Analysis of Autism Spectrum Disorder Like Behavior in Female Rats Using a Model of Maternal Stress and Terbutaline

By Taylor G. Crist

Department of Psychology and Neuroscience, University of Colorado Boulder

Defended April 5th, 2019

Thesis Advisor

Daniel S. Barth Ph.D, Psychology and Neuroscience

Defense Committee

Heidi E W. Day Ph.D, Psychology and Neuroscience

Ricardo H. Stephen Ph.D, Biochemistry

Abstract

Autism Spectrum Disorders (ASD) in humans is diagnosed 3.1 times more in males than in females (Loomes et al., 2017) in addition to a 30% bidirectional comorbidity with epilepsy (Lewis et al., 2018). This model of stress terbutaline and potential bacterial treatment, *M. vaccae,* hope to establish a method by which to study the comorbidity and better understand the mechanisms underlying the disorders and perhaps their overlapping genesis. This model has proved successful at producing ASD like behavior and seizures in male rats in recent years (Bercum et al., 2015).

Female Sprague Dawley rats underwent a stress paradigm protocol consisting of both maternal and developmental stress with injections of Terbutaline. Half of the treatment animals were also given injections of *Mycobacterium Vaccae* and the other half a vehicle solution. Behavioral tests were conducted to determine which groups of animals demonstrated ASD like behavior.

ASD like behavior was seen in the females rats with regard to sociability and social novelty preference. *M. vaccae* ameliorated this behavior as a treatment only in the test of sociability which was tested in the three chamber social task. Neither marble bury or defensive burying tasks revealed ASD like behaviors in treatment group rats. The demonstrated differences between males and females could possibly be the result of the physiological sex differences that produce the skewed ASD sex ratio. It is also possible that the same tasks used in measures of male behavior are not accurate measures of the same behavior in females and alteration of behavioral tasks to those more suited to test the behaviors in females may produce different results.

Further research to determine the model's depiction of epileptogeneis would be needed to identify it as a model of the bidirectional comorbidity. Use of different behavioral tasks to determine if data in females that differs from males is due to differences in behavior from the model or physiological sex behavioral differences would be necessary to substantiate the legitimacy of the model in both sexes.

Introduction

In the human population, there is a 30% bidirectional comorbidity between Autism Spectrum Disorders (ASD) and epilepsy (Lewis et al., 2018). This has led to the hypothesis that there is overlap in the genesis of each disorder and potential shared mechanisms (Lewis et al., 2018). The Diagnostic and Statistical Manual of Mental Health Disorders (DSM) defines the criteria for a diagnosis of ASD as persistent deficits in social communication across multiple contexts and repetitive patterns of behavior present in early development that significantly impair normal functioning and cannot be explained by another intellectual disability (American Psychiatric Association, 2013). Additionally, it has been determined that the male to female ratio of ASD diagnosis is likely 3.1 times higher in males than females (Loomes et al., 2017). The mechanisms are not well understood, but recent research involving humans suggests that genetic differences predispose males to ASD disorders and that these phenotypes may be modulated by hormones such as testosterone (Werling and Geschwind, 2013). Further research into potential differences between males and females in ASD is needed to better understand the epidemiological divide between the sexes.

Animals models of autism attempt to replicate repetitive behaviors and deficits in social interaction and communication as dominant defining characteristics indicative of ASD-like

behavior (Patterson, 2011). Models may also replicate other behaviors such as anxiety or sleep disorders, sometimes by using genetic variants containing missing genes linked to ASD (Andres, 2002). One model of autism that has emerged in recent years is the of Maternal Immune Activation in which dams are injected with a single dose of IL-6 which is thought to be a good model of the environmental risks contributing to ASD (Smith et al., 2007). Recently a new non-genetic model of ASD using chronic stress in addition to terbutaline has shown success in male rat populations at reproducing ASD-like behaviors and epileptic seizures (Bercum et al., 2015). Even though ASD affects more males than females, there is a striking gap in the knowledge in female ASD animal models. Animal models that reproduce the disproportionate sex ratio of ASD would be useful in determining the potential mechanisms and pathways leading to the genesis of ASD.

Recent studies suggest that ASD may be the result of immune system dysregulation. In humans with ASD abnormal counts of T helper cell type 1, lymphocytes, and serum immunoglobulin levels have been reported in conjunction with decreased T cell mitogen response and autoimmunity (Ashwood et al., 2006). Several immune response genes have been identified as abnormal in individuals with ASD behavior (Ashwood et al., 2006). Recent studies have shown an association with mothers who had infections during pregnancy, correlated with a hyperinflammatory response in the mother and Autistic child (Chez et al., 2007). It has been suggested that maternal immune activation induced elevations of IL-6 in mothers crossing the placenta is an essential contributing factor in autism and schizophrenia (Hsiao and Patterson, 2011). Croonenberghs also found higher levels of proinflammatory cytokine IL-6 in the whole blood of persons with ASD and suggests proinflammatory cytokine overproduction may play a role in the physiology of ASD (Croonenberghs et al., 2002). Peripheral blood cells in people with

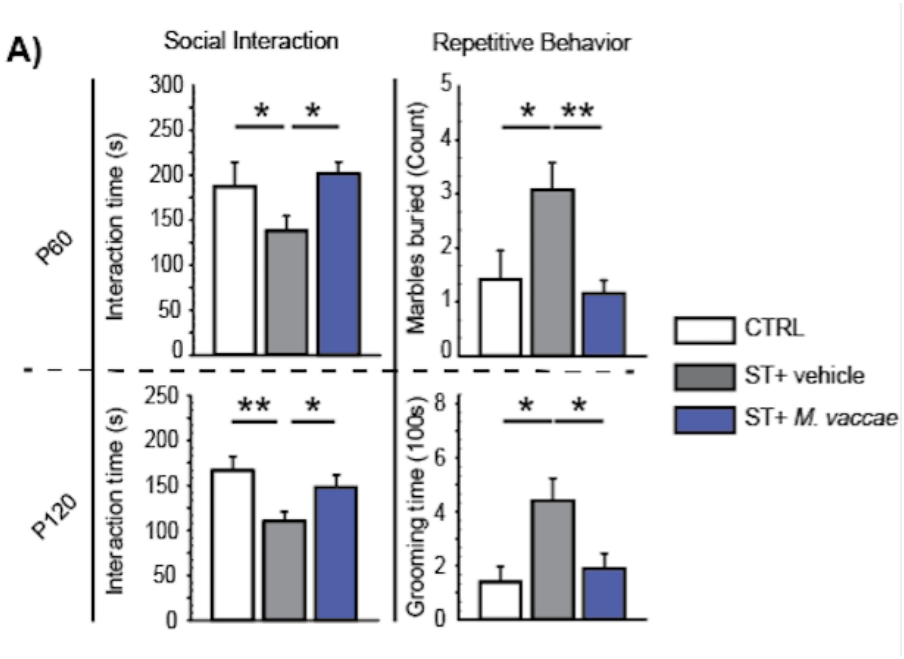
ASD demonstrate higher levels of proinflammatory/counter-regulatory cytokines without stimuli than controls, suggesting that ASD may create excessive innate immune responsiveness (Jyonouchi et al., 2001).

Maternal stress and terbutaline, both risk factors for ASD (Bercum et al., 2015), produce extended neuroinflammation in animal models. It is thought that inflammation in pregnant mothers may be the connection between stress and adverse neuropsychological disorders. Terbutaline administration to pups in postnatal days 2-5 has been found to be associated with increased microglial activation in pathways associated with behaviors similar to that of ASD (Zerrate et al., 2007). Terbutaline is given to the pups to simulate administration to human mothers in the third trimester since the third trimester in rats is ex utero. Additionally, stress induced maternal immune activation has been shown to increase the risk of abnormal behaviors in rats (Hantsoo et al., 2019). However, direct maternal immune activation models show differential effects between males and females through the increased presence of proinflammatory cytokines IL-6 and IL-1β in the placenta (Bronson and Bale, 2014). Therefore, the inflammatory effects of stress-terbutaline in this model may be different between males and females.

The Old Friend's hypothesis suggests that exposure to environmental bacteria has an integral role in immune system development. When exposure to these environmental microorganisms is decreased, in urban areas or as a nation develops, there is typically an increase in the prevalence of diseases modulated by inflammation. It is thought that reduced exposure may lead to dysfunction in an organism's ability to modulate stress and inflammation and may explain the increase in psychiatric disorders in developed nations (Rook et al., 2013). Autism is thought to be a condition affected by the Old Friend's hypothesis, as rates of ASD have been

suggested to be higher in urban than rural areas (Becker, 2007). One such old friend, *Mycobacterium vaccae,* has been shown to be centrally (Fonken et al., 2018) (Frank et al., 2018) and peripherally (Reber et al., 2016) anti-inflammatory and stress protective.

In males under the model of chronic stress and terbutaline previous studies have provided evidence for the immunization with heat-killed *M. vaccae* as a potential treatment for ASD like behaviors with a focus on repetitive behavior, social interaction, and the potential for seizures (Smith, Barth, Kubiak, personal communication, February 12th, 2019) (Fig. 1).



B)

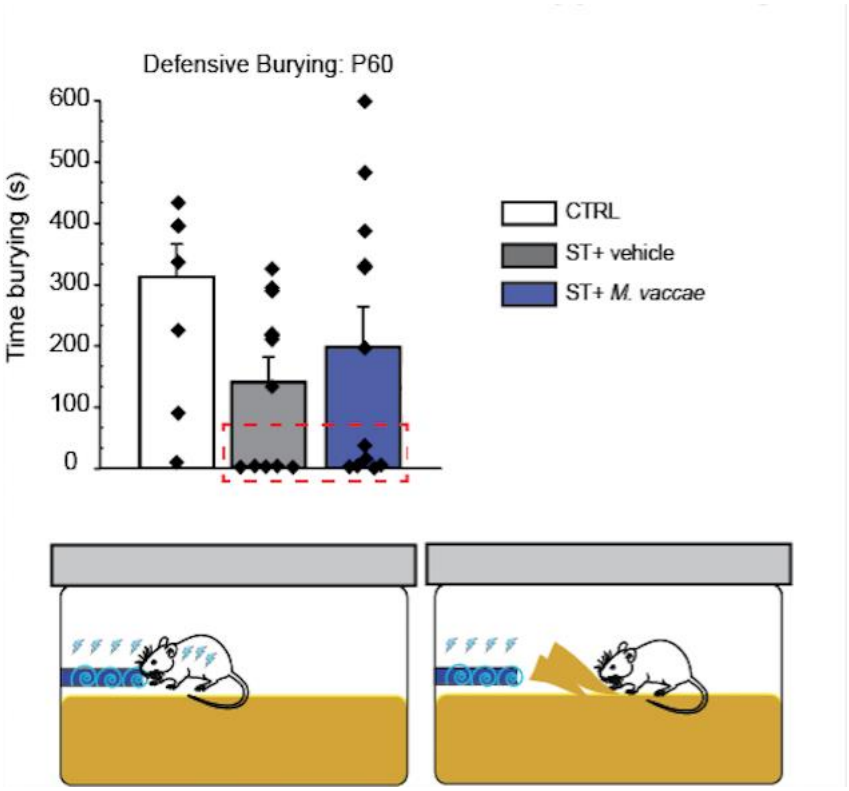


Figure 1. ST + Veh treated males in a previous study demonstrated decreased sociability and increased repetitive behavior which was returned to near normal when accompanied with treatment by *M. vaccae*. Previous study using the stress + terbutaline model assessed ASD like behaviors. **(A)** Three chamber social task as a measure of social interaction demonstrated significantly less social interaction in the ST + Veh group but a ST + MV group with no differences when compared to controls. Measure of repetitive behavior, marble bury and grooming, were significantly higher in the ST + Veh group and the effect appears to have been reduced by ST + MV treatment. **(B)** Both ST + Veh and ST + MV treatment groups demonstrated less defensive burying but not to a statistically significant extent. In both groups there was significantly more animals that did not bury at all with regard to Control.

Due to the large sex differences in ASD diagnoses a model of autism that replicated this difference would be ideal. In addition, since *M. vaccae* has been shown to ameliorate ASD like behavior in males it would be beneficial to know if this effect is sex specific for the given model. To test this, Female Sprague- Dawley rats underwent chronic maternal stress and developmental stress with injections of terbutaline. Half of the treatment animals received *Mycobacterium Vaccae* (ST + MV) as a potential treatment of ASD like behaviors and the other half a vehicle (ST + Veh). The control was an untreated control (Control). Female progeny then underwent behavioral testing to test sociability, social novelty preference, repetitive behavior, and coping mechanisms.

Methods and Materials

Animals and Treatment

Female Sprague-Dawley rats (n=12, Envigo) arriving at embryonic day (E) 2 weighing 200-220 grams were acclimatized to the facility for 24 hours before stress protocol was administered. Dams were housed in 26.67 x 48.26 x 20.32 cm sealed and ventilated static cages (Allentown Inc.). Animals were housed under standard conditions (temperature controlled; 20°C ± 1, relative humidity 22%) with 12-hour light/dark cycles (lights on 7:00am-7:00pm). Dams were given *ad libitum* food and water. All procedures were performed in accordance with University of Colorado Institutional Animal Care and Use Committee guidelines for humane use of laboratory rats in biological research.

Eight dams were randomly assigned to one of two groups, developmental stress + terbutaline + *M. vaccae* injections (ST+MV, n=4) or developmental stress + terbutaline + vehicle injections (ST + Veh, n=4). The other four dams were chosen to be a no stress control group

(Control, n= 4) due to potential stress cofounds of injections. On E2, E9, and E16 the experimental pregnant dams received either subcutaneous immunization with 0.1 mg whole heat-killed *M. vaccae* suspension (10 mg/mL solution; strain NCTC 11659, batch ENG 1, provided by Bio Elpida diluted to 1 mg/mL in 100 μL sterile borate-buffered saline (BBS) using 21-gauge needles or injections of 100 μL of the vehicle, BBS, in the morning. The control group was left unmanipulated.

Developmental Stressors

Several stress paradigms were combined to create a period of developmental stress mimicking the duration of human pregnancy, starting on embryonic day 3 (E3) until post-natal day 9 (PN9). Rats are an altricial species (Sengupta, 2013), meaning that many developmental events occur postnatally, with post-natal days 1-10 roughly equivalent to the third trimester in humans (Semple et al., 2013).

Chronic Maternal Stress. Experimental group dams were exposed to a repeated mild stress paradigm from E3-E20 (Figure 1). Dams were habituated to a sound-attenuated chamber for 5 minutes with a shock grid floor (Coulbourn Instruments) on E3. This was done to allow for habituation to the novel context . On E4, contextual fear was established by returning the dam to the same sound-attenuated chamber and performing the shock paradigm. After 60 seconds, 2x1mA shocks were delivered at 60 second intervals, followed by a 5-minute exposure to the environment post shock. The following two days (E5 and E6) the dams were returned to the same context for 5 minutes without shock. To avoid fear extinction, on E7, the shock paradigm was repeated followed by 2 days of context exposure (E8 and E9). On E10 the dams were left to

rest before beginning the 7-day shock paradigm again until they gave birth. During the trials, a blind observer recorded freezing time using a stopwatch. Dams were weighed daily to ensure they continued to gain weight to determine that the pregnancy had not been terminated.

Week 1	Context E3	Shock E4	Context E5	Context E6	Shock E7	Context E8	Context E9
Week 2	Rest E10	Shock E11	Context E12	Context E13	Shock E14	Context E15	Context E16
Week 3	Rest E17	Shock E18	Context E19	Context E20			

Figure 1. Calendar outline of maternal stress shock paradigm

Cross Fostering. On post-natal day 2 the pups from all litters were cross fostered within treatment group in a counterbalanced fashion to allow for uniform maternal care.

Terbutaline Injections. Terbutaline injections were given on PN 2-5 to the ST+ *M. vaccae* and ST+ vehicle groups using terbutaline sulfate (Sigma-Aldrich, St. Louis, MO) in doses of 10 mg/kg dissolved in saline (Zerrate et al., 2007; Slotkin and Seidler, 2013; Bercum et al., 2015). *M. vaccae* and vehicle solutions were prepared as stated above and administered interperitoneally on PN 7, 13, and 20 at 10:00 A.M. to the pups according to their maternal treatment group.

Limited Bedding. On PN 2-9 the dams and pups were exposed to a limited bedding paradigm described in previous experiments (Ivy et al., 2008; Rice et al., 2008; Molet et al., 2014). Animals were placed in custom chronic recording housing with 6 pieces of food (26 grams,

Tekland), *ad libitum* water, and 24/7 video was recorded (Axis M3104-L network Camera) for analysis of maternal care. At PN 10 animals were returned to their home cages with normal amounts of bedding and *ad libitum* food and water access (Ivy et al., 2008; Molet et al., 2014). *Maternal Separation.* On PN 2-9 pups were removed from their mother and litter mates and placed in individual cages (28.79 x 19.96 x 11.43 cm) in a separate room for three hours with one paper towel each day (Molet et al., 2014).

Behavioral Testing

Social Interaction

3 Chamber Social (PN 57). A three-chamber social test consisting of two trials was used as an assessment of social interaction and social novelty preference. The apparatus (91.4 x 41.9 x 41.9 cm) was divided in to 3 (30.5 x 41.9 X 41.9 cm) chambers with doorways to allow movement between chambers when doors were removed. The rat resided in the middle chamber with both side doors closed for a five-minute habituation at the beginning of the test. In the first trial, an age and weight matched female novel rat was placed into a 6-inch diameter cylindrical apparatus in the left chamber and an identical empty cylindrical apparatus placed in the right. The test rat was then allowed to explore all three chambers freely for ten minutes. In the second trial, the same familiar rat from the first trial is placed back in its original cylindrical apparatus in the left chamber and in the right chamber a new age and weight matched novel rat is placed in the previously empty cylindrical apparatus. For ten minutes, the test rat was allowed to explore freely with the doors open (Wöhr and Scattoni, 2013; Banerjee et al., 2014). Interaction with the novel animal, object, and familiar rat, time spent in each chamber, and time active were recorded

using Anymaze 4.99z. Between each of the trials the test rat was placed back in its home cage and the apparatus was cleaned with a 0.2% triclosan solution.

Repetitive Behavior

Marble Bury (PN 64). Prior to the test animals underwent a 2-day habituation period in static cages (26.67 x 48.26 x 20.32 cm, Allentown Inc.) with 5 cm of bedding for a half hour each day in the testing room. For the test, animals were placed into a static cage (identical to habituation cage) with 5cm of bedding and 18 clear glass marbles lined up in a 3x6 grid for 10-minutes. At the end of the trial, the number of marbles buried by the animal was recorded. A marble was considered buried if more than 2/3 of its volume was covered by the bedding. (De Boer and Koolhaas, 2003; Wöhr and Scattoni, 2013).

Stress Coping Measures

Defensive Burying Task (PN 106). For the two days prior to the test rats were habituated for 1 hour in a static cage identical to their home cage (26.67 x 48.26 x 20.32 cm, Allentown Inc.) with 5 cm of bedding. On the day of the experiment the rat was placed into a static cage for 15 minutes containing an electrified probe that was wired to deliver a 1mA shock when contacted. At the end of the trial the height of bedding was recorded in addition to number of shocks the animal received, time spent burying, rearing bouts, and latency to begin burying the probe from the first shock (Anderson et al., 2018).

Statistical Analysis

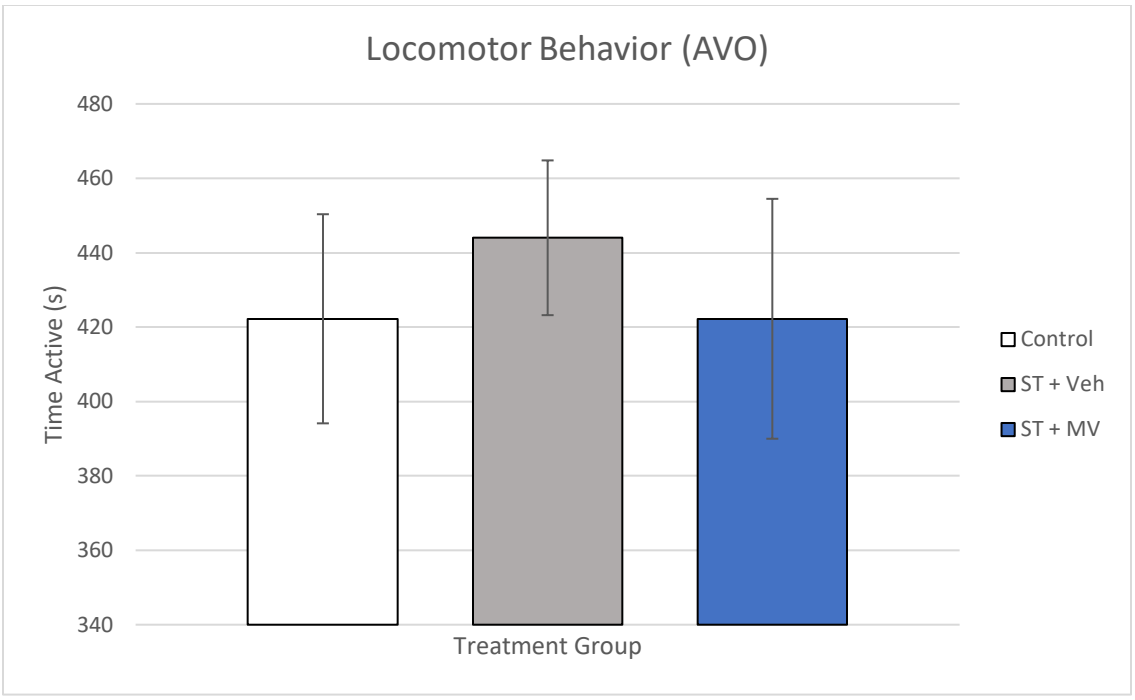
Results of behavioral tests were analyzed using one-way ANOVAs, corrected for unbalanced designs where applicable. Pairwise comparisons were performed where ANOVA's indicated significant group differences. A two-tailed $\alpha = .05$ was used to establish statistical significance.

Results

Three Chamber Social PN57

Sociability. Paired sample T-tests for each group to measure social preference found that that *M. vaccae* treated animals (Animal 239.55 ±16.77 s, Object 28.99 ± 11.00, $p < .001$) and control animals (Animal 211.58 ± 25.40, Object 53.26 ± 10.79, $p < .01$) exhibited a social preference while no statistical preference was observed in vehicle treated animals (Animal 206.21 ± 30.98 s, Object 71.49 ± 29.71, $p = 0.053$) (Fig. 2B). There was not a statistically significant difference in the time the animals were active during the test ($F_{2,21} = 0.165$, $p = 0.848$). Group averages were Control; 445.488 ± 27.472 s, ST + Veh; 448.775 ± 25.661 s, ST + MV; 428.438 ± 27.402 s (Fig. 2A). Overall, there were no differences between groups in time spent interacting with the novel animal ($F_{2,21} = .509$, $p = 0.607$) or with the object ($F_{2,21} = .711$, $p = 0.503$).

A



B

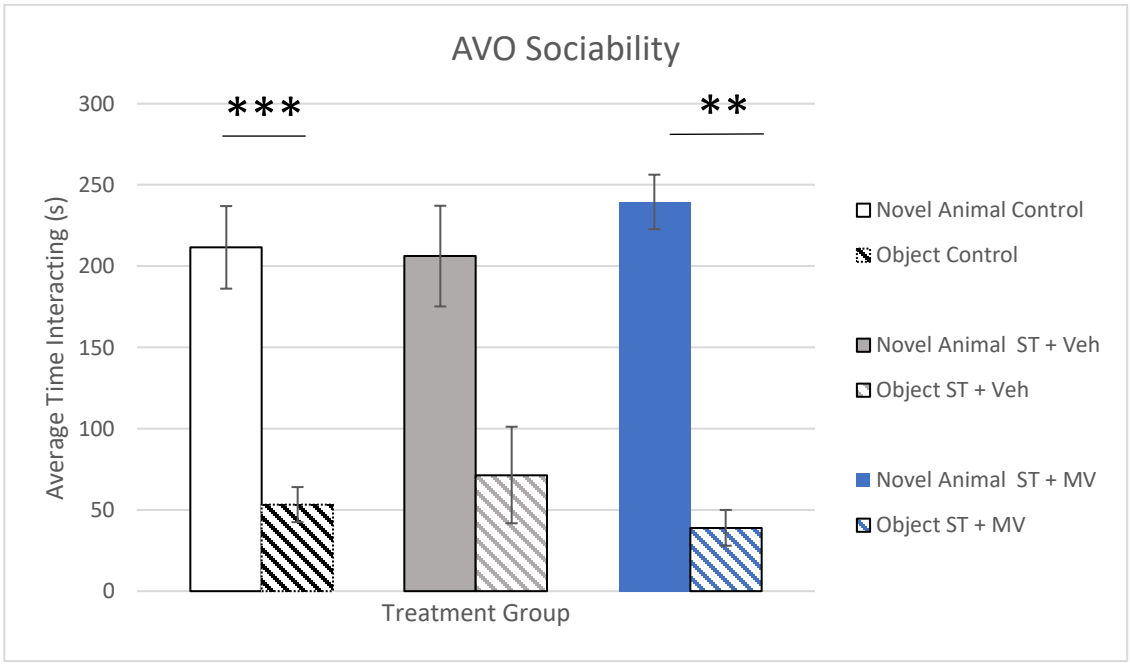
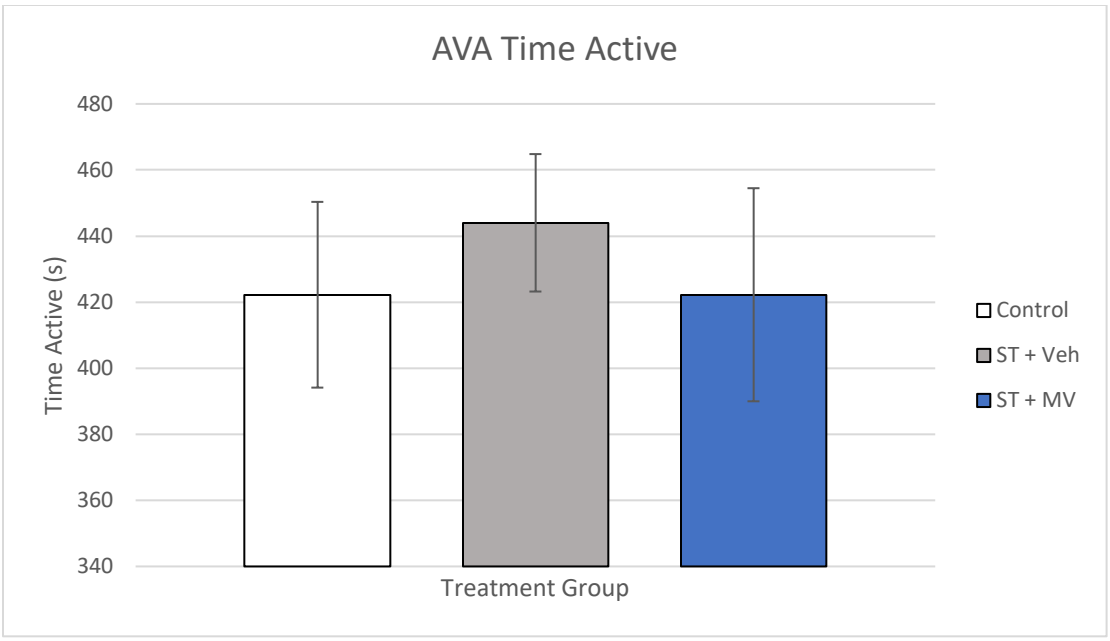


Figure 2. Vehicle treated animals do not show a preference for social interaction in the Three Chamber Social Test. Three chamber social task was performed at PN 57 to assess sociability and social novelty preference. **(A)** No locomotion differences were detected between groups when testing sociability ($F_{2,21}$= 0.165, p = 0.848). **(B)** Animals of the vehicle group did

not demonstrate a statistically significant preference for social interaction, $p = 0.053$, but the *M. vaccae* treated group did, $p < 0.001$. Bars represent means of each group, error bars represent ± SEM. Comparisons were made between untreated control (Control; n=8, white), vehicle treated stress-terbutaline (ST + Veh; n=8, grey), and *M. vaccae* treated stress-terbutaline (ST+ MV; n=8, blue) groups. *P<.05, **P<.01, ***P<.001.

Social Preference. Within the second stage of the three chamber social task performed at PN 57 to test social novelty preference there were no differences in locomotion among groups in this stage ($F_{2,21}$= 0.210 p = 0.813) (Fig. 3A). Group averages were Control; 422.238 ± 28.110 s, ST + Veh; 444.013 ± 20.799 s, ST + MV; 422.238 ± 32.245 s. ANOVA revealed no differences in interaction times among treatment groups (familiar animal $F_{2,21}$= 1.532, p = 0.240, novel animal $F_{2,21}$= 0.879, p = 0.430). Pairwise comparisons showed that only control animals demonstrated a preference, for the familiar animal (familiar animal 138.45 ± 12.23 s, novel animal 78.69 ± 15.83 s, $p < .001$). Vehicle treated animals (familiar animal 112.46 ± 17.99 s, novel animal 79.80 ± 15.83 s, p = 0.249) and *M. vaccae* treated animals (familiar animal 102.90 ± 13.76 s, novel animal 115.15 ± 33.15 s, p = 0.763) did not demonstrate a preference animal (Fig. 3B).

A



B

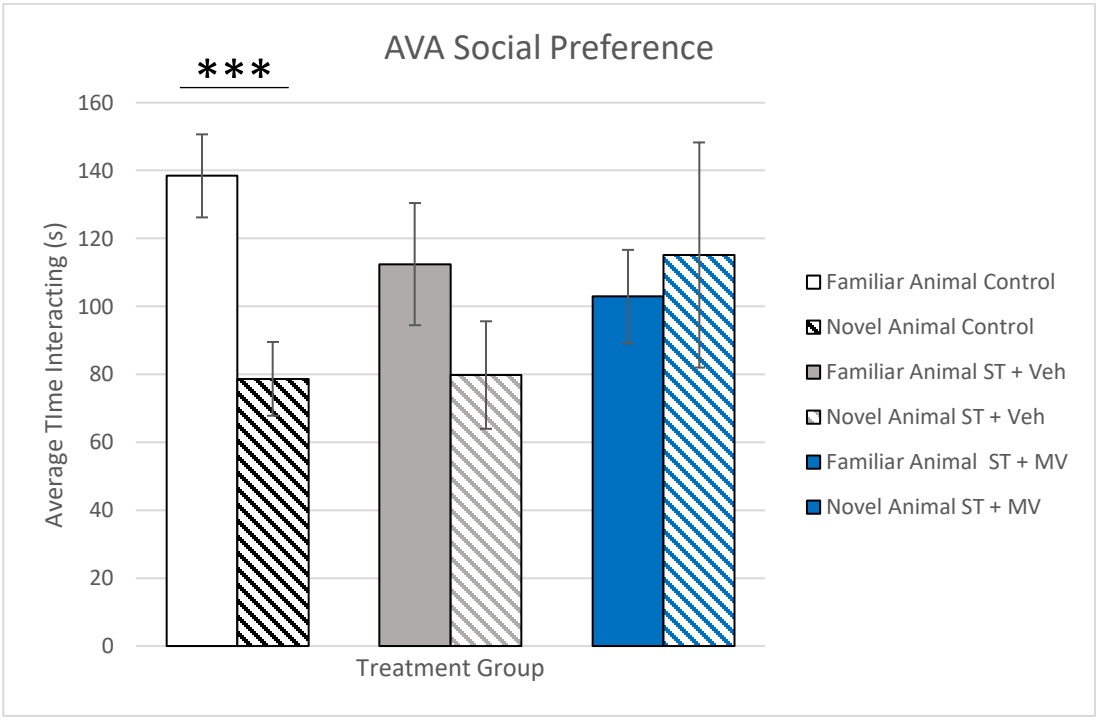


Figure 3. Animals treated with the stress paradigm, *M. Vaccae* and Vehicle, exhibit no social preference. Three chamber social task was performed at PN 57 to assess sociability and social preference. **(A)** No locomotion differences were detected between groups when testing social preference ($F_{2,21}$= 0.210 p = 0.813). **(B)** *M. vaccae* and vehicle treated animals did not

demonstrate a preference for either animal, novel or familiar, (p = 0.763, p = 0.249). Control animals exhibited a preference for the familiar animal (p < .001). Bars represent means of each group, error bars represent ± SEM. Comparisons were made between untreated control (Control; n=8, white), vehicle treated stress-terbutaline (ST + Veh; n=8, grey), and *M. vaccae* treated stress-terbutaline (ST + MV; n=8, blue) groups. *P<.05, **P<.01, ***P<.001.

Marble Bury

The marble bury task was carried out as a measure of repetitive behavior as related to ASD-like behavior at PN 64. No differences were detected in number of marbles buried between groups as indicated by an ANOVA, $F_{2,19}$= 0.010, p = 0.990. Control; 4.857 ± 1.262 marbles, ST + Veh; group 5.125 ± 1.695 marbles, ST + MV; 4.857 ±1.262 marbles (Fig. 4).

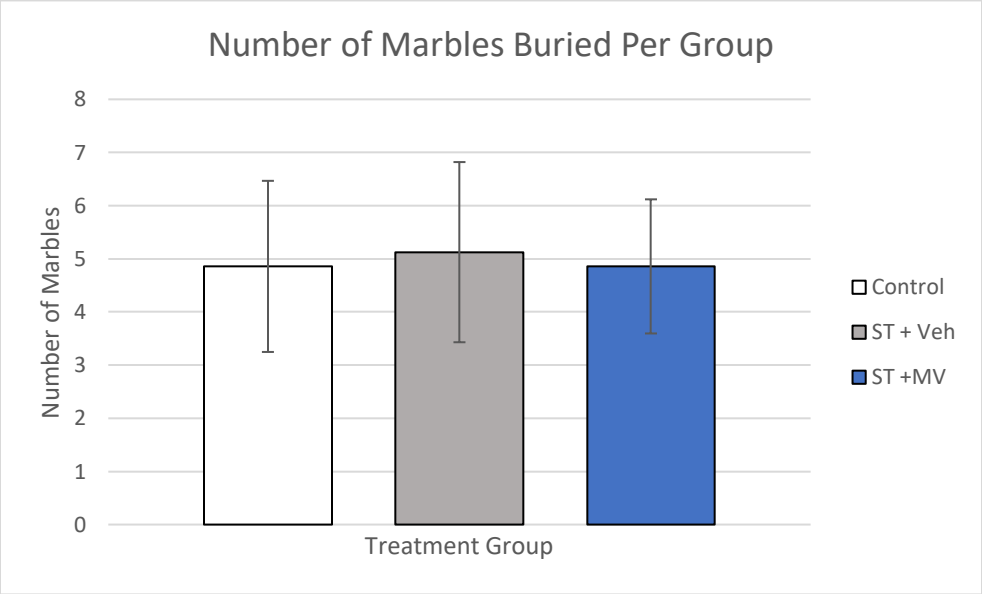


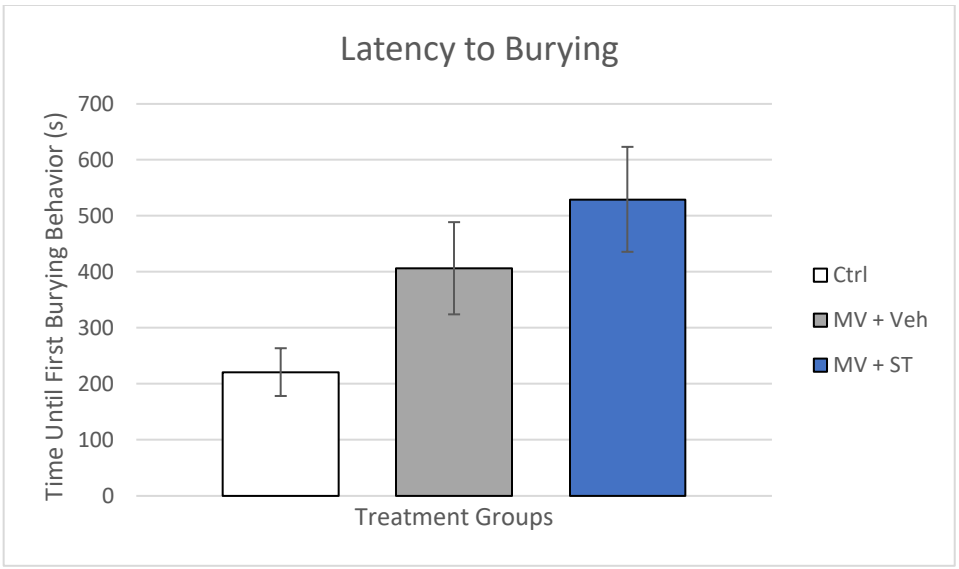
Figure 4. Treatment groups did not differ in number of marbles buried from untreated controls. Bars represent means of each group, error bars represent ± SEM. Comparisons were made between untreated control (Control; n=8, white), vehicle treated stress-terbutaline (ST +

Veh; n=8, grey), and M. vaccae treated stress-terbutaline (ST + MV; n=8, blue) groups. *P<.05, **P<.01, ***P<.001.

Defensive Burying.

Defensive burying task was carried out to assess the animals coping strategies and responses to stress to determine if they differ between groups. Among latency to bury after first shock there was no statistical significance, ($F_{2,13}$= 3.236, p = 0.0724). Group averages were Control, 221.095 ± 42.635 s; ST + Veh, 406.536 ± 82.313 s; ST + MV, 529.58 ± 93.724 s. (Fig. 5A). Animals that did not bury were not included in the calculations for latency. Time spent burying contained no significance between groups ($F_{2,20}$= .440, p = .651). Within the control group 2 of 8 animals did not bury, the group had a mean of 301.380 ± 81.588 s. In the ST + Veh group 3 of 8 animals did not bury, the group had a mean of 233.72 ±102.733 s. In the ST + MV treatment group 2 of 7 animals did not bury. This group had an average of 183.13 ±75.965 s. (Fig. 5B). There was no significance among number of shocks administered ($F_{2,20}$= 0.943, p = 0.406). There was no significance among depth buried by treatment group, ($F_{2,20}$= 0.378, p = 0.690.) There is no significance among groups in terms of number of rearing bouts, ($F_{2,20}$= 1.188, p = 0.326).

A



B

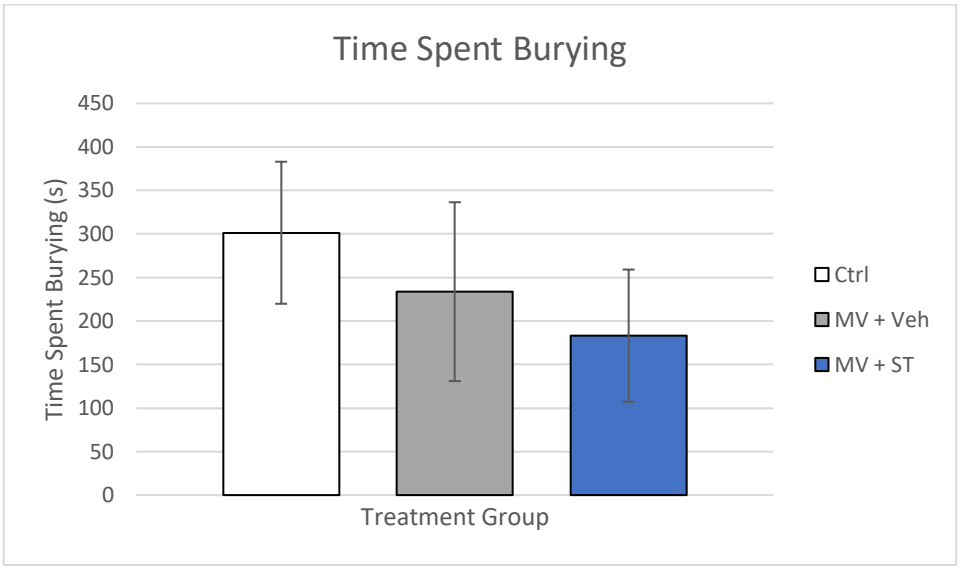


Figure 5. Stress terbutaline animals do not exhibit coping strategies different of that of untreated controls. Marble bury test performed at PN 106 was carried out to asses coping strategies and response to stress. **(A)** No differences were demonstrated among treatment groups for latency to begin burying the probe, $F_{2,13}$= 3.236, p = 0.0724. Animals that did not bury were not included in the calculations for latency. **(B)** Within the Marble Bury test no differences were detected for time spent exhibiting burying behavior, $F_{2,20}$= .440, p = .651. Animals that did not bury to any depth were calculated with a burying time of 0 seconds. Bars represent means of

each group, error bars represent ± SEM. Comparisons were made between untreated control (Control; n=8, white), vehicle treated stress-terbutaline (ST + Veh; n=8, grey), and M. vaccae treated stress-terbutaline (ST + MV; n=8, blue) groups. *P<.05, **P<.01, ***P<.001.

Discussion

Summary of results

Behavioral testing has shown that the group treated with ST + Veh did not demonstrate expected sociability as they did not interact more with the animal than the object statistically. It should be noted that they did interact with the animal more but just not in more in a statistically significant way. In addition it was determined that ST + MV and ST + Veh treatment resulted in lack of social preference. Marble bury and defensive burying tasks did not produce differences in behavior between groups.

Three Chamber Social

The fact that the locomotion time was not significantly different between the groups in either stage is a good indicator that potential differences in interaction are due to preferences for interaction and not locomotor defects. In the previous study of stress terbutaline in males the ST + Veh group had less interaction overall which was indicative of ASD like behavior.

Sociability

Lack of social preference is a hallmark of ASD. The new Social Motivation Theory of Autism puts a greater emphasis on avoiding social interaction in ASD as a way of avoiding social rejection (Chevallier et al., 2012). In our test of sociability, vehicle treated ST animals did

not demonstrate a preference for a novel animal over a novel object, indicative of Autistic-like behavior. Control animals and *M. vaccae* treated animals both showed a preference for sociability. Therefore we concluded that *M. vaccae* improved the social deficits seen in this model in female rats. However, there were no significant differences in the average time each group spent with the novel animal or the object. Additionally, while the ST + Veh group did not have statistical significance of a social preference for the animal, the averages indicate some difference may exist. Spending more time with the animal than the object is the expected outcome for untreated controls (Moy et al., 2004). While there is not a vast array of research on the subject, there is evidence that social behavior and anxiety may be correlated differently in each sex. Researchers found that social behavior may not be a good indicator of level of anxiety in either sex as the relationship between responses to stress did not always correlate with social behavior tests used more frequently such as the three chamber social (Boguszewski et al., 2013). However, it remains that social behavior is a significant factor in a diagnosis of Autism and the result of ST + Veh having no social preference is indicative of ASD like behavior.

In ASD like behavior mice, with modified dopamine neurons in the VTA, Bariselli and colleagues found decreased preference for social novelty stimuli in both stages, less interaction with animal and less interaction with novel animal (Bariselli et al., 2018).

Social Preference

The fact that stress-terbutaline groups do not display a social preference is consistent with the DSM V definition regarding deficits in social communication within ASD. Control animals usually show a social novelty preference, replicating previous research showing that several

untreated control strains of mice prefer the novel animal to the familiar animal during this task (Moy et al., 2004). The Integrin β3 Knockout Mice model of ASD like behavior have normal sociability and no social preference consistent with what was found in the ST + MV and ST + Veh groups of this study (Carter et al., 2011). Acetylcholine elevation in animals as a treatment of the BTBR T+tf/J model of autism showed equal activity but increased preference for the stranger animal (Karvat and Kimchi, 2014). The animals in this study also showed equal activity and the ST + MV group did have the greatest time interaction with a stranger animal, although not to a statistically significant extent. In recent years there has been criticism of Three Chamber Social Task as a measure of social behavior citing reliance on spatial navigation skills to demonstrate sociability (Netser et al., 2017). It is hypothesized that the task may not account for complex behavioral dynamics. Researchers showed that females may have reduced length of attention to the same stimuli as compared to males which may compromise comparisons between sexes in the task (Netser et al., 2017). Additionally, estrous cycle does not appear to interfere with performance in the task. At PN60 there was no differences in social interaction for female rats among the estrous phases and found that they investigated less than male rats overall (Markham and Juraska, 2007). The use of the Ultrasonic Vocalization (USV) task may also be valuable in determining social communication as an adjunct to account for deficits in communications that may be exacerbated in the three chamber social test (Scattoni et al., 2009).

Because *M. vaccae* treatment did not reverse all of the social effects of stress-terbutaline, it is unlikely that this treatment can act as a cure-all for ASD-like behavior. Another microbe based treatment, heat killed *L. reuteri,* appears to decrease social deficits in autistic animal models perhaps as a result of restored Oxytocin levels via vagal stimulation (Sgritta et al., 2019). This

effect only existed for the *L. reuteri* species and no other Lactobacillus species tested (Buffington et al., 2016). This may offer *L. reuteri* and *M. vaccae* as both potential microbiological treatments for autism or in combination.

Marble Bury

In the previous study of males, *M. vaccae* effectively prevented the appearance of repetitive behaviors within stress-terbutaline animals by reducing marble burying behavior. The lack of a difference in number of marbles buried between control and stress-terbutaline groups in the females could either indicate that this model may still produce ASD like behavior in female rats. However, it is more likely that marble burying behavior is not a good measure of repetitive behavior in females. Female burying behavior may not be an accurate measure of repetitive behavior because it has been shown to vary across the estrous cycles and is influenced by hormones, which was not controlled for in this study (Schneider and Popik, 2007). Due to the potential variability in results that this could cause, it would be interesting to test marble burying within stress-terbutaline animals.

An alternative test of repetitive activity could be useful in this study. Utilizing the valproic acid model, researchers tested locomotor and repetitive behavior activity by measuring beam breaks. Three or more times within a time interval was considered repetitive behavior. Pups treated with Valproic Acid had decreased locomotion and increased repetitive behavior (Schneider and Przewłocki, 2005).

Defensive Burying

Stressed females develop passive coping strategies and decreased exploration perhaps due to decreased HPA excitability (Smith et al., 2018). Previously, performance on the defensive burying task was closely associated with the development of spontaneous seizures. In females, there were no differences in burying between treatment groups. However, this may not be the only conclusion. In the previous study, only animals that went on to develop seizures failed to bury. This may indicate that burying was indicative of a protective mechanism and would be supported if few females rats develop seizures since 83% of females buried the probe. If later tests result in no difference in burying among rats that have seizures it could be estimated that males and females differ in their coping behaviors (Campbell et al., 2003)It has been found in the past that females tend to resort to passive coping strategies due to HPA excitability which may account for the lack of burying even if the stress paradigm had the same effects (Smith et al., 2018).

Defensive burying is also used as a measure of emotional processing relating to anxiety and panic. ASD like behavior would indicate emotional processing disruptions and would be expected to result in less burying of the probe (Fucich and Morilak, 2018). Consistency demonstrated across treatment groups in this experiment may indicate that the model does not provide the emotional processing disruptive behavior characteristic of autism. There remains the possibility that females do not display this characteristic as often as males diagnosed with autism.

Additionally, there may be reason to believe that defensive burying is not as accurate of a measure of anxiety as reported in previous studies (Anderson et al., 2018). It is hypothesized that defensive burying tasks are a better measure of panic than anxiety, which are controlled by different areas and pathways within the brain (Martin et al., 2009). Initially it was thought that

the burying task tested either fight or flight reactions but now it is hypothesized that there are many more reactions possible which may complicate the ability of this task to reliably predict anxiety responses of a treatment (Sharp, 2006). If defensive burying is not the best measure of anxiety an alternative test that could be run would be the elevated plus maze (Walf and Frye, 2007).

It has been determined that males are more greatly affected by maternal stress as indicated by findings of proinflammatory cytokines IL-6 and IL-1 in male placentas after early prenatal stress (Bronson and Bale, 2014). Additionally another study found that combinations of IL-6 and IL-1β cytokines enhance the risk for epilepsy in the maternal immune activation model (Pineda et al., 2013). This may indicate that the model, with a basis of maternal immune activation due to chronic stress, would impact males and females differently as seen in this study.

Conclusion

This study showed a difference in the behavior of the male and female rats of the maternal stress and Terbutaline model. It is possible that these effects may get at the underlying mechanisms of Autism and how it differentially affects males three times more often than females. On the other hand these differences may appear as a result of behavioral tests that should not be used across both sexes. Repeating the study with different tests that may be more suited to test the hallmark characteristics of ASD in females may give a better sense of the efficacy of the model. In particular marble bury task may not be a good measure of repetitive behavior if the treatment group sees the marbles as a threat due to heightened stress instead of just as something to bury.

Additionally, as this model is used to test the comorbity of Autism and Epilepsy further study on which animals and which groups developed seizures would shed more light on if the model is one of ASD behaviors only or the comorbidity.

As it cannot be well established that this model produced ASD like behaviors in the female rats the efficacy as *M. vaccae* as a treatment for ASD like behaviors cannot be estimated.

Acknowledgements

This thesis would not have been possible without Zach Smith who helped me revise drafts of this paper and assisted me with statistical analysis. Thank you to Dr. Dan Barth, Dr. Heidi Day, and Rebecca Kubiak for helping me pull all the pieces together and keeping me motivated.

Citations

American Psychiatric Assocation (2013) DSM5 Diagnostic Criteria 5th Ed. Autism Spectrum Disorder. :3.

Anderson EM, McWaters ML, McFadden LM, Matuszewich L (2018) Defensive burying as an ethological approach to studying anxiety: Influence of juvenile methamphetamine on adult defensive burying behavior in rats. Learning and Motivation 61:97–106.

Andres C (2002) Molecular genetics and animal models in autistic disorder. Brain Research Bulletin 57:109–119.

Ashwood P, Wills S, Van de Water J (2006) The immune response in autism: a new frontier for autism research. J Leukoc Biol 80:1–15.

Banerjee A, Engineer CT, Sauls BL, Morales AA, Kilgard MP, Ploski JE (2014) Abnormal emotional learning in a rat model of autism exposed to valproic acid in utero. Front Behav Neurosci 8:387.

Bariselli S, Hornberg H, Prevost-Solie C, Musardo S, Hatstatt-Burkle L, Scheiffele P, Bellone C (2018) Neuronal signature of social novelty exploration in the VTA: implication for Autism Spectrum Disorder. bioRxiv Available at: http://biorxiv.org/lookup/doi/10.1101/280537 [Accessed March 3, 2019].

Becker KG (2007) Autism, Asthma, Inflammation, and the Hygiene Hypothesis. Med Hypotheses 69:731–740.

Bercum FM, Rodgers KM, Benison AM, Smith ZZ, Taylor J, Kornreich E, Grabenstatter HL, Dudek FE, Barth DS (2015) Maternal Stress Combined with Terbutaline Leads to Comorbid Autistic-Like Behavior and Epilepsy in a Rat Model. J Neurosci 35:15894–15902.

Boguszewski PM, Szulawski M, Meyza K, Zagrodzka J (2013) Sex Differences in Anxiety and Social Behavior in Temperamentally Different Rats. Acta Neurobiologiae Experimentalis 73:44–45.

Bronson SL, Bale TL (2014) Prenatal Stress-Induced Increases in Placental Inflammation and Offspring Hyperactivity Are Male-Specific and Ameliorated by Maternal Antiinflammatory Treatment. Endocrinology 155:2635–2646.

Buffington SA, Viana Di Prisco G, Auchtung TA, Ajami NJ, Petrosino JF, Costa-Mattioli M (2016) Microbial reconstitution reverses maternal diet-induced social and synaptic deficits in offspring. Cell 165:1762–1775.

Campbell T, Lin S, DeVries C, Lambert K (2003) Coping strategies in male and female rats exposed to multiple stressors. Physiol Behav 78:495–504.

Carter MD, Shah CR, Muller CL, Crawley JN, Carneiro AMD, Veenstra-VanderWeele J (2011) Absence of Preference for Social Novelty and Increased Grooming in Integrin β3 Knockout Mice: Initial Studies and Future Directions. Autism Res 4:57–67.

Chevallier C, Kohls G, Troiani V, Brodkin ES, Schultz RT (2012) The social motivation theory of autism. Trends in Cognitive Sciences 16:231–239.

Chez MG, Dowling T, Patel PB, Khanna P, Kominsky M (2007) Elevation of tumor necrosis factor-alpha in cerebrospinal fluid of autistic children. Pediatr Neurol 36:361–365.

Croonenberghs J, Bosmans E, Deboutte D, Kenis G, Maes M (2002) Activation of the inflammatory response system in autism. Neuropsychobiology 45:1–6.

De Boer SF, Koolhaas JM (2003) Defensive burying in rodents: ethology, neurobiology and psychopharmacology. European Journal of Pharmacology 463:145–161.

Fonken LK, Frank MG, D'Angelo HM, Heinze JD, Watkins LR, Lowry CA, Maier SF (2018) Mycobacterium vaccae immunization protects aged rats from surgery-elicited neuroinflammation and cognitive dysfunction. Neurobiol Aging 71:105–114.

Frank MG, Fonken LK, Dolzani SD, Annis JL, Siebler PH, Schmidt D, Watkins LR, Maier SF, Lowry CA (2018) Immunization with Mycobacterium vaccae induces an anti-inflammatory milieu in the CNS: Attenuation of stress-induced microglial priming, alarmins and anxiety-like behavior. Brain Behav Immun 73:352–363.

Fucich EA, Morilak DA (2018) Shock-probe Defensive Burying Test to Measure Active versus Passive Coping Style in Response to an Aversive Stimulus in Rats. Bio Protoc 8 Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6157633/ [Accessed March 5, 2019].

Hantsoo L, Kornfield S, Anguera MC, Epperson CN (2019) Inflammation: A Proposed Intermediary Between Maternal Stress and Offspring Neuropsychiatric Risk. Biol Psychiatry 85:97–106.

Hsiao EY, Patterson PH (2011) Activation of the maternal immune system induces endocrine changes in the placenta via IL-6. Brain Behav Immun 25:604–615.

Ivy AS, Brunson KL, Sandman C, Baram TZ (2008) Dysfunctional nurturing behavior in rat dams with limited access to nesting material: a clinically relevant model for early-life stress. Neuroscience 154:1132–1142.

Jyonouchi H, Sun S, Le H (2001) Proinflammatory and regulatory cytokine production associated with innate and adaptive immune responses in children with autism spectrum disorders and developmental regression. J Neuroimmunol 120:170–179.

Karvat G, Kimchi T (2014) Acetylcholine Elevation Relieves Cognitive Rigidity and Social Deficiency in a Mouse Model of Autism. Neuropsychopharmacology 39:831–840.

Lewis ML, Kesler M, Candy SA, Rho JM, Pittman QJ (2018) Comorbid epilepsy in autism spectrum disorder: Implications of postnatal inflammation for brain excitability. Epilepsia 59:1316–1326.

Loomes R, Hull L, Mandy WPL (2017) What Is the Male-to-Female Ratio in Autism Spectrum Disorder? A Systematic Review and Meta-Analysis. J Am Acad Child Adolesc Psychiatry 56:466–474.

Markham JA, Juraska JM (2007) Social Recognition Memory: Influence of Age, Sex, and Ovarian Hormonal Status. Physiol Behav 92:881–888.

Martin EI, Ressler KJ, Binder E, Nemeroff CB (2009) The Neurobiology of Anxiety Disorders: Brain Imaging, Genetics, and Psychoneuroendocrinology. Psychiatr Clin North Am 32:549–575.

Molet J, Maras PM, Avishai-Eliner S, Baram TZ (2014) Naturalistic rodent models of chronic early-life stress. Dev Psychobiol 56:1675–1688.

Moy SS, Nadler JJ, Perez A, Barbaro RP, Johns JM, Magnuson TR, Piven J, Crawley JN (2004) Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. Genes Brain Behav 3:287–302.

Netser S, Haskal S, Magalnik H, Wagner S (2017) A novel system for tracking social preference dynamics in mice reveals sex- and strain-specific characteristics. Molecular Autism 8:53.

Patterson PH (2011) Modeling autistic features in animals. Pediatr Res 69:34R-40R.

Pineda E, Shin D, You SJ, Auvin S, Sankar R, Mazarati A (2013) Maternal immune activation promotes hippocampal kindling epileptogenesis in mice. Annals of Neurology 74:11–19.

Reber SO et al. (2016) Immunization with a heat-killed preparation of the environmental bacterium *Mycobacterium vaccae* promotes stress resilience in mice. Proceedings of the National Academy of Sciences 113:E3130–E3139.

Rice CJ, Sandman CA, Lenjavi MR, Baram TZ (2008) A Novel Mouse Model for Acute and Long-Lasting Consequences of Early Life Stress. Endocrinology 149:4892–4900.

Rook GAW, Lowry CA, Raison CL (2013) Microbial 'Old Friends', immunoregulation and stress resilience. Evol Med Public Health 2013:46–64.

Scattoni ML, Crawley J, Ricceri L (2009) Ultrasonic vocalizations: a tool for behavioural phenotyping of mouse models of neurodevelopmental disorders. Neurosci Biobehav Rev 33:508–515.

Schneider T, Popik P (2007) Attenuation of estrous cycle-dependent marble burying in female rats by acute treatment with progesterone and antidepressants. Psychoneuroendocrinology 32:651–659.

Schneider T, Przewłocki R (2005) Behavioral Alterations in Rats Prenatally Exposed to Valproic Acid: Animal Model of Autism. Neuropsychopharmacology 30:80–89.

Semple BD, Blomgren K, Gimlin K, Ferriero DM, Noble-Haeusslein LJ (2013) Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. Prog Neurobiol 0:1–16.

Sengupta P (2013) The Laboratory Rat: Relating Its Age With Human's. Int J Prev Med 4:624–630.

Sgritta M, Dooling SW, Buffington SA, Momin EN, Francis MB, Britton RA, Costa-Mattioli M (2019) Mechanisms Underlying Microbial-Mediated Changes in Social Behavior in Mouse Models of Autism Spectrum Disorder. Neuron 101:246-259.e6.

Sharp P (2006) The Laboratory Rat. Elsevier. Available at: https://linkinghub.elsevier.com/retrieve/pii/B9780120749034X50006 [Accessed March 3, 2019].

Slotkin TA, Seidler FJ (2013) Terbutaline impairs the development of peripheral noradrenergic projections: Potential implications for autism spectrum disorders and pharmacotherapy of preterm labor. Neurotoxicology and Teratology 36:91–96.

Smith BL, Morano RL, Ulrich-Lai YM, Myers B, Solomon MB, Herman JP (2018) Adolescent environmental enrichment prevents behavioral and physiological sequelae of adolescent chronic stress in female (but not male) rats. Stress 21:464–473.

Smith SEP, Li J, Garbett K, Mirnics K, Patterson PH (2007) Maternal Immune Activation Alters Fetal Brain Development through Interleukin-6. J Neurosci 27:10695–10702.

Walf AA, Frye CA (2007) The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat Protoc 2:322–328.

Werling DM, Geschwind DH (2013) Sex differences in autism spectrum disorders. Curr Opin Neurol 26:146–153.

Wöhr M, Scattoni ML (2013) Behavioural methods used in rodent models of autism spectrum disorders: current standards and new developments. Behav Brain Res 251:5–17.

Zerrate MC, Pletnikov M, Connors SL, Vargas DL, Seidler FJ, Zimmerman AW, Slotkin TA, Pardo CA (2007) Neuroinflammation and behavioral abnormalities after neonatal terbutaline treatment in rats: implications for autism. J Pharmacol Exp Ther 322:16–22.