The Effects of Immunization with Heat-killed *Mycobacterium vaccae* on Maternal Stress and Terbutaline Induced Changes in Microglial Density

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Abstract

The comorbidity of autism and epilepsy has been studied for many years. The exact mechanism for this comorbidity is still unknown, but it is hypothesized that inflammatory mediators, such as dysregulated microglia, serve a significant role. Maternal stress and terbutaline (ST) have previously been shown to result in prolonged CNS inflammation and can be used as an animal model for the comorbidity of ASD and epilepsy. Injections of a heat killed bacterium, *Mycobacterium vaccae*, has been shown to protect against stress-induced inflammation in the periphery and CNS. To test the potential mitigating effects of *M. vaccae* in this model, we measured microglial density across development in certain areas of the brain commonly associated with autism and epilepsy. Three experimental groups (ST+ *M. vaccae*, ST + vehicle, and control unstressed) were perfused at two time points (P15 and P30). Brains were stained for the marker Iba1 and cell counts were analyzed. Data showed significant differences in microglial density in the insula, motor cortex, and molecular layer of the dentate gyrus between the different groups, with *M. vaccae* appearing to have protective effect on the disruptions caused by stress and terbutaline for certain brain regions. These differences are important because they show that these disruptions could lead to the abnormalities in the brain regions that are associated with the two disorders.

Introduction

Autism spectrum disorder (ASD) is a developmental disability that affects 62 in 10,000 children globally (Elsabbagh et al., 2012). Despite its prevalence, much is still unknown about the neurological basis behind the disability. Emerging evidence implicates a combination of genetic, environmental, and immunological factors in the development of the disorder (El-Ansary & Al-Ayadhi, 2012). Recent research has shown neuroinflammation to be present in the cerebral cortex and white matter of those with ASD (Pardo, Vargas, & Zimmerman, 2009). The detrimental effects of neuroinflammation are thought to be mediated in part by glial cells releasing proinflammatory cytokines. The cytokines also influence the excitation of neurons, increasing the risk for hyperexcitability in the brain. This finding is important because epilepsy, a disorder of hyperexcitability, is found in 10-30% of individuals with ASD (Gabisa, Pomeroy, & Andriolac, 2005). More recently, it has been hypothesized that this comorbidity stems from a chronic imbalance of excitation and inhibition contributing to both behavioral deficits and seizure risk (Bozzi, Provenzano, & Casarosa, 2018).

Although the role of neuroglial cytokines in epilepsy is still unknown, it is hypothesized that neuroinflammatory processes may contribute to the comorbidity of ASD and epilepsy (Bozzi et al., 2018). Recently, a novel rat model of combined ASD and epilepsy resulting in neuroinflammation has been developed that uses a combination of maternal stress and terbutaline as developmental teratogens. Terbutaline is a drug administered to mothers to prevent preterm labor (Rodier, Miller, & Brent, 2011). In this model, either maternal stress or terbutaline leads to ASD-like behavior in rats, but not epilepsy. However, the combination of both leads to the comorbid ASD-like symptoms and epilepsy (Bercum et al., 2015).

Development is characterized by a complex interaction between genes and environment (Bale et al., 2010). Early developmental processes are vulnerable to disruptions from environmental stressors, creating lasting impacts on the individual. Certain early life experiences, such as disjointed maternal care, early life stress, infection, or inflammation can lead to changes within gene expression and increase the risk for neurodevelopmental disorders (Bale et al., 2010). These experiences have been used in animal models and have shown that the environment can have an influence on gene function and expression (Bale et al., 2010). Therefore, it is clear that negative early life experiences interfere with development and have lasting effects and impacts. Conversely, positive early life experiences can be very beneficial for development. Studies have shown environmental enrichment to be correlated with a decrease in anxiety and stress related behaviors (Benaroya-Milshtein et al., 2004).

The immune system has a prominent role in dealing with stress. It has been shown to alleviate consequences of childhood stressors, such as abuse or neglect (Johnson & Kaffman, 2018). However, early life stress can lead to immune and neuroimmune system dysregulation. An animal model for early life stress, composed of brief early separation of pups from mothers showed that early life stress was correlated with differences in number of microglia and differences in their functions (Johnson & Kaffman, 2018). Microglia are the innate immune cells of the nervous system. Microglia are typically responsible for synaptic pruning, synaptogenesis, myelination and axonal growth during development (Johnson & Kaffman, 2018). Due to the extent of their involvement, neuroinflammation or dysregulation of the immune system can have adverse effects on numerous different cognitive processes. Therefore, disruptions in these processes can lead to problems in development. Preventative strategies are important in restoring correct brain function, or inhibiting adverse effects of developmental stressors.

Closely linked to the immune system is the gut microbiome. The "Old Friends" hypothesis follows the observation that organisms in the microbiome play a key role in immunoregulation (Rook, Lowry, & Raison, 2013). A reduction in immunoregulatory commensal bacteria, or "Old Friends," due to modern hygiene and dietary changes could explain the increases in autoimmune and stress-related disorders in developed countries. Psychosocial stressors have a large impact early on in life and can lead to increases in inflammatory regulators, as well as modulate the microbiota in the body (Rook et al., 2013). This dysregulation has been linked to the appearance of numerous psychiatric disorders and has been found to have implications in ASD (Rook et al., 2013). Therefore, by ensuring the microbiota is regulated, these psychiatric disorders could potentially be prevented. A microorganism, *Mycobacterium vaccae* (*M. vaccae*), has been shown to have immunoregulatory and anti-inflammatory properties (Fonken et al., 2018; Reber et al., 2016; Zuany-Amorim et al., 2002). Administration of heat-killed *M. vaccae* has been shown to alleviate stress-induced inflammation and behavioral effects of chronic stress (Reber et al., 2016). This effect of *M. vaccae* involves the induction of an anti-inflammatory phenotype in the periphery and the CNS (Reber et al., 2016; Frank et al., 2018).

Stress-terbutaline has previously been shown to cause extended neuroinflammation. By characterizing the neuroinflammatory responses early in life, and targeting stress-induced neuroinflammation using an anti-inflammatory intervention (*M. vaccae*), it could be possible to prevent ASD/Epilepsy comorbidity in this model. Given this information, we hypothesized that the maternal stress and terbutaline model will cause dysregulation of microglia during brain development. These disruptions could interfere with developmental programming and have enduring effects on the development of the nervous system. However, *M. vaccae* could help prevent disturbances that lead to or result from increased neuroinflammation. To test this, we

measured the effect of *M. vaccae* on the proliferation of microglia across development in animals undergoing a stress + terbutaline procedure.

Materials & Methods

Animals & Treatment

Female Sprague-Dawley rats (n=24, Envigo) weighing 200-220 grams at embryonic day (E) 2 were used and given one day to acclimate to the animal facility before experimental manipulations were performed. The pregnant dams were individually housed in 26.67 x 48.26 x 20.32 cm sealed and ventilated static cages (Allentown Inc.). Animals were housed under standard conditions in a temperature-controlled environment (20°C ± 1, relative humidity 22%) with a 12h light/dark cycle (lights on from 7:00 A.M. – 7:00 P.M.) and *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.

18 Dams were randomly assigned to one of two experimental groups (developmental stress + terbutaline + *M. vaccae* (ST+MV, n=9) or developmental stress + terbutaline + vehicle (ST+VEH, n=9)). Due to the complexity of this chronic stress model, the potential confounding stressors of injection, and previous results demonstrating that behavioral effects of M. *vaccae* are only present during periods of stress, a completely undisturbed no-stress control group was chosen for comparison in this study (NSC, n=6). 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, at 10:00 am. The control group was left completely unmanipulated.

Developmental Stressors

To create a procedure mimicking chronic stress exposure during pregnancy in humans, we combined several established stress paradigms to create a singular "developmental stress" rat model starting on Embryonic Day 3 (E3) and continuing until post-natal day 9 (P9). Rats are an atrical species, meaning that many developmental events occur postnatally, with P1-10 considered roughly equivalent to the third trimester in humans (Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013).

Chronic Maternal Stress. Experimental dams were exposed to a repeated mild stress paradigm from E3-E20 (Figure 1). On E3, dams were habituated to a sound attenuated chamber for 5 minutes with a shock grid floor (Coulbourn Instruments) for exploration of the novel environment (Bercum et al., 2015). From E4-E9, animals underwent a 7-day context re-exposure stress paradigm. On E4, contextual fear was established by returning the pregnant dams to the same attenuated chamber. After an initial 60 s exposure, two 1mA shocks were delivered (at 60 s intervals) followed by an additional 5-minute exposure to the environment post-shock. The following 2 days, the dams were returned to the chamber for 5 min. To avoid complete fear extinction, the shock paradigm was repeated on E7, followed by 2 days of contextual environment exposure. On E10, the pregnant dams were left undisturbed, before beginning the 7-day context re-exposure paradigm again. This paradigm was repeated until dams gave birth. During each trial, freezing time was recorded via a blind observer using a stopwatch. The dams were weighed every day to ensure pregnancy was not terminated.

Wk1	Habituation E3	Shock E4	Context E5	Context E6	Shock E7	Context E8	Context E9
Wk2	Rest E10	Shock E11	Context E12	Context E13	Shock E14	Context E15	Context E16
Wk3	Rest E17	Shock E18	Context E19	Context E20			

Figure 1. Outline of mild stress paradigm schedule.

Cross Fostering. On post-natal day (P) 2, females were culled and pups from all litters were cross fostered within treatment group. Pups were counterbalanced across dams to promote ubiquitous maternal care.

Terbutaline Injection. Terbutaline injections were administered on P2-5 to the ST+MV and ST+VEH groups using terbutaline sulfate (Sigma-Aldrich, St. Louis, MO) in doses of 10 mg/kg dissolved in saline (Bercum et al., 2015; Slotkin & Seidler, 2013; Zerrate et al., 2007a), *M. vaccae* and vehicle solutions were prepared as stated above and administered interperitoneally on P7, 13, and 20 at 10:00 A.M. to the pups, according to their maternal treatment group.

Limited Bedding. On P2-9, the dams and pups were exposed to limited bedding paradigm as described in previous experiments (Ivy, Brunson, Sandman, & Baram, 2008; Molet, Maras, Avishai-Eliner, & Baram, 2014; Rice, Sandman, Lenjavi, & Baram, 2008). The animals were placed in a chronic video recording cage (Axis M3104-L network Camera) and were given 6 pieces of food (26 grams, Tekland) and *ad libitum* water. At P10, the animals were returned to their home cage with normal amount of bedding and *ad libitum* access to food and water (Ivy et al., 2008; Molet et al., 2014). Maternal behavior (nursing, pup grooming, self-grooming, and

eating/drinking) was observed at three time points: 11:00 A.M, 1:30 P.M., an 6:30 P.M. for an hour and a half (Ivy et al., 2008).

Maternal Separation. On P2-9, pups were removed from their mother and litter mates. The pups were placed in individual cages (28.79 x 19.96 x 11.43 cm) with one paper towel in a separate room from the dams for 3 hours each day (Molet et al., 2014).

Histology

Immunofluorescence: At P15 and P30, select rats were anesthetized with isoflurane and transcardially perfused with ice-cold saline, followed by 4% paraformaldehyde. Brains were removed and post-fixed for 24hrs in 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose and then frozen using dry ice and isopentane. Brains were sectioned onto Histobond® slides (Cat. No. 16,004-406; VWR, West Chester, PA, USA) at 20 µm using a cryostat (Leica CM 1950, Leica Biosystems, Buffalo Grove, IL, USA) and stored at -80°C. Slides were removed from refrigeration and allowed to reach room temperature before being washed in 1X PBS for 5 minutes. Slides were blocked with 10% NGS in 1X PBS with 0.2% Triton X (PBS + Tx) for 1 hour at room temperature. Slides were incubated at room temperature overnight in 1X PBS + Tx with the following primary antibodies: Iba1 (rabbit, Wako 019-19741, 1:1000. Slides were washed in 1X PBS 3 times for 5 minutes, then incubated for 2 hours in the dark in 1X PBS + Tx with the following secondary antibodies: 488 goat anti-rabbit (1:500) and 594 Goat anti-mouse (1:500). Slides were washed with 1X PBS, then coverslipped using Vectashield with DAPI (Vectashield). Images were taken using a Nikon Eclipse 90 with a Lumen Dynamics X-Cite 120 Fluorescence Light Source and analyzed using FIJI.

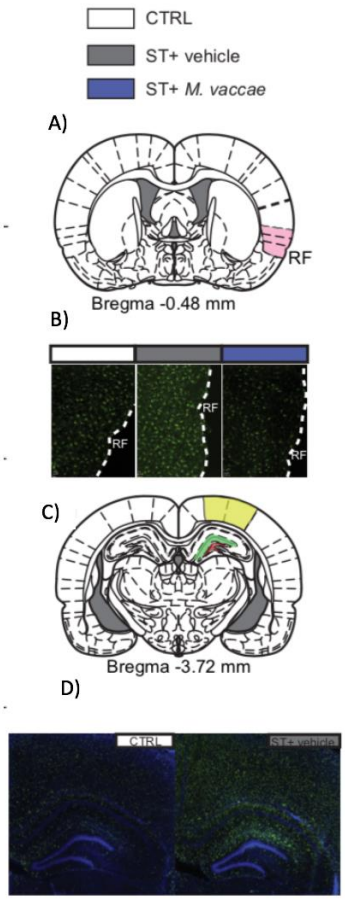

Figure 2. Stress-terbutaline causes increased microglial staining in specific brain regions and is mitigated by *M. vaccae*. A) Illustration of first rostrocaudal level used and brain region that was sampled for measurement of ionized calcium-binding adapter molecule 1 (Iba1) immunostaining with the rhinal fissure for reference (pink; insular cortex) B) Representative images at 10X of Iba-1 positive cells in the insular cortex at P15 with the rhinal fissure for reference for each group (RF). C) Illustration of second rostrocaudal level and brain regions that were sampled for Iba-1 staining (yellow; motor cortex, red; dentate hilus, green; dentate gyrus molecular layer) D) Representative images at 4X of Iba-1 positive staining within the hippocampus and cortex, co-stained with DAPI for subregion localization at P15 in control and ST+ vehicle animals.

Results

Brain Regions Associated with Autism

Microglia are highly sensitive to disruptions in response to challenges within the environment (Rohan Walker, Nilsson, & Jones, 2013). Stress has been shown to disrupt typical microglia function and structure (Rohan Walker et al., 2013). Terbutaline has also been related to

increases in microglia (Zerrate et al., 2007b). Therefore, when combining stress and terbutaline, it was hypothesized that there would be disturbances in microglia density. Microglia are typically responsible for synaptic pruning, synaptogenesis, myelination, and axonal growth (Zerrate et al., 2007). Therefore, disruptions within these functions could potentially have numerous implications on typical brain development.

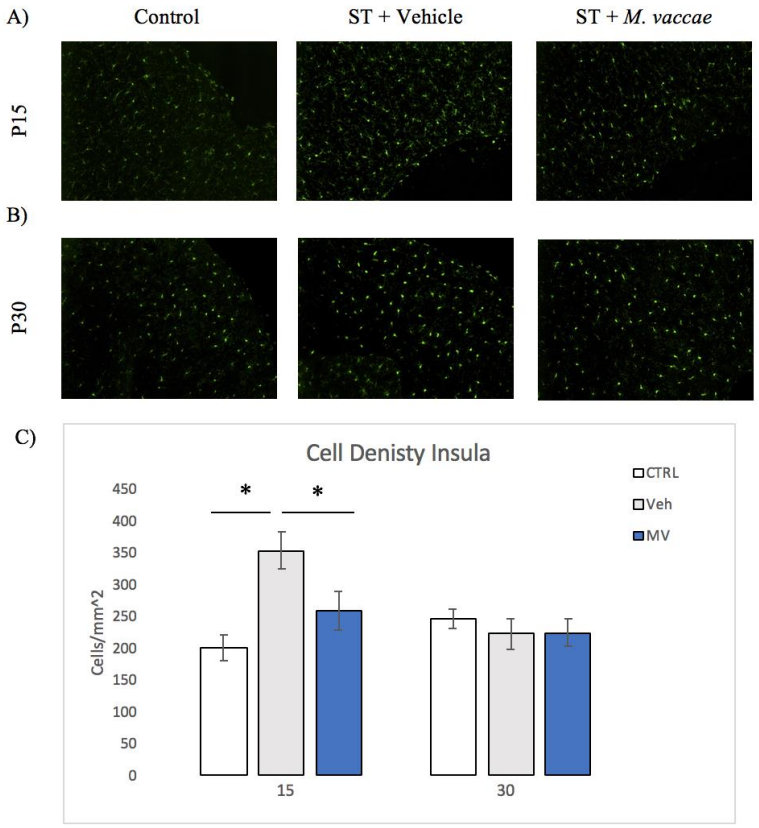

Figure 3. *M. vaccae* prevents stress + terbutaline induced increases in microglial cell density in the insular cortex of rat pups at P15. A) Representative images of Iba-1 staining in the insula of control, ST+ vehicle, and ST+ *M. vaccae* pups at P15. B) Representative images show differences in microglial cell density do not persist to P30. C) Cell density analysis of control and treatment groups demonstrated significantly higher microglial density counts in vehicle treated stress + terbutaline animals compared to both unmanipulated control animals and *M. vaccae* treated stress + terbutaline animals at P15. There were no statistical differences found between control and *M. vaccae* treated ST animals. This stress + terbutaline induced increase in microglial density appeared to be transient within the insula, as no group differences were observed differences did not persist to P30. Error bars represent mean $\pm$ SEM. *p<0.05, **p<0.01

The insular cortex is a region of the brain that is involved in many different functions, such as integrating cognitive, sensory, and emotional information (Gogolla, Takesian, Feng, Fagiolini, & Hensch, 2014). Abnormalities within the development of this brain region have been found in animal models of ASD (Gogolla et al., 2014). A between groups ANOVA test was run to look for differences in microglial cell density between control, ST+ vehicle, and ST+ *M. vaccae*, for P15 and P30. A significant difference was found between the control, ST+ vehicle, and ST+ *M. vaccae* groups for P15 ($F_{(2,15)}$=8.17, $p < 0.01$). A pairwise t-test was run to look for individual differences and showed there were significant differences between ST+ vehicle (353.97 ± 29.86 cells/mm^2) and control (201.04 ± 20.79 cells/mm^2, p<.01) and ST+ vehicle and ST+ *M. vaccae* (258.48 ± 30.63 cells/mm^2, p<.05). There was not a significant difference between ST+ *M. vaccae* and the controls. Further, there were no significant differences found within the insular cortex between the control (246.69 ± 14.58 cells/mm^2), ST+ vehicle (222.62 ± 23.65 cells/mm^2), and ST+ *M. vaccae* (224.21 ± 21.36 cells/mm^2) groups on P30 ($F_{(2,13)}$=0.49, p = 0.619).

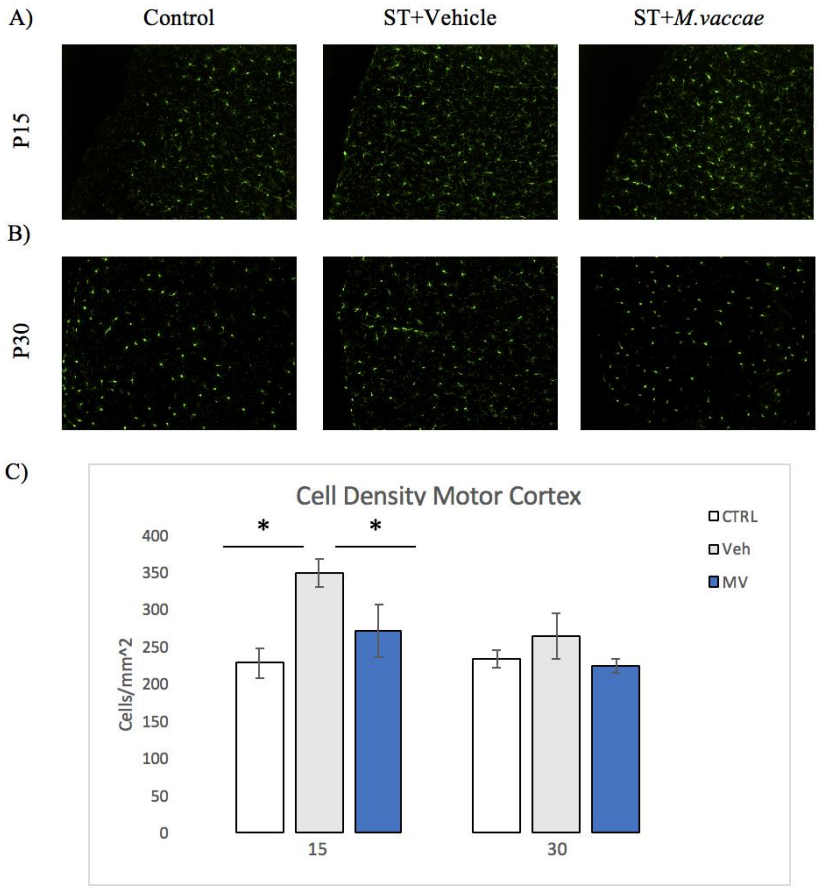

Figure 4. *M. vaccae* prevents stress + terbutaline induced increases in microglial cell density in the motor cortex of rat pups at P15. A) Representative images of Iba-1 staining in the motor cortex of control, ST+ vehicle, and ST+ *M. vaccae* pups at P15. B) Representative images show differences in microglial cell density do not persist to P30. C) Cell density analysis of control and treatment groups demonstrated significantly higher microglial density counts in vehicle treated stress + terbutaline animals compared to both unmanipulated control animals and *M. vaccae* treated stress + terbutaline animals at P15. There were no statistical differences found between control and *M. vaccae* treated ST animals. This stress + terbutaline induced increase in microglial density appeared to be transient within the motor cortex, as no group differences were observed and differences did not persist to P30. Error bars represent mean $\pm$ SEM. *$p<0.05$, **$p<0.01$

The motor cortex is another region that has been studied and given attention in its relation to ASD. Motor impairments can be seen in animal models of ASD, and such impairments have been attributed to abnormalities within the motor cortex (Reynolds, Millette, & Devine, 2012). A between groups ANOVA test was run to look for differences in microglial cell density between

control, ST+ vehicle, and ST+ *M. vaccae*, for P15 and P30. A significant difference was found between the control, ST+ vehicle, and ST+ *M. vaccae* groups for P15 ($F_{(2,14)}$=6.93, $p < 0.01$). A pairwise t-test was run to look for differences and showed there were significant differences between ST+ vehicle (350.28 ± 19.14 cells/ mm^2) and control (228.51 ± 19.32 cells/ mm^2, p<.01). There was not a significant difference between ST+ *M. vaccae* (271.87 ± 34.52 cells/ mm^2) and ST+ vehicle or the controls. Further, there were no significant differences found within the motor cortex between the control (233.79 ± 11.28 cells/ mm^2), ST+ vehicle (265.87 ± 30.85 cells/ mm^2), and ST+ *M. vaccae* (224.41 ± 8.75 cells/ mm^2) on P30 ($F_{(2,13)}$=1.27, p = 0.312).

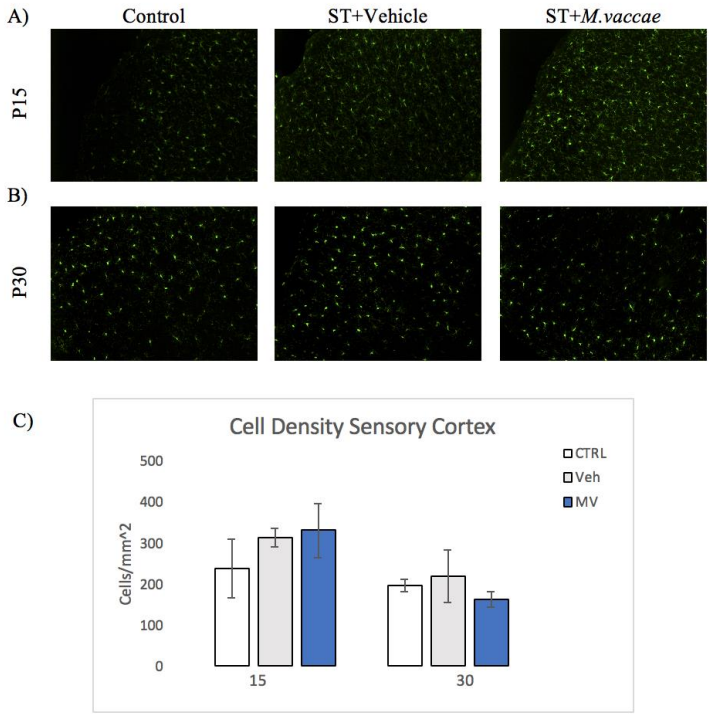

Figure 5. There is no statistical difference in microglial cell density in the somatosensory cortex of rat pups at P15 or P30. A) Representative images of Iba-1 staining in the somatosensory cortex of control, ST+ vehicle, and ST+ *M. vaccae* pups at P15. B) Representative images show no differences in microglial cell density at P30. C) Cell density analysis of control and treatment groups demonstrated no significant difference in microglial density counts in vehicle treated stress + terbutaline animals, unmanipulated control animals, or *M. vaccae* treated stress + terbutaline animals at P15. Error bars represent mean ± SEM. *p<0.05, **p<0.01

The sensory cortex is also a region of the brain that has been studied for how it relates to what appears to be abnormal sensory stimulated behavior in individuals with ASD. It is hypothesized that abnormalities within the sensory cortex could be attributed to the core features associated with ASD (Marco, Hinkley, Hill, & Nagarajan, 2011). Therefore, a between groups, ANOVA test was run to look for differences in microglial cell density between control, ST+ vehicle, and ST+ *M. vaccae*, for P15 and P30. There was not significant difference found between the control (238.28 ± 71.42 cells/mm^2), ST+ vehicle (313.12 ± 22.0 cells/mm^2), and ST+ *M. vaccae* (332.06 ± 66.70 cells/mm^2) groups for P15 ($F_{(2,14)}$=0.689, p =0.518). Further, there were also no significant differences found within the sensory cortex between the control (196.22 ± 14.87 cells/mm^2), ST+ vehicle (220.74 ± 64.51 cells/mm^2), and ST+ *M. vaccae* (163.35 ± 19.69 cells/mm^2) on P30 ($F_{(2,14)}$=0.579, p = 0.57).

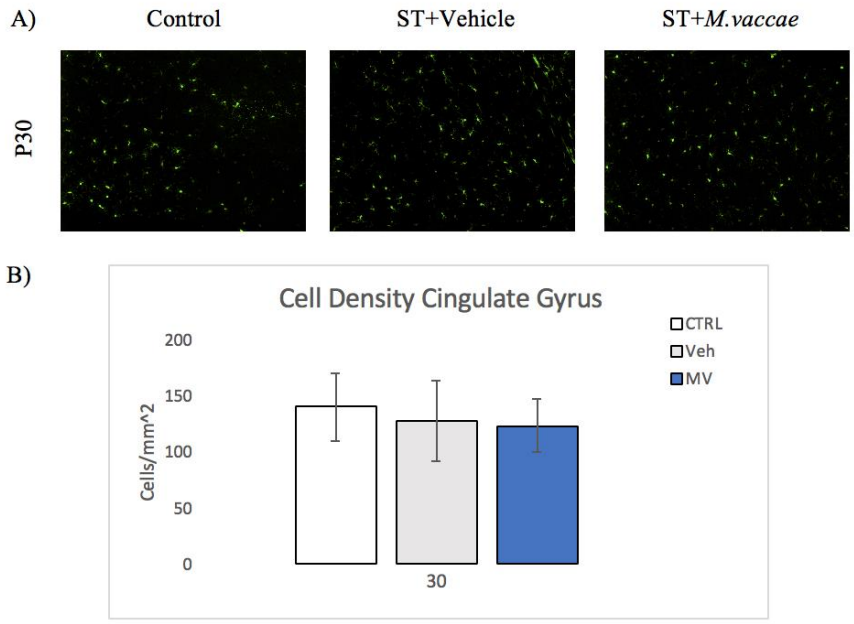

Figure 6. There is no statistical difference in microglial cell density in the cingulate gyrus of rat pups at P30. A) Representative images of Iba-1 staining in the cingulate gyrus of control, ST+ vehicle, and ST+ *M. vaccae* pups at P30. B) Cell density analysis of control and treatment groups demonstrated no significant difference in microglial density counts in vehicle treated stress +

terbutaline animals, unmanipulated control animals, or *M. vaccae* treated stress + terbutaline animals at P30. Error bars represent mean ± SEM. *p<0.05, **p<0.01

A final structure that is thought to be involved in autism is the cingulate gyrus. This region of the brain is involved in regulation of emotions and behavior. Many studies have shown that the cingulate gyrus smaller in individuals with autism (Lord, Cook, Leventhal, & Amaral, 2000). Although it is smaller, it also tends to be more densely packed with neurons, as compared to a typically developing brain (Lord et. al, 2000). A between groups, ANOVA test was run to look for differences in microglial cell density in the cingulate gyrus between control, ST+ vehicle, and ST+ *M. vaccae*, for P30. There was no significant difference found between the control (140.22 ± 29.81 cells/ mm^2), ST+ vehicle (127.84 ± 36.54 cells/ mm^2), and ST+ *M. vaccae* (123.58 ± 23.14 cells/ mm^2) groups for P30 ($F_{(2,14)}$=0.085, p = 0.92).

Brain Regions Associated with Epilepsy

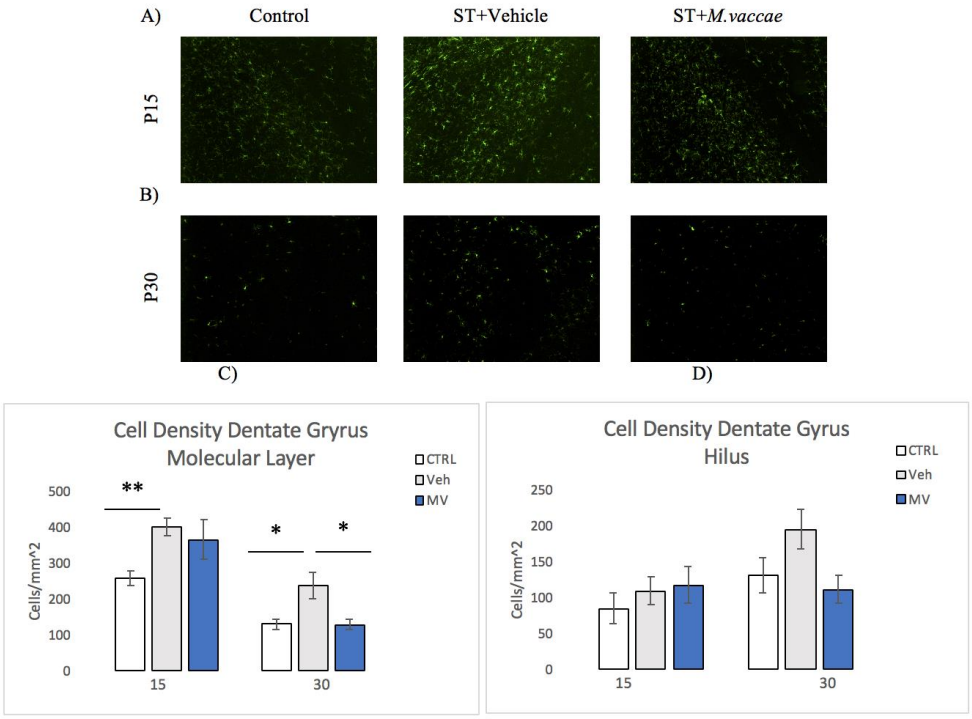

Figure 7. Stress + terbutaline increases in microglial cell density in the dentate gyrus molecular layer of rat pups at P15. These differences persist for ST+ vehicle pups to P30. A) Representative images of Iba-1 staining in the dentate gyrus of control, ST+ vehicle, and ST+ *M. vaccae* pups at P15. B) Representative images show differences in microglial cell density do persist to P30 for ST+ vehicle pups. C) Cell density analysis of control and treatment groups demonstrated significantly higher microglial density counts in Vehicle treated stress + terbutaline animals compared to unmanipulated control animals at P15. There were no statistical differences found between M. *vaccae* treated ST animals and control or ST+ vehicle animals. This stress + terbutaline induced increase in microglial density appeared to persist within the ST+ vehicle animals, as there was a statistically significant difference between ST+ vehicle and both control and *M. vaccae* animals at P30. D) Cell density analysis of the dentate gyrus hilus demonstrated there were no significant differences between *M. vaccae* treated ST animals, control animals, or ST+ vehicle animals for P15 or P30. Error bars represent mean ± SEM. *p<0.05, **p<0.01

The dentate gyrus is considered an important region and has been heavily studied with its relation to epilepsy. The dentate gyrus appears to have strong implications in epilepsy. There is an emerging theory that decreased inhibition of granule cells of the dentate gyrus lead to increased activity and seizures (Krook-Magnuson et al., 2015). A between groups ANOVA test was run to look for differences in microglial cell density between control, ST+ vehicle, and ST+ *M. vaccae*, for P15 and P30 in the dentate gyrus molecular layer and hilus. A significant difference was found between the control, ST+ vehicle, and ST+ *M. vaccae* groups in the molecular layer for P15 ($F_{(2,15)}$=4.13, $p < 0.05$). A pairwise t-test was run to look for specific differences. There were significant differences between ST+ vehicle (401.58 ± 25.44 cells/mm^2) and control (257.19 ± 19.64 cells/mm2, p<.01). There was not a significant difference between ST+ *M. vaccae* (366.26 ± 55.54 cells/mm^2) and ST+ vehicle or the controls. There were also significant differences in microglial density found within the molecular layer of the dentate gyrus between the control, ST+ vehicle, and ST+ *M. vaccae* groups on P30 ($F_{(2,14)}$=5.74, $p < 0.05$). A pairwise t-test was run to look for specific differences. There were significant differences between ST+ vehicle (232.58 ± 37.67 cells/mm^2) and control (129.93 ± 13.38 cells/mm^2, p<.05) and ST+ vehicle and ST+ *M. vaccae* (129.24 ± 14.23 cells/mm^2, p<.05). There were no

significant differences found between the control (85.28 ± 521.28 cells/mm^2), ST+ vehicle (109, 51 ± 20.08 cells/mm^2), and ST+ *M.vaccae* (117.77 ± 26.07 cells/mm^2) groups in the dentate gyrus hilus for P15 ($F_{(2,14)}$=0.56, p= 0.58) or the control (131.45 ± 24.26 cells/mm^2), ST+ vehicle (195.14 ± 27.42 cells/mm^2), and ST+ *M.vaccae* (111.55 ± 18.74 cells/mm^2) groups on P30 ($F_{(2,14)}$=2.26, p= 0.14).

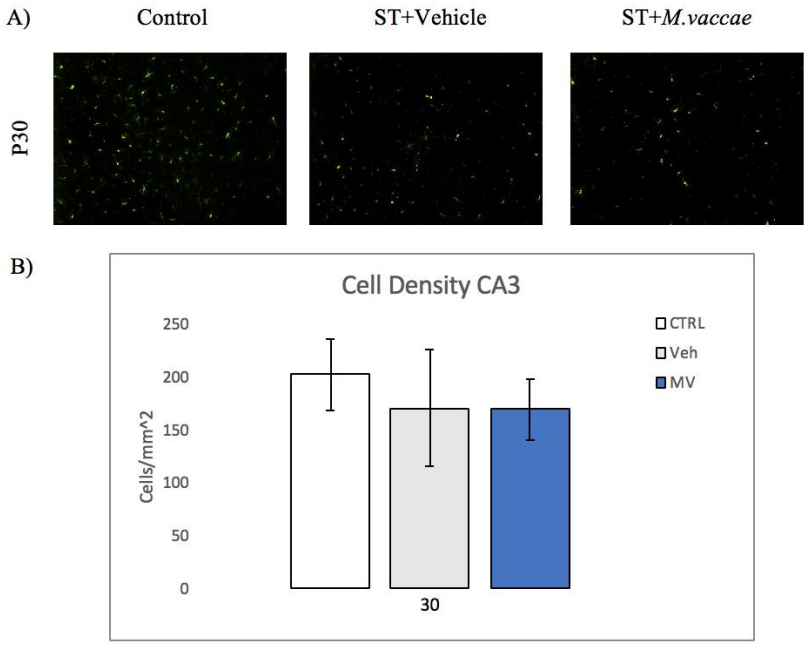

Figure 8. There is no statistical difference in microglial cell density in the region CA3 of rat pups at P30. A) Representative images of Iba-1 staining in CA3 of control, ST+ vehicle, and ST+ *M. vaccae* pups at P30. B) Cell density analysis of control and treatment groups demonstrated no significant difference in microglial density counts in vehicle treated stress + terbutaline animals, unmanipulated control animals, or *M. vaccae* treated stress + terbutaline animals CA3 at P30. Error bars represent mean ± SEM. *p<0.05, **p<0.01

A region of the hippocampus, CA3, is another region in which abnormalities appear to have implications in epilepsy. Specifically, it appears that neuronal networks within the CA3 region are hypoactive in those with epilepsy. This hypoactivity changes the typical hippocampus output signals, which may have an effect on the differences in brain activity which are seen in epilepsy (Biagini et al., 2005). A between groups ANOVA test was run to look for differences in

microglial cell density for CA3 between control, ST+ vehicle, and ST+ *M. vaccae*, for P30. There was not a significant difference found for microglial density CA3 between the control (202.59 ± 33.31 cells/ mm^2), ST+ vehicle (170.73 ± 55.13 cells/ mm^2), and ST+ *M. vaccae* (169.68 ± 29.01 cells/ mm^2) groups for P30 ($F_{(2,13)}$=0.23, p = 0.78).

Discussion

Results showed that there were differences in microglial density between the three groups in certain brain regions. Specifically, microglial density was significantly higher in the insular cortex in the ST + vehicle compared to the other two groups for P15. Differences within microglial density did not persist to P30 for the insular cortex. Further, microglial density was also significantly higher in the motor cortex for the ST + vehicle treatment group, as compared to the ST + *M. vaccae* and controls on P15. Similar to the insular cortex, differences did not persist to P30. Lastly, microglial density was significantly higher in the molecular layer of the dentate gyrus in the ST + vehicle group as compared to controls. There were no significant differences in microglial density between ST + *M. vaccae* and ST+ vehicle or the controls. Unlike the motor and insular cortex, these differences did persist to P30. Microglial density was significantly higher in the molecular layer of the dentate gyrus for the ST + vehicle treatment group compared to the ST + *M. vaccae* and controls on P30.

It is hypothesized that activation of microglia can have potential negative effects on the brain, due to the fact that microglia can release proinflammatory cytokines (Dheen, Kaur, & Ling, 2007). Although it is clear there are significant differences in microglial density in certain brain regions between the two treatment groups and the control group, it is important to note that these microglia might not be active. Chronic microglial activation has been associated with

numerous neurodegenerative diseases (Dheen et al., 2007). However, since we only looked at the density of microglia and not whether they were active, we cannot speak to whether that leads to increased inflammation. Knowing that microglia are typically responsible for synaptic pruning, synaptogenesis, myelination, and axonal growth, it is possible that differences within microglial density could lead to abnormalities within these functions (Johnson & Kaffman, 2018).

Autism spectrum disorder is a neurodevelopmental disorder that is typically characterized by differences in sensory processing and integration, as well as motor impairments (Lord et al., 2000). Motor impairments can be seen through difference in motor speed and performance (Reynolds et al., 2012). Results showed no differences between the groups in the sensory cortex for P15 or P30. However, there was a significant difference in microglial density in the motor cortex for P15. Previous studies have found that increased white matter was positively correlated with increased motor impairment in individuals with ASD (Mostofsky, Burgess, & Gidley Larson, 2007). Since microglia are responsible for myelination, it is possible that an increase in microglia could affect myelination. However, further studies would need to be done to test whether the microglia were contributing to increases in white matter. Although the differences in microglial density did not persist to P30, the impacts of the increased density at P15 could have endured.

The cingulate gyrus is a brain region associated with emotions and behavior. It is often looked at with regards to ASD because individuals with ASD often have difficulties regulating emotions and abnormalities with behavior (Lord et al., 2000). Previous research has found that this area tends to be smaller in individuals with ASD; however, the area is more densely packed with neurons (Lord et al., 2000). These data, however, showed no differences in microglial density in the cingulate gyrus for P30.

The insular cortex is another important brain region associated with ASD. The insular cortex is responsible for integration of processes such as emotions, cognition, and sensory information (Gogolla et al., 2014). Animal models of autism have shown this brain region to be impaired in the ASD animals. Further, studies have shown this impairment is due to an inhibition/excitation imbalance within the insular cortex, which can be restored by inhibitory manipulations during early developmental critical periods (Gogolla et al., 2014). Other research has found abnormalities within the function and connections of the insula in those with ASD (Odriozola et al., 2016). Some researchers believe the region is hypoactive in individuals with ASD (Uddin & Menon, 2009). Overall, it is unclear whether the region is overactive or underactive; however, there does seem to be evidence for an imbalance of excitation and inhibition. The data showed there were differences within this region between the ST + vehicle and both the control and ST + *M. vaccae*. The microglial density was significantly higher in the ST + vehicle group. If microglia are directly affecting neuronal firing by facilitating excitatory activity, this could be a possible explanation for excitation/inhibition imbalances. Alternatively, microglia could also be responsible for differences in synaptic pruning, axonal growth, or myelination. Because inhibitory networks are still developing during this early period, differences within any of these functions could lead to abnormalities of the typical functions the insular cortex.

The dentate gyrus is considered an important brain region in epilepsy, because it is typically responsible for inhibiting hippocampal overexcitation (Krook-Magnuson et al., 2015). It is hypothesized that decreased inhibition of granule cells in the dentate gyrus is related to onset of spontaneous seizures (Krook-Magnuson et al., 2015). The dentate gyrus is unique in that the granule cells of the dentate gyrus continue to develop throughout life (Pleasure, Collins, &

Lowenstein, 2000). This is important because it means that, unlike other brain regions, the dentate gyrus remains highly plastic throughout life. Further research has also shown that stress may inhibit granule cell production and, therefore, alter the development of the dentate gyrus (Tanapat, Galea, & Gould, 1998). The data showed differences in microglia density in the molecular layer of the dentate gyrus between ST + vehicle and control animals for P15 and differences between ST + vehicle and both ST + *M. vaccae* and the controls for P30. There were significantly more microglia in the ST + vehicle group. Given typical microglial function, disruptions within those functions could have led to an imbalance of excitation and inhibition within the dentate gyrus, therefore causing a dysregulation to inhibitory control over the hippocampus. There were no differences found within the dentate hilus of the dentate gyrus. Animal models have found methods for decreasing seizures, by increasing inhibition on the granule cells (Krook-Magnuson et al., 2015). Based on this evidence, it was not necessarily expected that there would be differences within granule layer because the abnormalities appear to be in inhibitory control of such cells in epilepsy, but not in cell function itself.

CA3 is a region of the hippocampus which has also been hypothesized to be involved in epilepsy. The granule cells of the dentate gyrus are the communication pathway to CA3 (Scharfman & Bernstein, 2015). Therefore, abnormalities with inhibition of the granule cells will affect downstream firing of the CA3. There were no differences found in microglial density within CA3 region. However, if there is an imbalance within regulation of the granule cells, then there will also be an imbalance within regulation of CA3. Therefore, despite not seeing a difference in microglia density, it does not mean the CA3 region would be unaffected.

Overall, we can see there is an effect of maternal stress and terbutaline on microglial density in certain brain regions. These effects can be prevented in some brain regions via

immunization with *M. vaccae*. It is important to understand that the increased microglial density does not necessarily mean that the brain region will be overactive or underactive. However, the results simply imply the microglia could be related to any one of the abnormalities found within the different brain regions for each disorder. These results are important because they give insight to just how early in development differences start to occur. By being able to detect differences earlier, intervention methods can begin to be studied to try to alleviate or inhibit such changes from occurring. The earlier we can detect differences, the earlier we can act in taking steps to prevent these differences and potentially reverse adverse consequences.

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