which further supports the theory that schizophrenia has a developmental etiology (Hikida et al., 2007; Kvajo et al., 2008). Lesion models involve causing damage to small regions of the brain early in life that cause cognitive and behavioral traits that are reflective of the symptoms of schizophrenia, such as impairments in working memory produced by neonatal lesioning of the hippocampus (Lipska & Weinberger, 2000). Developmental animal models include exposure to teratogens, chemicals that disrupt typical development, during gestation in order to produce effects that mimic the pathology of schizophrenia. The prenatal immune activation model is based on maternal exposure to the

influenza virus during pregnancy (Brown & Derkits, 2010; Buka et al., 2001). The MAM E17 model involves maternal exposure to methylazoxymethanol acetate on day 17 of pregnancy in rodents (Lodge & Grace, 2009).

Compared to other models, the MAM E17 developmental disruption model reflects the complex behavioral, physiological, and neuroanatomical features of schizophrenia most accurately (Modinos et al., 2015). Drug-induced models of schizophrenia fail to address the developmental aspect of the disorder. Genetic models have not shown that there is a specific genetic alteration that always leads to the development of schizophrenia, although many genes have been linked to a greater likelihood of developing the disorder (Harrison & Weinberger, 2005). Lastly, lesion models lack validity because although the behavioral and cognitive effects may reflect schizophrenia, there is no evidence that people diagnosed with schizophrenia develop lesions in their brains. Other animal models may reflect a portion of the pathological profile of schizophrenia, but the MAM developmental model provides the most comprehensive array of symptoms that mirror schizophrenia. The MAM model shows that prenatal exposure to MAM leads to an overactive dopamine system, abnormal functioning in the hippocampus and frontal lobes, progressive cortical reduction, increased neuronal density, increased response to stimulants, and cognitive dysfunction, all of which are traits of schizophrenia (Lodge & Grace, 2009, 2010, 2012).

The MAM Model of Schizophrenia

The MAM model of neurodevelopmental disruption uses rodent subjects to make inferences about the development of schizophrenia in humans and has received wide empirical support. This model was first developed by neuroscientists Dr. Daniel J. Lodge and Dr. Anthony A. Grace (2010) and is commonly used in research today.

Methylazoxymethanol acetate (MAM) is a DNA methylating agent that targets the central nervous system and disrupts the division of cells. DNA methylation is an epigenetic mechanism that involves directly interfering with the DNA and results in modification of gene expression (Moore et al., 2012). MAM is a controlled substance that humans do not typically encounter in the environment but can be found in the seeds of some cycad plants (Nair & van Staden, 2012). To produce the intended effects, it is necessary to expose rodent subjects to MAM on gestational day 17 (E17), which is the point during the embryonic period when cortical structures are being developed and subcortical structures are mostly complete in their development (Lodge, 2013; Moore et al., 2006). If MAM is exposed to subjects at any other time, such as embryonic day 15 (E15), the resulting neuroanatomical effects are severe and reflect global brain damage but do not reflect the disruption to specific brain areas associated with schizophrenia (Lodge, 2013). When MAM is administered on E15, the reduction of the whole brain, hippocampus, and cerebellum volume is far more severe than what would reflect the morphological traits of schizophrenia (Johnson et al., 2006). When this neurotoxin is exposed to rodents on gestational day 17, it induces behavioral, physiological, and anatomical symptoms that mirror the pathology of schizophrenia (Lodge & Grace, 2009).

The behavioral effects of exposure to MAM are similar to the cognitive and negative symptoms observed in schizophrenia. Research shows that male rats exposed to MAM on E17 show decreased social interaction and difficulties with learning and cognitive flexibility tasks (Lodge & Grace, 2009). Impaired cognitive flexibility is the tendency to continue making incorrect responses during tasks, this is often referred to as perseverative responding and is also seen in individuals affected by schizophrenia (Everett et al., 2001). These behavioral effects are

linked to the abnormalities in brain functioning that are caused by exposure to the teratogen MAM.

Administration of MAM on embryonic day 17 also impacts the physiology of rodent brains. Male mice exposed to MAM are observed to have decreased size and function of the hippocampus and show decreased function of the prefrontal cortical region (Chalkiadaki et al., 2019). The hippocampus and prefrontal cortical region are important for working memory and visuospatial tasks, which can be measured in rodents using water mazes. The male mice exposed to MAM made more mistakes in the mazes, which is indicative of the decreased function and size of these cortical regions (Chalkiadaki et al., 2019). These effects are similar to the neurophysiological abnormalities typically seen in individuals diagnosed with schizophrenia.

The structural effects of developmental disruption caused by MAM also reflect structural abnormalities seen in humans with schizophrenia. Male MAM E17-treated rodents are found to have significantly reduced cortical volume in regions that parallel the cortical reduction observed in humans affected by schizophrenia (Chalkiadaki et al., 2019; Lodge et al., 2009; Moore et al., 2006). Chin and colleagues (2010) found that male rodents exposed to MAM also have increased ventricle size. Ventricular enlargement and cortical reduction are both features typical of schizophrenia (Haijma et al., 2012; Johnstone et al., 1976; Vita et al., 2006; Wright et al., 2000). There is evidence that males diagnosed with schizophrenia have more severe ventricular enlargement than females (Arango et al, 2008; Nopoulos et al., 1997), but this has not been studied using the MAM model.

Using the MAM Model to Study Sex Differences in Anatomy

To examine the anatomical sex differences in the MAM model of schizophrenia, this study compares data from 27 rodents. Evidence shows that there are sex differences in the pathology of schizophrenia, but few studies have used animal models to examine the significance or cause of these sex differences. The variables analyzed included the average volume of the left and right lateral ventricles in cubic millimeters, the postmortem brain weight in milligrams, and the body weight measured in grams over a span of 12 weeks. In this study we investigate the following question: are there significant sex differences in average ventricular volume and average brain weight between of rodents exposed to the teratogen MAM? The rodents will be divided into four groups based on sex and treatment: females exposed to MAM, males exposed to MAM, control females, and control males. We hypothesized that the male rodents exposed to the teratogen MAM would have an average ventricular volume significantly greater than females exposed to MAM and the male controls.

Method

Preparation of Tissue Samples

The tissue samples used in this research project were derived from a past study conducted by Dr. Stephanie Penley, Professor of Psychology at Wheaton University, with IACUC approval (#95-2016). No live animal subjects were involved in the following research.

Dr. Stephanie Penley's study "E17 MAM Exposure and Working Memory" (in preparation) was conducted at Wheaton College in Massachusetts from 2016 to 2019. This research involved nine female Sprague-Dawley (albino) rats, also referred to as "dams". On gestational day 17 (GD17, E17, or 17 days into pregnancy) five of the dams received an intraperitoneal, or abdominal, injection of methylazoxymethanol acetate (MAM). MAM is a

toxin that causes long-term behavioral and neuroanatomical effects by disrupting the division of cells within the central nervous system. Following birth, the MAM-exposed and control pups were culled and sorted into litter sizes of 10, with an even number of males and females in each litter. This was necessary to control for the maternal behavior and hormonal levels among the pups. A final sample size of 22 was used for the MAM groups and 18 for the control groups with equal number of males and females were included in each condition (control male = 18, control female = 18, MAM-male = 22, MAM-female = 22). The rats were single housed during pregnancy and weaning in BPA-free polycarbonate cages. After weaning, the animals were pair housed. The temperature was maintained at $21 \pm 2^\circ\text{C}$, food and water were available ad libitum and the lights were maintained on a 14:10 light cycle with lights going on at 7:00 a.m.

The dams produced a total of 80 offspring, each with a unique identification number. Due to time constraints, the tissue samples from only 27 of the total 80 offspring were analyzed. All offspring were weaned from their mothers on postnatal day 21 (P21 or 21 days after birth). The body weight of each rodent was recorded in grams for 12 successive weeks beginning on P21 until P95. Every day the activity levels, food consumption, and general well-being of all animal subjects were monitored by Dr. Penley and colleagues.

After reaching developmental maturity around postnatal day 95, the rodents were euthanized by receiving a lethal dose of pentobarbital, a barbiturate that inhibits the central nervous system. The subjects were then given a transcardial perfusion of 0.9% saline and 4% paraformaldehyde; this procedure utilizes the vascular system of the anesthetized animal subject to circulate a chemical that acts as a fixative and preservative for tissue. Following chemical euthanasia and absence of all signs of life, cervical dislocation (decapitation) was used as a secondary method of confirming the sacrifice of all animal subjects.

Surgery was then performed to remove the brains of all animal subjects. The brains of each subject were stored in paraformaldehyde and 10% sucrose (cryoprotectant) in preparation to be divided into sections for analysis. Using a cryostat machine, the brains of the animal subjects were sectioned into sequential coronal slices. A cryostat machine freezes the preserved tissue at -24°C and uses a microtome, a sharp rotating blade within the machine, to cut the tissue into extremely thin slices. Coronal sections, which divide the tissue vertically from front to back, were used to aid in the visualization and measurement of brain structures. Each section of brain tissue is 50 micrometers thick. Adhering to the technique established by Simpson and Vicario (1991), every fifth section was stored for analysis. Using a sampling interval of 5 (every fifth section) to measure volume rather than all sections has been shown to be significantly more efficient without compromising accuracy (Gundersen & Jensen, 1987). Sections being kept for microscopic analysis were mounted on glass slides with a clear fixative (Permount) and Nissl stained. Nissl staining involves the application of (0.1%) Thionin to tissue samples. Thionin is a chemical dye that improves the visualization of brain structure by coloring the DNA and RNA of each nerve cell a dark blue.

Out of the 80 offspring produced, 27 were chosen randomly and analyzed in the following study. In order to remain unbiased, the sex and treatment of the subjects were not disclosed during the data collection phase of the study. For the purpose of data analysis, the 27 subjects were divided into four groups: Group 1 included 8 female offspring exposed to MAM (MAM-females), Group 2 included 7 male offspring exposed to MAM (MAM-males), Group 3 included 5 non-exposed female offspring (control females), and Group 4 included 7 non-exposed male offspring (control males). Data from all subjects was collected using the circling method, as described below.

Measurement of Lateral Ventricles

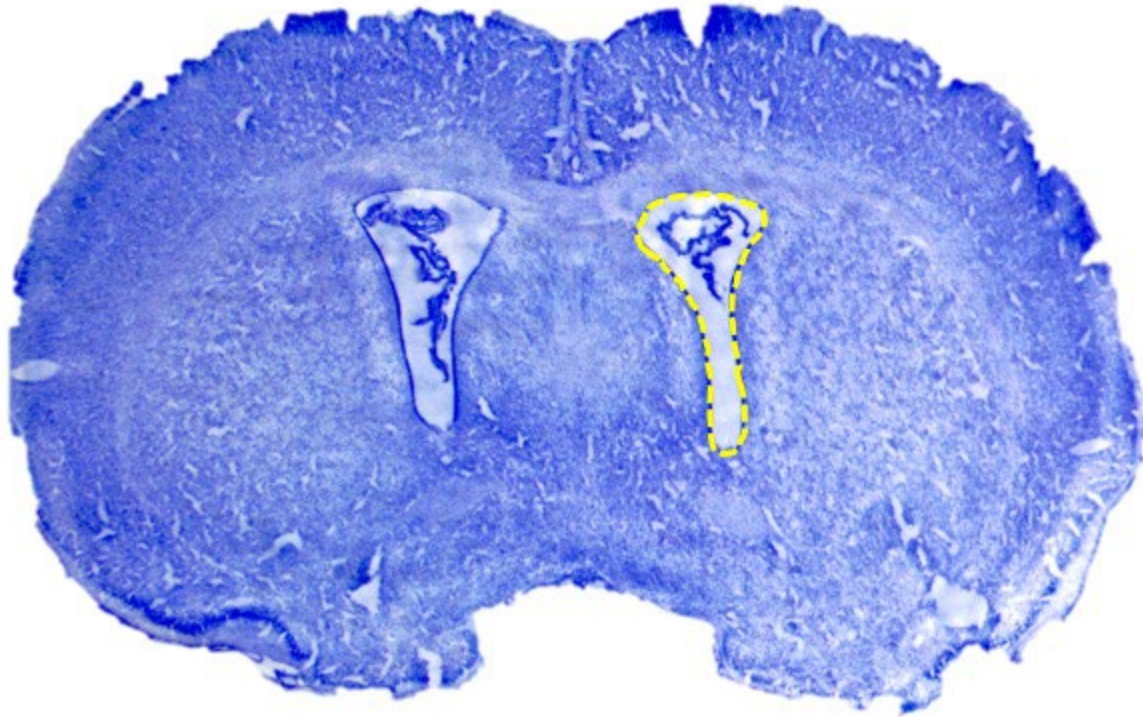
The circling method refers to a technique involving the use of image processing software to carefully trace the border of a specific region of the brain (Grisham & Arnold, 1995; Grisham et al., 2008; Simpson & Vicario, 1991). The software AmLite was paired with a digital microscope to take photographs of each section of tissue. ImageJ, the software used for microscopic analysis in the current study, is able to calculate the area of a circled region in millimeters squared. After measuring the area of the lateral ventricles in each successive section of the brain, the sum of these areas is multiplied by both the thickness of the slice and the sampling interval. The thickness of the slices (50 μm or 0.5 mm) is multiplied by the sampling interval (5) to produce the multiplier 0.25 mm. The stereological (three dimensional) formula used to estimate the volume of the entire lateral ventricle is: $\text{VOLUME mm}^3 = \text{SUM OF AREAS mm}^2 * 0.25 \text{ mm}$.

The lateral ventricles are one set of the four cerebral ventricles within the brain's ventricular system. Ventricles are interconnected cavities containing choroid plexus, which produces the cerebrospinal fluid that fills the ventricular system and carries nutrients throughout the brain. Anatomical points on the rodent skull (Bregma, the coronal suture, and the sagittal suture) and internal brain structures were used to determine the point within the brain where measurement of the lateral ventricles will begin and end. Bregma is the intersection of the coronal suture and sagittal suture on the top of the rodent skull. The coronal suture is a line from left to right on the skull that marks the joining of the front-facing frontal bone from the parietal bones on the left and right sides of the skull. The sagittal suture of the skull intersects perpendicularly with the coronal suture then recedes to the back of the skull and marks the joining of the parietal bones on either side of the skull. Using the Bregma intersection as a

reference point, the distance that the lateral ventricles span within the brain can be described using millimeters. The point at which measurement of the lateral ventricles began is approximately Bregma +2.76 mm (2.76 mm in front of Bregma), where the lateral ventricles begin to appear in the serial coronal sections of the typical rat brain (Paxinos & Watson, 2014). The established end point for measurement is the point at which the lateral ventricles of the typical rodent brain are no longer visible, approximately Bregma -4.92 mm (4.92 mm behind Bregma). These operational definitions help reduce the subjectivity of measurements. The lateral ventricles will be distinguished from other brain structures based on the relative position, shape, and cytoarchitecture. The borders of the ventricles are defined by a dense line of cells that appear darker than the surrounding neurons. Inside this border the ventricles are mostly acellular, except for small amounts of dark-pigmented choroid plexus cells. In the instance that the lateral ventricles on one section of tissue were damaged or otherwise unable to be measured, the ventricular volumes of the previous slide and next slide were averaged and used in data analysis. Figure 1 below depicts a coronal section of a rodent brain viewed under a microscope where the lateral ventricle is circled in yellow.

Figure 1

Coronal Section of Rodent Brain Highlighting the Lateral Ventricles



Note: This image depicts a coronal section of a rodent brain at approximately Bregma -0.12 mm. Highlighted in yellow is the left lateral ventricle (seen on the right side as the brain is facing forward). Within the lateral ventricles is the choroid plexus, which is stained dark blue. The choroid plexus is a type of epithelial cell that produces the cerebrospinal fluid that fills the ventricles (Lun et al., 2015).

Data Analysis

Separate analyses were run to examine the effects of sex and treatment on brain weight, body weight and lateral ventricular volume. As established by previous studies, nested ANOVA tests were used to examine the interaction between sex and treatment effects (McClure et al., 2006) after establishing that there were no significant birth cohort (dam) effects. To compare brain weight differences, a 2 (sex) x 2 (treatment) nested ANOVA design was used. For ventricular volume analysis, a nested design repeated measure ANOVA, 2 (sex) x 2 (treatment) x

2 (repeated brain volume measurement) was used. Finally, for body weight a 2 (sex) x 2 (treatment) by 12 (repeated week measurement) ANOVA was used.

Results

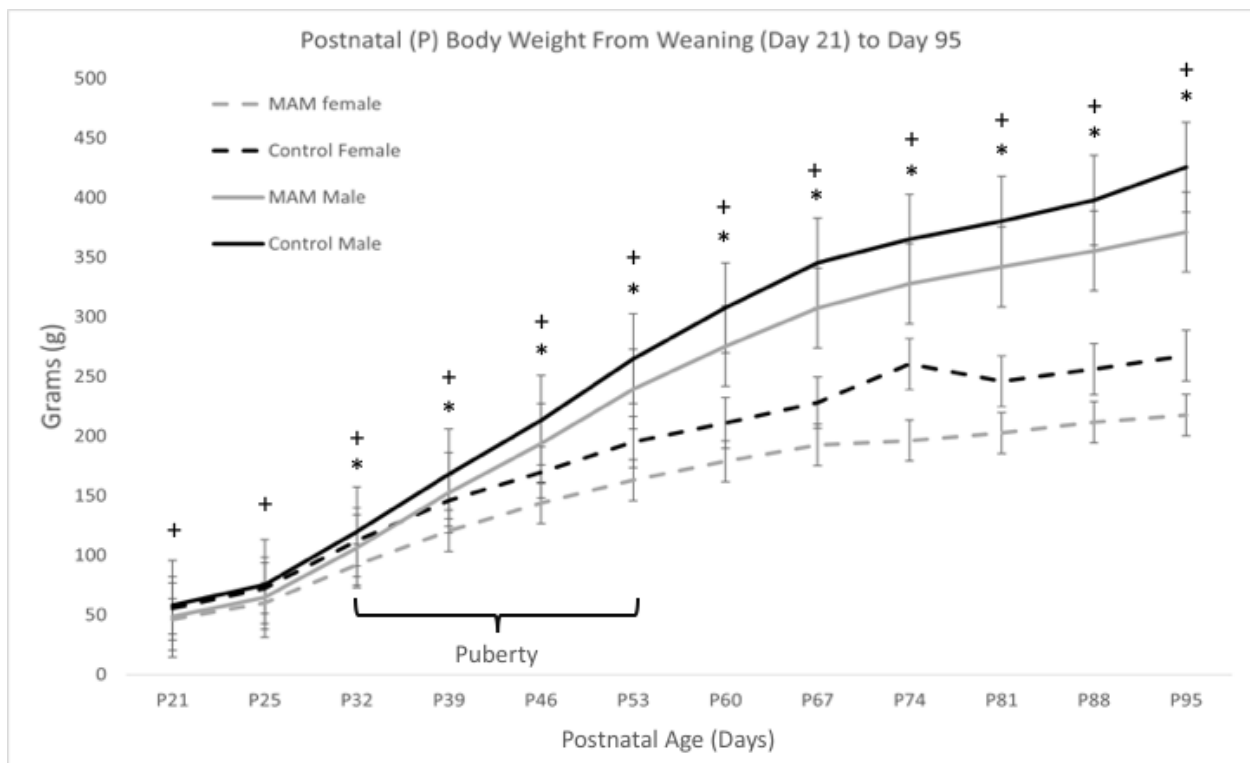
Data analysis showed that the body weights of the rats were affected by sex, treatment, and time. As previously stated, the rats were weighed once a week for a 12-week period. All rats showed an increase in body weight across those 12 weeks. A 2 (sex) x 2 (treatment) x 12 (weeks) design repeated measures ANOVA determined that there was a significant effect of time (in weeks) on the body weight of all rodents, indicating that they increased in weight over time, $F(11, 253) = 1389.73, p < .001, \eta^2 = .984$. There was also a significant effect of sex on body weight $F(1, 23) = 155.91, p < .001, \eta^2 = .871$, and a week by sex interaction, $F(11, 253) = 129.82, p < .001, \eta^2 = .850$, indicating that male and female rats grew at different rates. The female rodents showed more than a 300% increase in body weight through the developmental period (MAM-females = 371% increase and control females = 383%). As is expected based on typical adult body size differences between males and female rats, male rats showed a greater percentage increase in body weight from weaning (P21) through the end of the 12-week period (P95), increasing more than 600% in body weight (MAM-males = 666% increase, control males = 633% increase). The greater percentage increase in body weight of MAM-males may be due to lower initial body weight at P21 (see Figure 2.)

Exposure to MAM had a significant impact on body weight over time. There was a significant effect of treatment on body weight ($F(1, 23) = 24.09, p < .001, \eta^2 = .512$) as well as a week by treatment interaction ($F(11, 253) = 8.68, p < .001, \eta^2 = .274$), indicating that MAM had significant effects on body weight over time. After finding significant effects of both sex and treatment as well as significant sex by week and treatment by week interactions, post hoc

analyses were run. In line with previous studies, separate 2 (sex) x 2 (treatment) ANOVAs were run to analyze the body weights at each of the 12 weeks (see McClure et al, 2006). From P21 to P95, there was a significant effect of treatment on body weight, all $p < .001$. Beginning at puberty (P32), there was a significant effect of sex on body weight at each week, all $p < .001$. Each week, control males had the highest body weight (P21 through P95). The control females had the second highest body weight on P21, P25, and P32, but after that the MAM males consistently had the second highest body weight (P46 through P95). MAM females had the lowest body weight each week. This data supports the hypothesis that sex and exposure to MAM impact body weight over time. Figure 2 (below) displays the change in body weight among all groups over time.

Figure 2

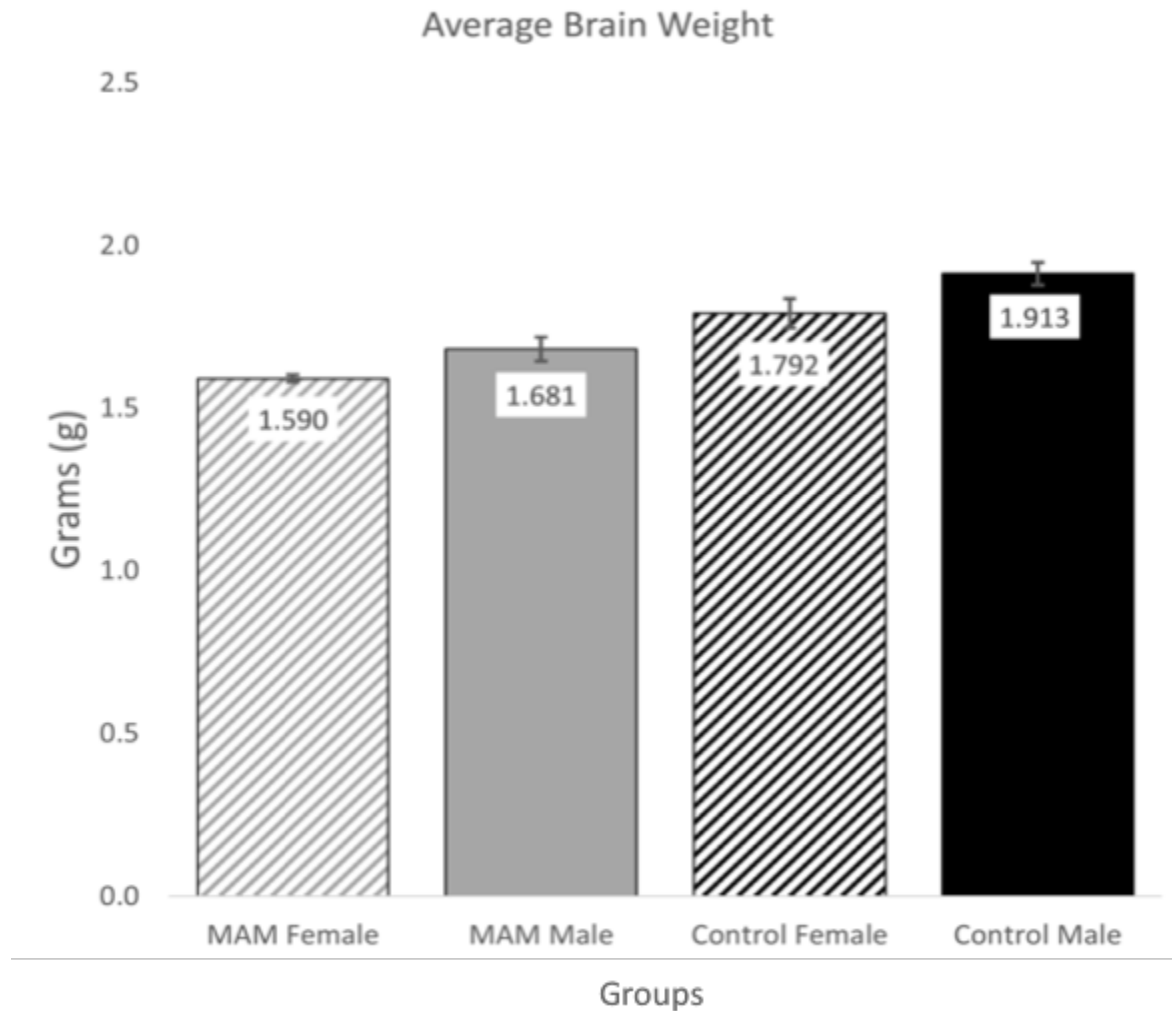
Postnatal (P) Body Weight from Weaning (Day 21) to Day 95



Note: This graph depicts the changes in average body weights of the four groups over a period of 12 weeks, beginning at weaning on P21 (postnatal day 21) and ending on P95. The x-axis represents postnatal age in days. The y-axis represents body weight in grams. The puberty period for female rats is typically P32-P34, the puberty range for male rats is typically P45-P48 (Sengupta, 2013). After puberty, the average body weights of both control groups remain greater than both MAM groups, respectively. Within the control groups, the females had consistently lower average body weight. This difference is also found in the MAM groups. MAM-females have the lowest average body weight at each of the 12 weeks. Asterisks represent a significant effect of sex. Crosses represent a significant effect of treatment.

Similarly, brain weight was also impacted by exposure to MAM (treatment) and sex. To compare brain weight differences, a 2 (sex) x 2 (treatment) nested ANOVA design was used. The 2 x 2 ANOVA revealed that there was a significant effect of sex on brain weight, $F(1, 23) = 11.53, p < .001, \eta^2 = .334$. We also found a significant effect of treatment on the brain weight of the rodents, $F(1, 23) = 45.54, p = .002, \eta^2 = .664$. Control males had the greatest brain weight ($M = 1.91$ g, $SEM = 0.04$). Male rodents exposed to MAM had an average brain weight that was 12.2% less than control males, with an average brain weight of 1.68 g ($SEM = 0.02$). As predicted by differences in body weight, control females ($M = 1.79$ g, $SEM = 0.04$) had 6.4% lower brain weight than control males. Similarly, MAM-females ($M = 1.58$ g, $SEM = 0.02$) had an average brain weight that was less than that of control females, with a decrease of 16.9%. Finally, MAM-males had a 5.4% increase compared to MAM-females. These results support the prediction that brain weight is influenced by both sex and exposure to MAM. Figure 3 (below) shows the average brain weights of all groups as well as the standard error of the means.

Figure 3

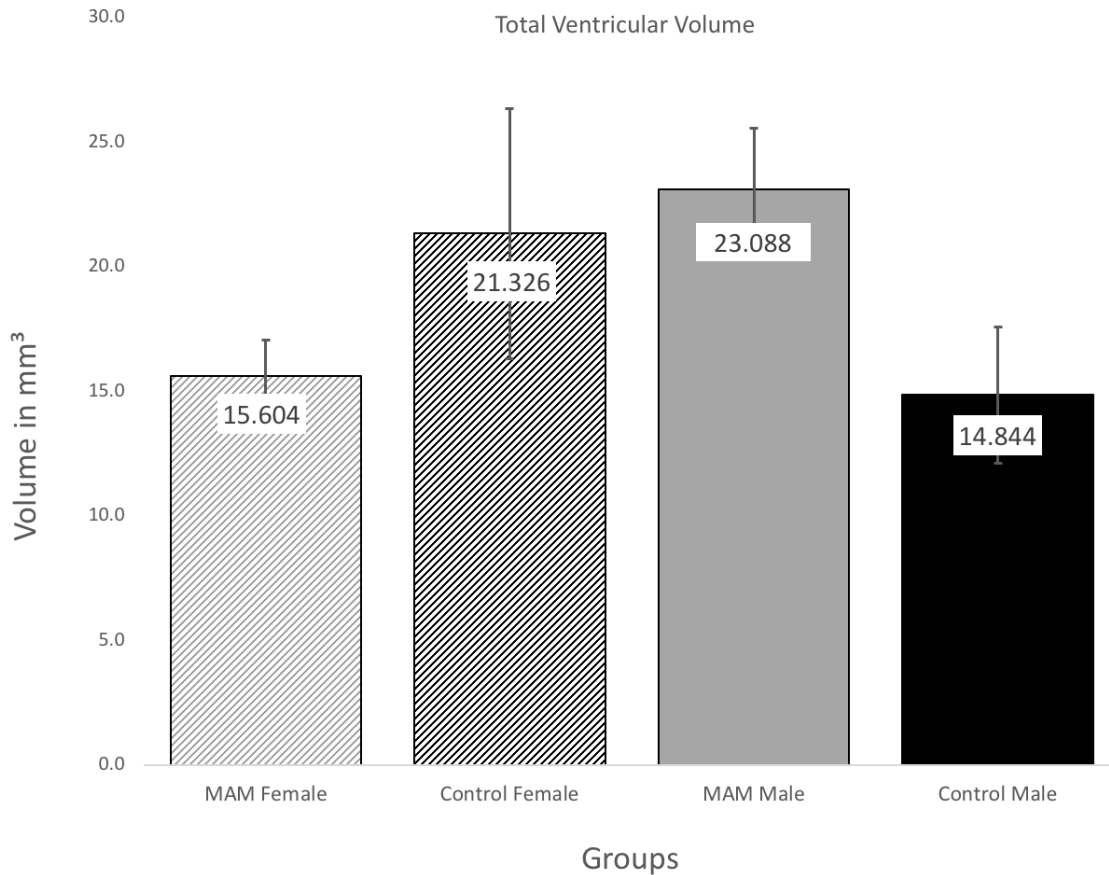
Average Brain Weight

Note: This graph depicts the effects of both sex and exposure to MAM on average brain weight of rats. The x-axis represents the four groups (MAM-females, MAM-males, control females, and control males). The y-axis represents brain weight in grams. The brain weights of both control groups are higher than both MAM groups, respectively. Within the control groups, females had lower average brain weight. Similarly, within the MAM groups, MAM-females had lower average brain weight.

Statistical analysis showed that sex and treatment had an interactive effect on ventricular volume. For ventricular volume analysis, a nested design repeated measure ANOVA 2 (sex) x 2 (treatment) x 2 (repeated brain volume measurement) was used to examine the influence of MAM and sex on ventricular volume. The repeated measures analysis showed no significant effect of volume measurement across left and right hemispheres for sex or treatment. There was also no significant effect of sex or treatment between groups, however there was a sex by treatment interaction, $F(1, 23) = 7.496, p = .012, \eta^2 = .246$. Since there were no differences between left and right hemispheres, we compared sex and treatment differences of the total ventricular volume. The average ventricular volumes of MAM-males ($M = 23.09 \text{ mm}^3, SEM = 2.27$) was 32% greater than MAM-females ($M = 15.60 \text{ mm}^3, SEM = 1.35$). Further, the MAM-males had a greater ventricular volume than control males ($M = 14.84 \text{ mm}^3, SEM = 2.53$), with MAM-males having 36% greater average ventricular volume than control males. Interestingly, the ventricular volume of control females ($M = 21.33 \text{ mm}^3, SEM = 4.43$) was greater than that of MAM-females. This data indicates that exposure to MAM affects the ventricular volume of male and female rodents differently. Figure 4 (below) shows the average ventricular volumes of all groups as well as the standard error of the means.

Figure 4

Total Ventricular Volume



Note: This graph depicts the effects of both sex and exposure to MAM on average ventricular volume of rats. The x-axis represents the four groups (MAM-females, MAM-males, control females, and control males). The y-axis represents average ventricular volume in mm³. This figure combines left and right ventricular volume because no meaningful difference was found between the hemispheres. There was a significant interaction of sex and treatment on ventricular volume. This data suggests that exposure to MAM does not have the same effect on the ventricular volume of male and female rats.

Discussion

This study examined differences in the anatomy of male and female rats following exposure to MAM on embryonic day 17. We are particularly looking at differences in the neuroanatomical features that mimic schizophrenia. While several studies have examined the impact of MAM, no other studies have made direct comparisons between males and females exposed to MAM, and so this study offers novel insights to the field. Understanding sex differences in the MAM model can help us gain a better understanding of the neurodevelopmental model of schizophrenia and contribute to possible treatments.

As explained before, research has shown that people diagnosed with schizophrenia tend to have reduced brain volume, increased ventricular volume, and lower body weight throughout development (Haijma et al., 2012; Johnstone et al., 1976; Lane & Albee, 1966; Vita et al., 2006; Wahlbeck, 2001; Wright et al., 2000). There is evidence that these anatomical abnormalities may be the result of prenatal injury. For example, the hippocampal neurons of people diagnosed with schizophrenia tend to show a pattern of disorganization that can only be the result of a disruption in the neuromigration that occurs during the second trimester of pregnancy (Scheibel & Kovelman, 1981). Similarly, male rodents that experience prenatal injury by being exposed to MAM have also been shown to have disorganized hippocampal cells (Moore et al., 2006). Even more, male rodents exposed to MAM have been shown to experience reductions in brain volume as well as enlarged ventricles (Chalkiadaki et al., 2019; Chin et al., 2010; Lodge et al., 2009; Moore et al., 2006). In line with previous findings, this study supports evidence that exposure to MAM impacts brain volume, ventricular volume, and body weight of rats in a way that mimics the anatomical traits correlated with schizophrenia. However, past research has not examined sex differences in the anatomical effects of being exposed to MAM. Exposure to MAM affected the body weight and brain weight of female and male rodents in a similar way, which suggests that

gross measures may not be able to tease apart the subtle sex differences in the anatomical effects of being exposed to MAM. In addition, this study found that exposure to MAM impacts the ventricular volume of female and male rodents differently. This finding indicates that sex differences in the neuroanatomical effects of MAM may be more subtle and should inform how future studies examine these variables.

As previously stated, individuals diagnosed with schizophrenia are more likely to have lower body weights at birth, before puberty, and after puberty (Lane & Albee, 1966; Sugawara et al., 2018; Wahlbeck, 2001). In this study, the data indicates that exposure to MAM reduces body weight at birth, before puberty, and after puberty in a way that is modulated by sex. Significant reductions in growth (as measured by body weight over time) were seen following exposure to MAM for both male and female rats. While sex differences in weight arose during puberty, with male rats increasing weight more rapidly than their female counterparts, the effect of MAM was maintained throughout development and into adulthood. Upon weaning, around 21 days after birth (P21), both control groups had greater average body weight than both MAM groups. Until puberty, the differences in body weight were primarily due to exposure to the teratogen MAM. The puberty period in rodent development begins around postnatal day 32 (P32) (Sengupta, 2013). After puberty, the differences in body weight were the result of both exposure to MAM and sex. After P32, both the control males and MAM-males had greater average body weights than control females and MAM-females, respectively. From P32 until P95, there are statistically significant effects of sex on the average body weights at each point of measurement. This is evidence that the anatomical effects of MAM are present before and after puberty, which replicates findings from Chin et al (2010). Lower body weight was consistently seen as an effect

of exposure to MAM in both males and females, which is further evidence of the developmental disruption caused by MAM.

In addition to changes in body weight, evidence shows that reduced brain weight is also associated with schizophrenia (Cahn et al., 2002). Reduced brain weight can provide indirect evidence of cortical reduction, a consistently observed trait of schizophrenia (Haijma et al., 2012; Vita et al., 2006). Research using the MAM model of schizophrenia have also found reductions in brain volume of male rodents (Chin et al., 2010; Lodge & Grace, 2009; Moore et al., 2006). This study found that both sex and treatment influence brain weight. As is typical, male rats had greater brain weights than female rats, which is reflective of differences in body weight. Significant reductions in brain weight were seen in both MAM-males and MAM-females. Importantly, previous studies did not include female rodents when examining the effects of MAM on brain weight. The current study reveals that MAM caused similar reductions in the brain weights of both male and female rodents. This data parallels findings of cortical reduction in MAM-males, but findings on cortical reduction in MAM-females have not been replicated.

Ventricular enlargement is another anatomical feature that is typically found in people diagnosed with schizophrenia (Johnstone et al., 1976; Vita et al., 2006; Wright et al., 2000). Studies have shown that males diagnosed with schizophrenia tend to have greater ventricular enlargement than females diagnosed with schizophrenia (Arango et al, 2008; Nopoulos et al., 1997). Previous research has not used the MAM model to compare severity of ventricular enlargement between female and male animals. The data analysis shows that there is an interaction of sex and MAM treatment on the ventricular volume of rodents. The ventricular volume of MAM-males increased while the ventricular volume of MAM-females decreased (see Figure 3.) This data supports research conducted by Chin and colleagues (2010) which found

that male rodents exposed to MAM experienced significant ventricular enlargement in both hemispheres of the brain. The current findings suggest that female rodents, however, did not experience significant ventricular enlargement. This is evidence that MAM impacts the ventricular system of male and female rodents differently.

These results demonstrate that the difference in ventricular enlargement between male and female rodents exposed to MAM on E17 may mirror the difference in ventricular enlargement between male and female patients diagnosed with schizophrenia. This means that the MAM-E17 model may be ideal for helping us to understand the anatomical differences associated with schizophrenia. Further, it can allow for an avenue to specifically explore sex differences following neonatal injury. This is important because there is evidence that cortical injury during development affects males differently and tends to be more severe (Herman et al., 1997; Hill & Fitch, 2012).

One possible explanation for these findings may be the theory that estrogens act as a protective factor against neurodegenerative conditions such as schizophrenia. Previous meta-analytic research has proposed a hormonal component in the development and pathology of schizophrenia as an explanation of why females diagnosed with schizophrenia typically have less severe neuroanatomical features and tend to be diagnosed later in life, when estrogen levels are lower (Leung & Chue, 2000). This leads to the hypothesis that higher estrogen levels can protect against neurodegeneration and brain injury. To study this, the neuroprotective qualities of estrogens have been demonstrated using other animal models of brain damage (Dluzen et al., 1996; Miller et al., 1998; Sawada et al., 1998). There is evidence that hormones have the potential to impact the severity of neonatal brain injury (Hill & Fitch, 2012). The interaction between estrogens and the effects of prenatal injury have also been analyzed using the MAM

model. Researchers have found that the estrogen cycle influences the functioning of the dopaminergic system within the brains of female rodents exposed to MAM on E17, which can be seen by recording the activity of neurons (Perez et al., 2014). The estrogen cycle also influences other schizophrenia-like traits that are seen in female rodents exposed to MAM such as hypersensitivity to stimulant drugs and abnormal social interaction (Perez et al., 2019). This evidence implies that hormones influence the behavioral and physiological effects seen in the MAM model of schizophrenia. No research to date has investigated whether hormones influence the structural effects of MAM exposure.

The results of this study, as well as previous research (Hill & Fitch, 2012; Perez et al., 2014, 2019), implicate that neuroanatomical sex differences may be due to differences in sex hormones between MAM-males and MAM-females. It is unclear whether estrogen plays a protective role or testosterone plays a maladaptive role when it comes to prenatal injuries. This presents two avenues for future research: manipulating either estrogen or testosterone levels in ovariectomized MAM-females. An ovariectomy is a procedure that involves removing the ovaries of female rats, which then produces estrogen-deficient females. Since estrogen levels vary depending on the individual and time during estrous cycle, ovariectomizing all female subjects will act as a control measure. Replicating the design of this study, the anatomical effects of MAM can then be compared between estrogen-deficient MAM-females and MAM-females that have been administered a specific dose of estrogen. A replication of this study comparing the effects of MAM in estrogen-deficient and estrogen-treated female rodents, instead of male and female rodents, would be better able to isolate the potential protective effects of estrogen.

If estrogen acts as a neuroprotective factor, then the estrogen-deficient MAM-females would experience more severe structural effects of prenatal injury. In this case, further studies

can then attempt to replicate the protective effects of estrogen using male rodents exposed to MAM. However, if estrogen does not modulate developmental disruption, then there would not be significant differences between estrogen-deficient and estrogen-treated MAM-females. Future studies should then focus on the effects of manipulating testosterone levels in estrogen-deficient MAM-females to gain insight on the possible maladaptive effects of testosterone. Either outcome will help to shed light on the sex differences in the anatomy of MAM-exposed rodents that were revealed by this study. This research could help to reveal the ways in which the sex differences in the MAM model of schizophrenia reflect the sex differences in the pathology of schizophrenia in humans, potentially improving the validity of the MAM model. In addition, exploring this topic further could reveal important interactions between hormones and brain injury that could influence the study and treatment of other types of neurodegenerative disorders.

Limitations of this study include the accuracy of the measurement methodology. The circling method of measurement provides a quick and valid estimation of three-dimensional volume, but this method is not exact as it is based on tracing the perimeter of the region being measured. Because each individual that uses the circling method of measurement will interpret the border of the region differently, there is some subjectivity in this method. Additionally, because it is reliant on outlining specific subjective borders, this method increases variability in area measurements (Rosen & Harry, 1990). Going forward, a more precise measurement methodology could be utilized to improve the reliability of results, such as Cavalieri's point-counting method. Point-counting is a stereological technique that is used to measure brain structures by counting the points on a grid that fall within the region being measured. This method depends on Cavalieri's estimator of morphometric volume, which has previously been shown to be a highly accurate mathematical measure of brain regions (Rosen & Harry, 1990).

Cavalieri's estimator requires the points being counted to be evenly spaced, so a grid is laid over each slide of tissue being measured using ImageJ, the same software used in the circling method. Each point on this grid that falls within the region being measured is counted to provide a calculation of the area. The area of the region on each slide is then combined to produce an estimate of volume. This method of measurement is also used in measuring structures within the human brain (Arango et al., 2008). The point-counting method is slightly more accurate than the circling method but takes considerably more time. The measurement method used in this study is valid, but a more accurate method of measurement could reduce the variability of the measurements, allowing for more subtle differences to be revealed.

Additionally, some sections of the tissue samples used were not able to be measured due to damage that occurred while the tissue samples were being processed. In this case, an average of the previous and following sections were used in data analysis. To avoid the potential influence of results caused by damage, future studies could use magnetic resonance imaging (MRI) images for measurement. MRI measurement takes place while the animal subject is still alive, which could help avoid the issue of damaged or missing tissue samples. The use of MRI to measure the ventricular volume of live rodents exposed to MAM has been used in previous studies (Chin et al., 2010). This method also offers better access to information about how the structures within the brain may change during development. Using this method, future researchers could conduct a longitudinal study that tracks differences in behavioral and structural effects of developmental disruption caused by MAM over time.

The generalizability of these results is also limited by the relatively small sample sizes. With small samples it is difficult to determine whether the results represent the population being studied. This study found that control females had greater average ventricular volume than

control males and MAM-females, respectively. This group also had the greatest variability (see Figure 4.) Although not statistically significant, this data was unexpected and may be due to small sample sizes and the variability of the circling method. In addition, data analysis revealed no significant effect of sex or treatment on ventricular volume. Further research with larger samples may be able to reduce the variability within groups and find significant differences where this study did not.

Conclusion

Significant effects of treatment and sex were found on the body weight and brain weight of male and female rodents exposed to MAM. In addition, an interaction was seen between sex and MAM exposure on ventricular volume. As predicted, rodents exposed to MAM experienced a disruption in their development which led them to have lower body weight than control rodents. The developmental disruption also affected the brain weight of the rodents exposed to MAM; both MAM-males and MAM-females had lower brain weights than control males and control females, respectively. However, exposure to MAM did not impact the ventricular volume of male and female rodents in the same way.

This data contributes to a clearer understanding of how the sex differences in the MAM model of schizophrenia reflect the sex differences in the pathology of schizophrenia. This study is one of the first to directly compare female and male rodents exposed to MAM and contributes novel data to the study of schizophrenia. Humans diagnosed with schizophrenia and rodents exposed to MAM on E17 are found to have similar physical abnormalities (Chalkiadaki et al., 2019; Chin et al., 2010; Lodge et al., 2009; Lodge & Grace, 2010, 2012; Moore et al., 2006). The MAM model has been studied extensively, but few studies have focused on sex differences within this model of schizophrenia. As previously discussed, schizophrenia presents differently

in females and males (Arango et al., 2008; Canuso & Pandina, 2007; Gur et al., 2000; Leung & Chue, 2000; Nopoulos et al., 1997; Rietschel et al., 2015). Further research using the MAM model with both female and male subjects could help researchers gain insight on how sex impacts the development and symptoms of schizophrenia, ultimately leading to improved treatment and diagnostic tools. As further research strengthens the validity of the MAM E17 model of schizophrenia, opportunities to use this model to study new early-intervention or drug therapies arise. As previously stated, very few studies that used the MAM model have included female subjects. The results of this study show that exposure to MAM impacts male and female rodents differently, so studies that test new treatments for schizophrenia using the MAM model should include both male and female subjects. This would help predict potential sex differences in how humans diagnosed with schizophrenia would respond to new treatments. Future research focusing on female rodents within the MAM model and the potential modulating effects of estrogen could develop novel therapeutic approaches specifically for female patients of schizophrenia. Finally, gathering more information on the role that estrogens play in the MAM model could potentially lead to hormone-based treatments for schizophrenia as well as other neurodegenerative disorders, such as Alzheimer's disease or Parkinson's disease.

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