The effect of prenatal *Mycobacterium tuberculosis* infection on offspring neurodevelopment and autistic-like behaviours in a Valproic acid mouse model of autism

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PREFACE

The experimental work described in this thesis was conducted at the Africa Health Research Institute and University of KwaZulu- Natal (Durban, South Africa), under the supervision of Dr. Thabisile Mpofana, Dr Nontobeko. E. Mvubu and Professor Adrie J. C. Steyn.

This work has not been submitted in any form for any degree to any tertiary institution, where use has been made of the work of others, it is duly acknowledged in the text.

7 May 2021

Wadzanai Manjeese **Date**

As the candidate's supervisor, I agree to the submission of this thesis

7 May 2021

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(Supervisor)

7 May 2021

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(Co-supervisor)

7 May 2021

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Professor Adrie. J. C. Steyn **Date**
(Co-supervisor)

DECLARATION

I **Wadzanai Manjeese (218074724)** declare that:

i. The research reported in this thesis, except where otherwise indicated, is my original work.

ii. This thesis has not been submitted for any degree or examination at any other university.

iii. This thesis does not contain other persons' data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.

iv. That my contribution to the project was as follows:
Identification of research topics, experimental design, execution, data analysis and interpretation, manuscript and thesis write-up.

v. This dissertation does not contain other persons writing unless specifically acknowledged as being sourced from other researchers. Where other sources have been quoted, their words have been rewritten but the general information attributed by them has been referenced.

vi. This dissertation does not contain text, graphics, or tables copied and pasted from the internet unless specifically acknowledged and the source being detailed in the dissertation and the reference sections.

7th May 2021

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Wadzanai Manjeese **Date**

DEDICATION

To my late brother, Chipo Manjeese

My number 1 cheerleader who encouraged and pushed me in my career amid fighting his own battle.

PUBLICATIONS AND PRESENTATIONS

List of published peer reviewed articles

Manjeese W, Mvubu NE, Steyn AJC and Mpofana T. *Mycobacterium tuberculosis*-Induced Maternal Immune Activation Promotes Autism-Like Phenotype in Infected Mice Offspring *International Journal of Environmental Research and Public Health.* 2021 Apr 23;18(9):4513. doi: 10.3390/ijerph1

Manjeese W, Mvubu NE, Steyn AJC and Mpofana T. M*ycobacterium tuberculosis* causes a leaky blood-brain barrier and neuroinflammation in the prefrontal cortex and cerebellum regions of infected mice offspring. *International Journal of Developmental Neuroscience*. 2021 May 1. doi: 10.1002/jdn.10116.

List of submitted peer-reviewed articles under review

Manjeese W, Mvubu NE, Steyn AJC and Mpofana T. Gestational *Mycobacterium tuberculosis* exposure affects pregnancy and leads to developmental delays in infected mice offspring. Submitted to *Experimental Physiology*

List of conference presentation

Manjeese W, Mvubu NE, Steyn AJC and Mpofana T. The impact of prenatal infections on Autism Spectrum Disorder. IBRO-UCT African Advanced School on Neuroimmunology and the Gut-Brain Axis. Cape Town, South Africa (1-18 December 2018).

Manjeese W, Mvubu NE, Steyn AJC and Mpofana T. Gestational *Mycobacterium tuberculosis* infection affects pregnancy and leads to developmental delays in infected mice offspring. UKZN College of Health Sciences Research Day (November 2020)

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ADHD	Attention deficit hyperactivity disorder
AREC	Animal Research Ethics Committee
ASD	Autism Spectrum Disorder
BBB	Blood-brain barrier
CER	cerebellum
CNS	Central Nervous System
CNV	Copy number variation
CRH	Corticotropin releasing hormone
DNA	Deoxyribonucleic acid
E	Embryonic day
EB dye	Evans Blue dye
HAC	Histone acetylase
HDAC	Histone deacetylase
HPA	Hypothalamic pituitary axis
IBA-1	Ionized calcium binding adaptor molecule 1
IFN-γ	Interferon gamma
IHC	Immunohistochemistry
IL-6	Interleukin 6
IL-1β	Interleukin 1 Beta
IL-10	Interleukin 10
LPS	Lipopolysaccharide
MAP	*Mycobacterium avium ss. paratuberculosis*
MBP	Myelin basic protein
MHC1	Major histocompatability complex 1
MIA	Maternal immune activation
Mtb	*Mycobacterium tuberculosis*
NDD	Neurodevelopmental disorder
nNOS	neuronal Nitric oxide synthase

NRXN1	Neurexin 1
NRXN2	Neurexin 2
NLGN1	Neuroligin 1
NLGN2	Neuroligin 2
NLGN3	Neuroligin 3
NLGN4	Neuroligin 4
NLGN5	Neuroligin 5
PDD-NOS	Pervasive developmental disorder not otherwise specified
PND	Post-natal day
Poly I:C	Polyinosonic: polycytidylic acid
PSD	Post-synaptic density
ROS	Reactive oxygen species
SHANK1	SH3 multiple ankyrin domain 1
SHANK2	SH3 multiple ankyrin domain 2
SHANK3	SH3 multiple ankyrin domain 3
SNP	Single nucleotide polymorphism
TNF-α	Tumour necrosis factor alpha
TB	Tuberculosis
VEGFA	Vascular endothelial growth factor
VPA	Valproic acid

ABSTRACT

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by restricted repetitive patterns, communication challenges and lack of social skills. ASD has no distinct biomarkers, with symptoms overlaping with related developmental disorders like Schizophrenia. Maternal immune activation (MIA) is when the maternal immune system is invaded by a pathogen causing an immune response that interferes with the normal fetal brain development process. *Mycobacterium tuberculosis* (*Mtb)* infections are common during pregnancy and are known to affect fetal health, often causing spontaneous abortions and low birth weights. Valproic acid (VPA) is an anticonvulsant and mood stabilizer associated with ASD when administered during pregnancy. Gestational VPA exposure of mice on Embryonic day 12.5 (E12.5) induces ASD-traits in offspring, as such, this study employed VPA as a positive control. This study investigated the effects of prenatal exposure to *Mycobacterium tuberculosis (Mtb)* (singularly and in combination with VPA) on developmental delays and offspring behaviour. Pregnant mice were divided into saline, VPA, *Mtb*, and VPA+*Mtb;* treatments were administered on E12.5. Developmental milestones were measured between post-natal day 7 (PND 7) and 28. Offspring were subjected to neurobehavioural studies to test for social interaction and repetitive behaviours on PND 35. Ionised calcium binding molecule 1 (IBA-1) and Glial Fibrillary acid protein (GFAP) expression in the prefrontal cortex (PFC) and cerebellum regions were analysed using immunohistochemistry (IHC). The effect on the BBB's function was determined using Evans blue dye-albumin extravasation method on PND 35. Additionally, cerebellar tissues were homogenized and processed for molecular analyses of *NRXN1*, *NRXN2*, *NLGN1*, *NLGN2* and *SHANK3* expression. Changes in expression patterns of *NRXNs* and *NLGNs* causes an imbalance in the excitation and inhibition of neurons, a feature associated with ASD. The *Mtb* treated group had significantly low litter count and high fetal resorption compared to saline treated group. Neuroinflammation was evident in the *Mtb* offspring at PND 35 as shown by a significant increase in GFAP and IBA-1 expressing astrocytes and microglia in the PFC and cerebellum compared to saline group. The BBB's integrity was compromised as shown by the increased permeability to EB-dye in the PFC and cerebellum of *Mtb*, VPA and VPA+*Mtb* offspring. The *Mtb* offspring also displayed systemic inflammation and altered ASD-linked behaviours**.** *NRXN1* and *NLGN1* were overexpressed in the cerebellum of *Mtb*-induced MIA offspring compared to saline offspring. Dual exposure to VPA and *Mtb* restored *NRXN1* expression levels, reduced astrocyte and microglia injury in the PFC, rescued social behaviours and restored normal eye-opening patterns in offspring. The

study demonstrates impaired fetal development which persists into the post-natal period. The impaired development was accompanied by neuroanatomical changes and behavioural patterns consistent with ASD pathophysiology. These findings might be attributed to *Mtb*-induced maternal system inflammation in pregnancy that induces fetal inflammation via the placenta and BBB of a developing fetus causing insult in the brain. Immune dysregulation and synaptic defects are hallmarks of ASD. We therefore conclude that prenatal *Mtb* infection predisposes offspring to a higher risk of neurodevelopmental challenges later in life and dual exposure to VPA and Mtb rescues some of these challenges.

Keywords: Pregnancy, *Mycobacterium tuberculosis*, valproic acid, developmental delay, immune dysregulation, neurobehaviours, gene expression, Autism

THESIS OUTLINE

This PhD thesis has been compiled into an article format and presented as a thesis by manuscript.

Chapter One

Provides background information with a literature review of topics underpinning this study. Study aims and objectives, hypotheses and potential benefits of this research are also highlighted.

Chapter Two

This forms the first part of the PhD experimental research that evaluated the gestational *Mycobacterium tuberculosis* infection effects on pregnancy and development of infected mice offspring.

Chapter Three

Forms part of experimental research that evaluated the effects of prenatal *Mycobacterium tuberculosis* exposure on neuroinflammation and BBB integrity in the cerebellum and prefrontal cortex regions of offspring. This forms part of the PhD experimental work that was published by *International Journal of Developmental Neuroscience*.

Chapter Four

Reports on the effect of *Mycobacterium tuberculosis*-induced maternal immune activation on immune dysregulation, synaptic gene expression and Autism-associated behaviours. This forms part of the PhD experimental work that was published by *International Journal of Environmental Research and Public Health*.

Chapter Five

Provides a synthesis of all the manuscripts and highlights the conclusion of research findings. It also highlights limitations and makes recommendations for future studies

CHAPTER ONE
INTRODUCTION AND LITERARURE REVIEW

1.1 Background

Neurodevelopmental disorders (NDDs) are a group of complex developmental disorders that are associated with a dysfunction of the Central Nervous System (CNS) often presenting during child's early years of life (Bitta et al., 2017; E. Klimkeit, 2015). They arise from disturbances in the process of brain development which can manifest as cognitive challenges, learning difficulties, language impairment and behavioural deficits. Common neurodevelopmental disorders include ASD, intellectual disabilities, cerebral palsy, epilepsy, schizophrenia and Attention deficit Hyperactivity Disorder (ADHD). Sub-Saharan Africa has seen more than 70% increase in the incidence of such NDDs over the past two decades (Olusanya et al., 2018).

1.2 Autism Spectrum Disorder

Autism spectrum disorder (ASD) is a group of similar neurodevelopmental disorders that include Asperger's syndrome, Autistic disorder and Pervasive developmental disorder not otherwise specified (PDD-NOS) (Park et al., 2016). These disorders were previously diagnosed separately, but because of their closely similar symptoms, they have been grouped to form a spectrum of disorders. The symptoms of ASD include lack of social skills, speech and communication deficits, repetitive behaviours and restricted interests (e.g. flapping of hands and body rocking) (Bitta et al., 2017; Won et al., 2013). Lack of eye contact is also a very common non-verbal communication trait in ASD patients. Distinct biomarkers of this condition have not been found; hence diagnosis is based on these behavioral traits. These symptoms manifest between 2 and 3 years of age often occurring with comorbidities, such as sleeping and eating problems, epilepsy, irritability, gastrointestinal challenges, anxiety and hyperactivity (Won et al., 2013). Because ASD symptoms are heterogeneous, diagnosis is difficult especially for medical practitioners without prior experience with the condition, hence most children live without a proper diagnosis.

1.2.1 Prevalence of ASD

The prevalence of ASD has increased over the past two decades as a result of improved diagnostic criteria and awareness among medical practitioners (Park et al., 2016). Males are four times more likely to be diagnosed with ASD than females. The Centre for Disease Control reported 0.2% increase in ASD cases over two years and that one in every 59 children under eight years is diagnosed with ASD in America (CDC- Autism Developmental Disability Monitoring, 2018). The Global burden of disease study (2016) estimates that more than one

million children living with ASD are from resource limited countries. The prevalence of Autism in sub-Saharan Africa is unknown, but three in five children are at risk of ASD (Olusanya et al., 2018). The condition remains under diagnosed in the region because of lack of awareness among medical practitioners and psychiatric caregivers. There is also a lot of stigma surrounding ASD as society regards these children as "bewitched" or a result of sinful acts that angered God rather than as suffering a medical condition. As a result, the first point of call for an African autistic child is the traditional healers rather than medical facilities, which delays diagnosis and management of the disorder (Tilahun et al., 2017). The few families who make it to the clinic are severe with the condition.

The prevalence of developmental disorders in South African children below 5 years of age is estimated to be 9% (Olusanya et al., 2018). Like most countries in Sub-Saharan Africa, diagnosis of ASD remains a challenge in South Africa due to scarcity of funds and lack of qualified child psychiatrists in the few mental health facilities available (Wetherston et al., 2017). The situation makes it difficult for children living with ASD to access treatment and behavioural therapy hence most cases remain undiagnosed.

1.2.2 Treatment of ASD

Currently there are no pharmacological treatments to ASD, however, there are approved drugs that are used to manage the condition. The antipsychotics, antidepressants and anti-convulsants, currently administered to ASD patients (Risperidone and Aripiprazole) help with managing the comorbidities associated with ASD and not the core symptoms (Park et al., 2016). Autistic individuals are prone to other health complications, especially those occurring because of injuries encountered in their daily routines (Tilahun et al., 2017)

1.3 Aetiology of ASD

ASD is a complex disorder whose causes are not yet fully understood, but research has pointed to a combination of genetic and environmental risk factors that interact to disrupt a developing brain (Figure 1.1). Several mechanisms are involved which converge on related genes or pathways of neuronal circuits (Gadad et al., 2013; Huguet & Bourgeron, 2016; Wisnioweiecka-Kowalnik & Nowakowska, 2019).

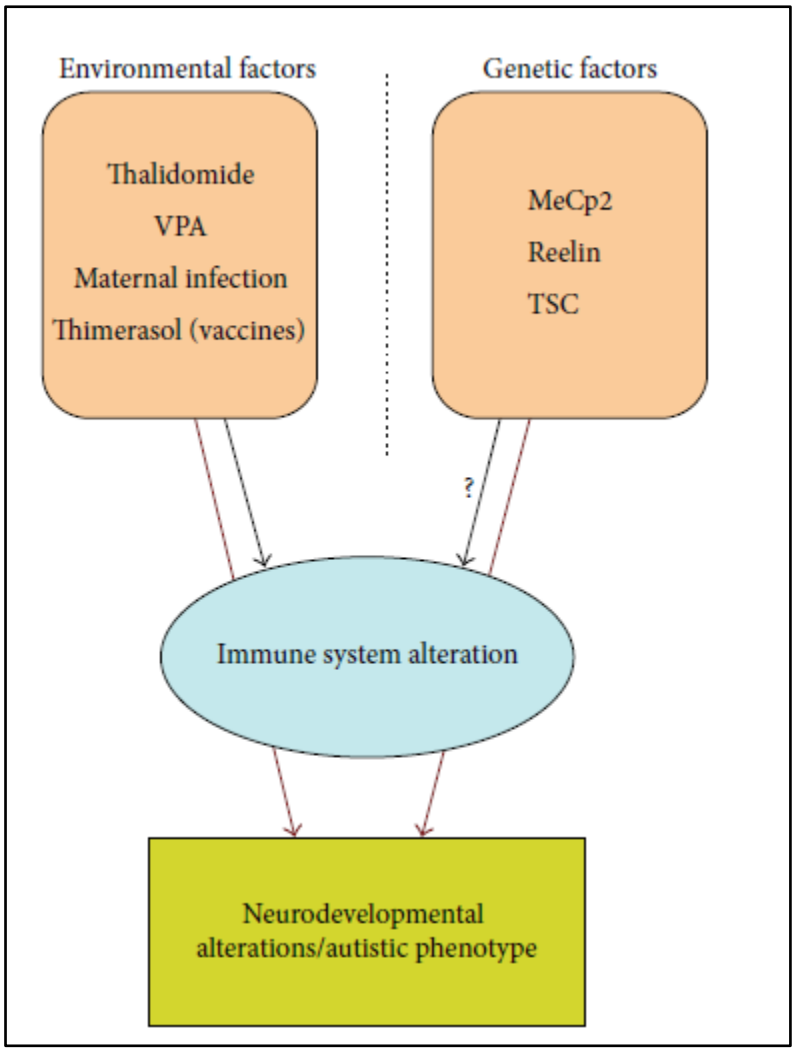

Figure 1. 1: Environmental and Genetic risk factors may alter the immune system to induce Autism (Gadad et al., 2014)

1.3.1 Genetic Risk factors of ASD

ASD is a genetically heterogenous condition that has gene heritability, copy number variations (CNV), single nucleotide polymorphisms (SNPs), chromosomal abnormalities and epigenetic changes contributing to ASD development (Kalkbrenner et al., 2014; Lyall et al., 2017; Park et al., 2016). Research in twins anchor the role of genetics in ASD aetiology. When one identical twin is diagnosed with ASD, there is a 90% chance the other twin has ASD; whereas there is a 10% chance with fraternal twins (Patel et al., 2018; Ratajczak, 2011; Won et al., 2013). Genes associated with ASD behavioural deficits account for about 10-20% of ASD cases (Rylaarsdam & Guemez-Gamboa, 2019), while CNVs account for about 10 % of ASD cases (Geschwind, 2011). The most inheritable genetic elements are common variants (49.8

%), and a few of them (2.6 %) are rare variants (Figure 1.2). The de novo variants (9.5 %) are regarded as environmental risk factors because they are suggested to arise from environmental risk factors of ASD (Huguet & Bourgeron, 2016).

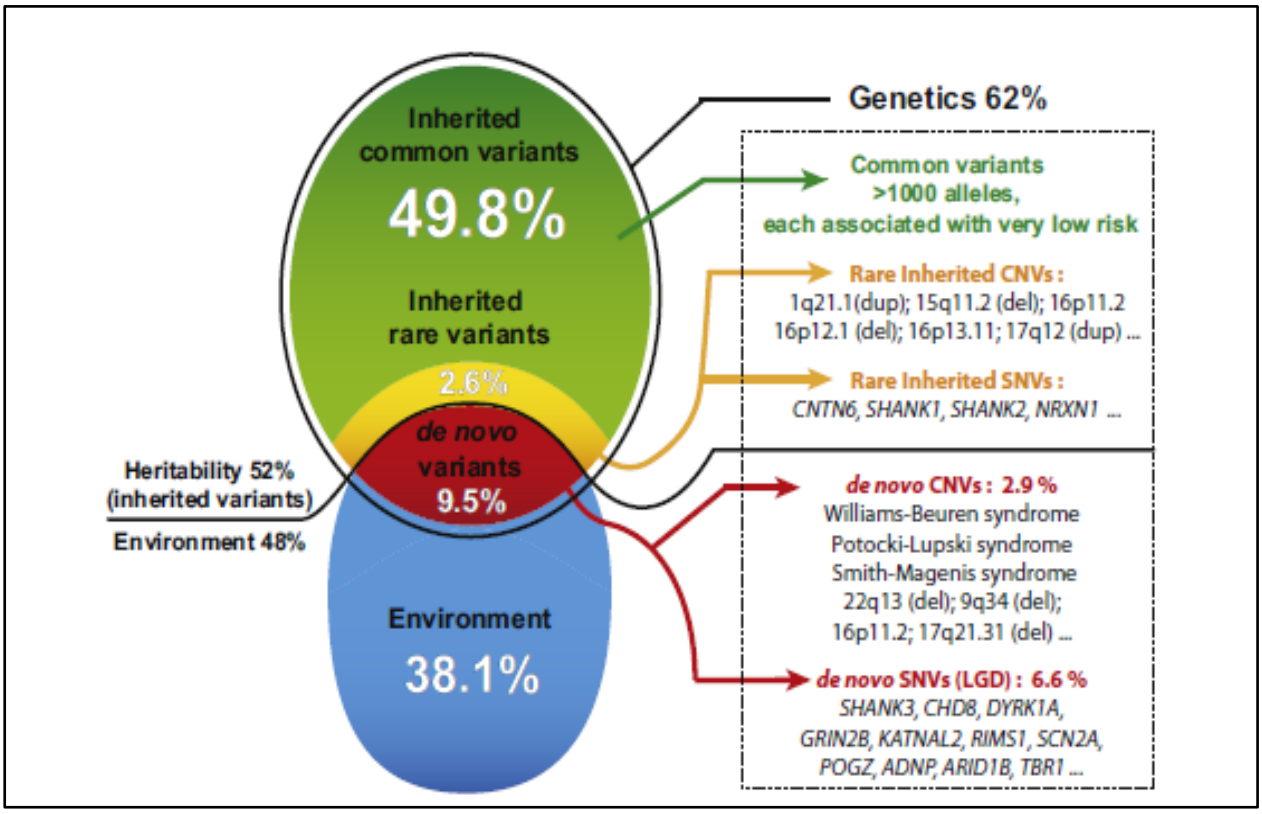

Figure 1. 2: Genetic and environmental contributions to ASD (Huguet & Bourgeron, 2016).

The ASD risk genes are known to converge on biological pathways that are involved in the regulation of neuronal activity (Huguet & Bourgeron, 2016; Wisnioweiecka-Kowalnik & Nowakowska, 2019). Synaptic cell adhesion molecules such as Neuroligins and Neurexins are involved in synapse formation. Neurexins are pre-synaptic cell adhesion molecules encoded by *NRXN1*, *2* and *3* genes while *NLGN*s are post-synaptic cell adhesion molecules encoded by *NLGN1*, *2*, *3* and *4* in mammals, *NLGN 5* exists in humans only (Ding et al., 2015; Guang et al., 2018). Mutations and deletions in these genes are implicated in the pathophysiology of ASD (Almandil et al., 2019; Kim et al., 2008; Lein, 2015). *NLGN1* and *NLGN2* are expressed on excitatory and inhibitory synapses, respectively, while *NLGN3* and 4 are found in both synapses. Changes in expression patterns of *NRXNs* and *NLGNs* influence the excitation and inhibitory balance of neurons, which impairs information processing, a feature common in ASD patients (Guang et al., 2018; Huguet & Bourgeron, 2016). Scaffolding proteins encoded

by *SHANK1, SHANK2, SHANK3* are important in the organisation of post synaptic density (PSD) and mutations in particularly *SHANK3* are associated with intellectual disabilities, developmental delays and language difficulties (Durand et al., 2007; Guang et al., 2018; Soler et al., 2018).

1.3.2 Environmental Risk factors

The environment can influence the process of brain development, which increases the risk of brain disorders. The impact depends on the timing, frequency and type of environmental factor encountered. Pregnancy is regarded as an environment to a growing fetus and maternal exposure to air pollutants, malnutrition, alcohol, stress, infections and some pharmacological drugs increase the risk of developmental disorders (Horvath & Bjørke-monsen, 2015; Kalkbrenner et al., 2014; Smith et al., 2007; Zerbo et al., 2016). Exposure to some medications during pregnancy increases the risk of ASD, In the 1950s, the anti-nausea drug, Thalidomide was reported to increase the incidence of Autism in children born to mothers who had used it during pregnancy (Gadad et al., 2013). VPA is an anti-convulsant and mood stabilizer administered for epilepsy and depression, but when administered during pregnancy, it increases the incidence of ASD children (Bambini-junior et al., 2014; Gadad et al., 2013). Parental age also influences the brain development of a child through genetics. When both parents are at an advanced age, the incidence of chromosomal abnormalities is increased, which can lead to ASD in the child born (Bölte, Girdler and Marschik, 2019).

1.4 The immune system in pregnancy

A mother's immune system should be able to accept a growing fetus, which is foreign to the maternal system. The survival of pregnancy depends on the ability of the maternal system to adapt to different immune requirements of pregnancy stages. A successful pregnancy goes through implantation, placentation, fetal growth and parturition. These stages demand different immune states for survival, implantation and placenta development require a pro-inflammatory immune state, mediated by T-Helper 1 (TH-1) pathway, which then shifts to an anti-inflammatory state to maintain the growth of the foetus via a T-Helper 2 (TH-2) pathway (Figure 1.3) (Hussein et al., 2011; Mor & Cardenas, 2010; Morelli et al., 2015). Pro-inflammatory cytokines such as IFN-γ and TNF-α are known to induce miscarriages, while IL-10 reverses the inflammation in mice (Morelli et al., 2015). The anti-inflammatory state encountered during fetal growth makes the mother susceptible to infections and the risk is higher in women who are already immunocompromised (Morelli et al., 2015).

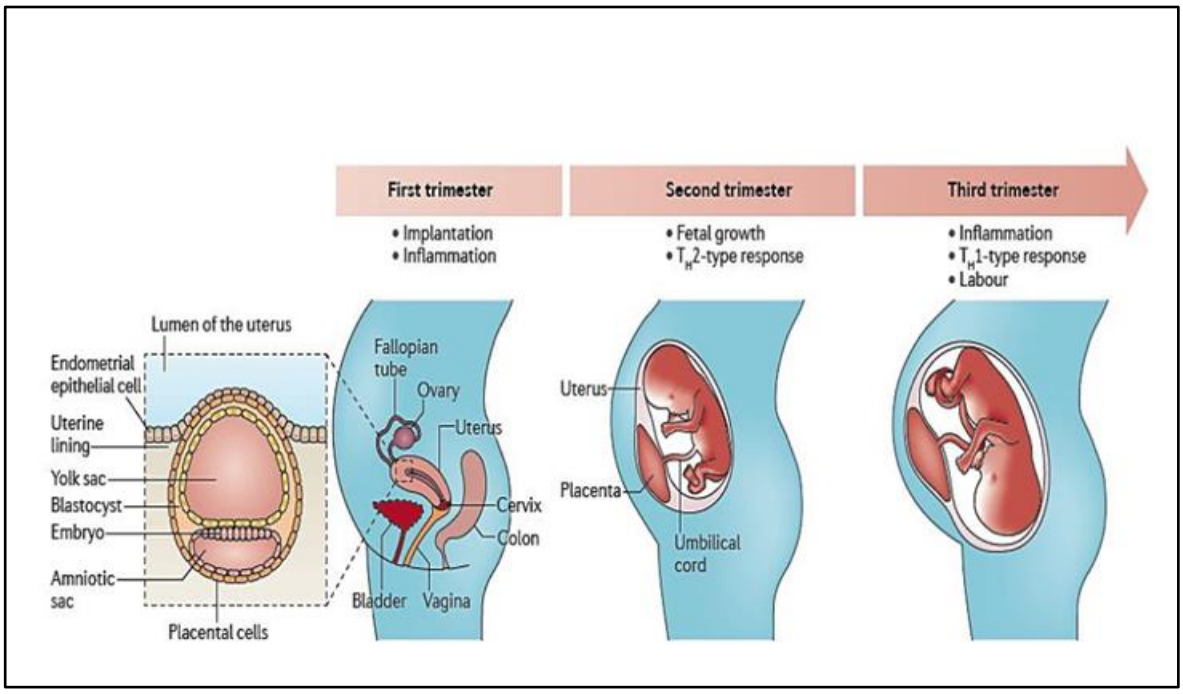

Figure 1. 3: Immunological states of pregnancy (Mor et al., 2017).

1.4.1 Maternal infections during pregnancy

Maternal Immune Activation (MIA) is a phenomenon where the maternal immune system is invaded by a pathogen that triggers an immune response, interfering with the normal fetal brain development process (Madore et al., 2016). Infection during pregnancy induces an inflammatory response causing a dysregulation in maternal cytokines. The cytokine dysregulation in the fetal compartment can compromise the integrity of the Blood-Brain Barrier (BBB), giving it an increased permeability that allows peripheral cytokines to infiltrate the fetal brain (Meltzer & Water, 2017). Microglia are activated in response to the increase in cytokines in the fetal brain resulting in neuroinflammation. Inflammatory cytokines such as IL-6, TGF-β and TNF-α are elevated in ASD patients (Patterson et al., 2008; Vargas et al., 2005). This indicates that neuroinflammation persists through postnatal development of the brain affecting neuronal function and behaviour phenotypes of the individual.

Evidence from clinical studies (Brown et al., 2000; Lee et al., 2015) supported by experimental animal models (Bauman et al., 2014; Careaga et al., 2017; Schwartzer et al., 2013; Yang, 2013) have regarded MIA as a factor that increases the risk of NDDs such as ASD. In California, a high risk of ASD was reported in babies born in winter and influenza infections during pregnancy were suggested to be the cause (Zerbo et al., 2011). Herpes Simplex Virus 2, Rubella, *Taxoplasma gondii*, Cytomegalovirus and Influenza virus infections during pregnancy

are associated with an increased risk of NDDs in children born (Atladdottir et al, 2010; Patterson, 2011; Sørensen et al., 2009). More than four decades ago, exposure to congenital rubella encephalitis during pregnancy increased ASD risk in children by 12.5% (Desmond, 1969). There was a marked incidence of low birth weight, pre-mature births and infant deaths in children born to pregnant women who contracted Influenza during the 2009 Influenza (H1N1) pandemic (Doyle et al., 2013). Recent evidence strongly associates ASD with low birth weight and preterm parameters, indicating impaired developmental patterns (Schieve et al., 2016).

The timing of infection during pregnancy has an impact on severity and diversity of NDDs (Hornig et al., 2017; Horvath & Bjørke-monsen, 2015). Hospitalized pregnant women with bacterial infections in the second and third trimester were at a high risk of delivering a child with ASD (Zerbo et al., 2016). Among Swedish children born between 1984 and 2007, 3.7% of the ASD diagnosed children were born to mothers who had been hospitalized due to gestational infections (Lee et al., 2015). Similarly, a study in Norway recorded frequent maternal exposures to infection to have doubled the ASD risk in children born (Hornig et al., 2017). Second trimester respiratory infections increased the risk of impaired neurodevelopment by 2.06% (Brown et al., 2000). Although specific infectious agents were not identified in this study, tuberculosis is suggested to have been a contributor to the respiratory infections recorded because it is a major respiratory disease.

1.5 MIA Mechanism in ASD

1.5.1 The role of cytokines in MIA

Inflammatory response during pregnancy due to infection contributes tremendously to impaired neurodevelopmental processes, regardless of the specific microorganism. Cytokines regulate the immune system as well as brain development, hence they are expressed in minute levels in the brain to ensure normal neurodevelopment (Horvath & Bjørke-monsen, 2015; Knuesel et al., 2014). Pro-inflammatory cytokines are upregulated in response to infection, while anti-inflammatory cytokines regulate the inflammatory response to regain homeostasis. When a pathogen invades the maternal system during pregnancy, specific pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α increase in the maternal blood, which can enter the fetal compartment via the placenta. IL-6 can cross the BBB and trigger a fetal immune response because the brain recognises an upregulation of pro-inflammatory cytokines as a sickness signal (Knuesel et al., 2014; Lombardo et al., 2017; Meltzer & Water, 2017; Parker-Athill & Tan, 2011). Interleukin 6 is known to have a crucial role in MIA. Smith *et al*. (2007) found that

behavioural deficits of MIA offspring are not displayed when IL-6 is suppressed during pregnancy (Smith *et al.*, 2007). Supporting these findings, co-administration of IL-6 antibody in a Poly-IC model of MIA normalised the gene expression and social behaviour changes common in MIA progeny, while a single injection of IL-6 at mid-gestation of pregnant dams was enough to cause behavioural and social deficits in the pups (Smith et al., 2007). The balance between pro and anti-inflammatory cytokines is crucial to a developing brain.

Cytokines interact with other immune molecules such as Major Histocompatibility Complex 1 (MHC1) that are found on neurons. These molecules regulate synaptic plasticity necessary in synaptogenesis. A prolonged inflammatory response in the brain may alter the expression of MHC1, causing improper formation of synapses in a developing brain (Jiang et al., 2018).

1.5.2 MIA and the Hypothalamic Pituitary Adrenal (HPA) axis

The HPA axis is the pathway responsible for stress responses in the body. Maternal cytokines released in response to an infection can induce the fetal hypothalamus to release Corticotropin releasing hormone (CRH) and arginine vasopressin, which stimulate the anterior pituitary gland and adrenal cortex to release Adrenocorticotropic Hormone (ACTH) and glucocorticoids, respectively (Figure 1.4) (Jiang et al., 2018; Ratnayake et al., 2013). Glucocorticoids assist in normal brain development by maintaining intra-uterine homeostasis through suppression of pro-inflammatory cytokines and stimulating anti-inflammatory cytokines (Ratnayake et al., 2013). However, exposure of the fetal brain to excess glucocorticoids can activate microglia leading to exaggerated expression of inflammatory cytokines which interfere with the normal stress response mechanisms of the HPA axis (Dunn, 2000; Duque & Munhoz, 2016).

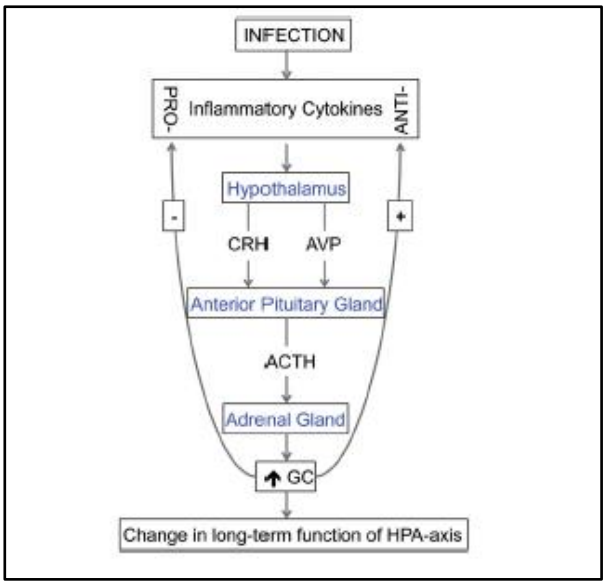

Figure 1. 4: Cytokine activation of the HPA axis – Corticotropin releasing hormone (CRH), arginie vasopressin (AVP), Adrenocorticotropic Hormone (ACTH), Glucocorticoid (GC) (Ratnayake et al., 2013)

1.6 ASD and Mycobacterial infections

TB is chiefly caused by the slow growing rod-shaped bacterium, *Mtb* (Mathad & Gupta, 2012). Women have an increased risk of suffering from TB during pregnancy due to immunological changes, which present an opportunity for mycobacterial infection establishment (Bates et al., 2015; Gupta, 2009). Dow (2011) proposed a possible link between Autism and mycobacterial infections, where *Mycobacterium avium* ss. *paratuberculosis* (MAP), a pathogenic bacterium that causes inflammatory bowel disease triggers autoantibodies to the brain via a mycobacterial stress induced protein, HSP65. The behavioral and social deficit phenotypes in ASD subjects are possibly a result of an anti-Myelin Basic Protein (MBP) antibody induced inflammation (Dow, 2011).

1.7 Animal models of ASD

1.7.1 Valporic Acid ASD model

Rodents prenatally exposed to VPA display impaired social behaviours, neural circuits and altered biochemical pathways that are very similar to those seen in ASD patients. Prenatal exposure to VPA in rodents is a widely used model in ASD studies, which delays the maturation of neurons leading to the delayed physical development seen in ASD phenotype (Deckmann et al., 2018). The VPA model involves a single injection of a high dose at day 12.5 of gestation. Researchers use a range of different high doses (500-800mg/kg) of VPA, which

results in anatomical and pathological outcomes similar to those of ASD in humans (Roullet & Crawley, 2012).

Table 1. 1: VPA rodent models

VPA dose/ adminstration	Exposure timing	Effects on offspring	Species/ strain	References
400 mg/kg; 300 mg/kg s.c	E10; E12.5	↓sociability and preference for social novelty, ↑repetitive behaviour	Sprague dawley rats and mice	(Kim *et al.*, 2014)
600 mg/kg i.p	E12.5	↓body weight, delayed eye opening, altered ofalctry discrimination, delayed swimming behaviour, ↓prepulse inhibition (PPI), ↑sensitivity to pain, ↑hyperactivity		(Schneider and Przewłocki, 2005)
600 mg/kg	E12.5	Delayed eye opening, ↓body weight, ↑congenital malformations	Wistar rats	(Ruhela *et al.*, 2017)
500 mg/kg i.p	E12.5	↓ dendritic spines	mice	(Yamaguchi *et al.*, 2017)
600 mg/kg	E12.5	↓social play, ↓social interaction, ↑self-grooming, ↑glucose metabolism	mice	(Campolongo *et al.*, 2018)
600 mg/kg i.p	E12.5	↓social interaction, ↑ expression of miR 134-5p	Wistar rats	(Hirsch *et al.*, 2018)
600 mg/kg i.p	E12.5	↑corticosterone levels, ↑glial activation in females, ↓social interaction in males	CFI mice	(Kazlauskas *et al.*, 2019)
500 mg/kg i.p	E12.5	↓sociability, impaired social discrimination abilities, ↑dopamine receptor gene expression	Wistar rats	(Schiavi *et al.*, 2019)

1.7.2 VPA mechanism of action in ASD

VPA has various mechanisms of disturbing the development and function of neurons. One way is by altering expression of synaptic molecules, which affects the activity of synapses (Nicolini & Fahnestock, 2018).

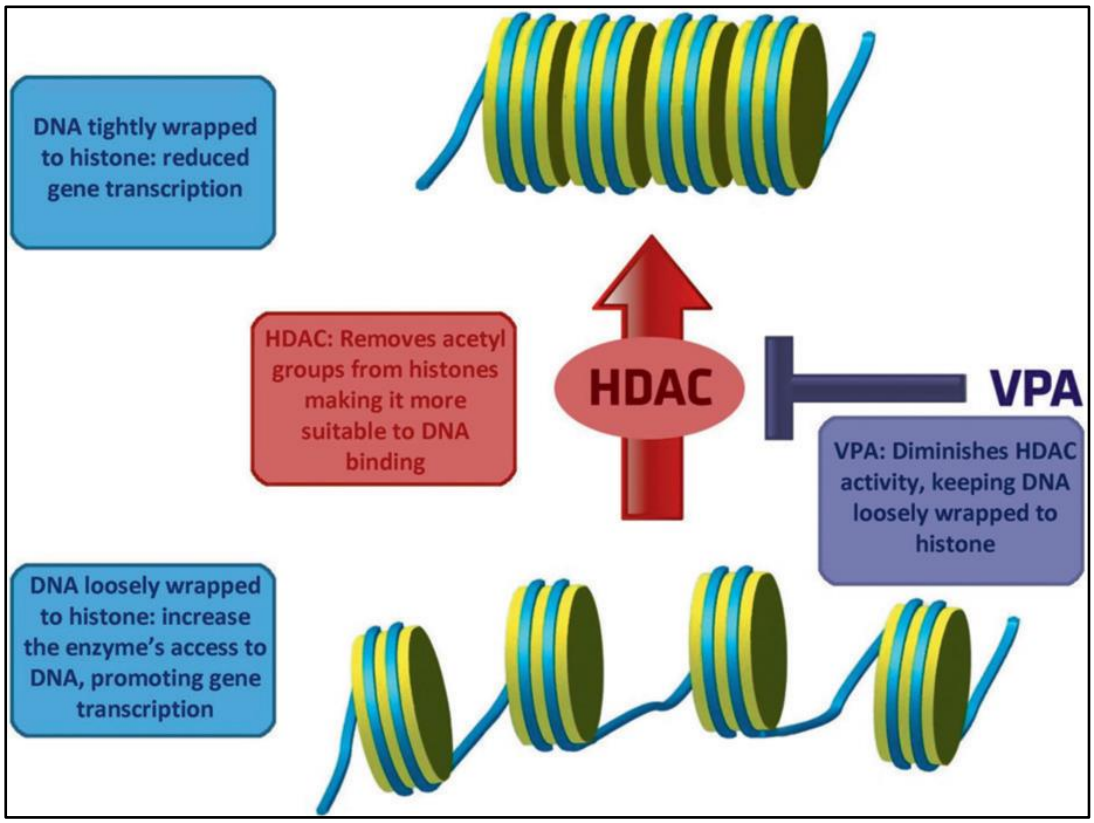

Figure 1. 5: VPA inhibition of Histone deacetylase (HDAC) activity (Bambini-junior et al., 2014).

Histones are proteins that bind to DNA to compress and pack it tightly. Post-translational modifications of these histone molecules (acetylation and deacetylation) allow for regulation of gene expression (Bambini-junior et al., 2014). Histone deacetylase (HDAC) removes acetyl groups from histones, which increases the histone-DNA binding affinity while reducing gene expression (Figure 1.5). Concurrently, histone acetylase (HAC) adds acetyl groups to histone, reducing the histone-DNA binding affinity allowing transcription factors to access DNA, hence increasing gene expression (Bambini-junior et al., 2014; Deckmann et al., 2018). Essentially, HDACs are crucial in immune response mechanisms where gene expression of immune signalling molecules are tightly controlled. A disturbance of HDAC enzyme activity interferes with immune response mechanisms. VPA inhibits HDAC activity through binding to the

enzyme's active site (Lloyd, 2013). When HDAC activity is inhibited, the expression of MHCII molecules is increased, which also increases pro-inflammatory cytokine production by CD68 cells while decreasing CD4 immune cell response (Deckmann et al., 2018).

Folic acid deficiency is another proposed mechanism of VPA action in ASD. Folic acid is essentially an inactive form of vitamin B9, which is important in the synthesis of nucleic acids (Lloyd, 2013). Methionine synthase methylates methionine from homocysteine in the presence of vitamin B9 and B12. Methionine is a precursor in DNA methylation, thus it controls the regulation of gene expression in important stages of brain development. A deficiency in folic acid interferes with this regulated gene expression. Pregnancy increases the demand for folic acid, thus creating a deficiency. VPA when administered during pregnancy further reduces the folic acid levels and reduced folic acid in the embryo increases oxidative stress causing altered protein synthesis (Lloyd, 2013).

1.8 Maternal Immune Activation models

Rodents and non-human primates have been used in neurodevelopmental studies to understand behavioral and cellular changes that are associated with such disorders (Bauman et al., 2014; Careaga et al., 2017). Monkeys are expensive to use in research because of their long gestation which lengthens research duration and their large size that demands large quantities of food. For these reasons, rodents are the preferred animal to use in neuroscience because of their small size that allows researchers to use many of them and rapid generation of results owing to their short gestation.

The most common MIA models involve the injection of pregnant rodents with synthetic viral RNA Polyriboinosinic: polyribocytidilic (Poly I:C) and bacterial endotoxin lipopolysaccharide (LPS) to induce inflammation. Poly:IC and LPS induced MIA models are non-infectious and widely used because they do not require implementing high biosafety measures and their effects on neurodevelopment are well characterized (Table 1.2) (Knuesel et al., 2014; Tohidpour et al., 2017).

Table 1. 2: Non-infectious MIA models

MIA agent/ adminstration	Exposure timing	Species/ strain	Effects on offspring	References
IL-6 (i.p)	E12.5	C57BL6/J mice	Prepulse inhibition deficits (PPI)	(Smith et al., 2007)
Poly I.C	E10.5; E12.5; E14.5	C57BL6/J mice	↓sociability, ↑self-grooming, ↓vocalisations	(Malkova et al., 2012)
LPS (i.p)	E15	Sprague dawley rats	↑stress and cell death gene expression, ↑inflammation, ↓social interaction and exploration	(Oskvig et al., 2012)
Poly I.C and LPS (i.p)	E11.5; E12.5	C57BL6/J mice	↓locomotion in Poly I.C male offspring, ↑ repetitive behaviour	(Xuan and Hampson, 2014)
Poly I.C (i.p)	E12,5	C57BL6/J mice	↓spine density, impaired inhibitory and excitatory synaptic function, ↑repetitive behaviours	(Coiro et al., 2015)
Poly I.C (i.v)	E17	C57BL6/J mice	↑IL-6, ↑IL-1β, ↑TNF-α, altered BDNF expression	(Giovanoli et al., 2015)
LPS i.p	E15	C57BL6/J mice	↓vocalisations, ↑repetitive behaviours, ↓CX3CR1 gene expression	(Fernández de Cossío et al., 2017)

LPS (i.p)	E9.5	Wistar rats	↑repetitive behaviours, ↓dopamine metabolites	(Konefal & Stellwagen, 2017)
LPS (i.p)	E12	C57BL6/J mice	↑IL-6, IL-1β and TNF-α, ↑ neuroinflammation in amygdala	(O'Loughlin et al., 2017)
LPS (i.p)	E12.5	C57BL6/J mice	↓expression of mTOR gene, ↓ expression of synaptic molecules	(Lombardo et al., 2017)
Poly I.C (i.p)	E12.5; E17.5	C57BL6/J mice	↑hyperexcitation in the hippocampus	(Gao et al., 2019)
Poly I.C (i.p)	E12.5	C57BL6/J mice	↓social interaction in males, impaired motor function, ↓purkinje cell numbers in males, ↓cortical neuronal numbers	(Haida et al., 2019)

i.p- intraperitoneal; i.v- intravenous

1.9 Infectious MIA models

1.9.1 Influenza viruses

Amongst all microorganisms used in MIA animal models, the Influenza viruses are the most documented. Fatemi's group pioneered influenza-induced MIA studies in mice to assess its impact on a developing brain. They found that prenatal exposure to Influenza virus H1N1 increases neuronal Nitric oxide synthase (nNOS) immunoreactivity, reduces cortical thickness and Reelin expression in the brain (Fatemi et al., 1998; Fatemi et al., 1999). They also reported altered expression of genes linked to neurodevelopment and anatomical changes in different brain regions of mice exposed to influenza virus *in utero* (Fatemi et al., 2009). (Shi et al., 2005) also reported altered brain development in offspring born to dams infected with Influenza virus H1N1 during pregnancy. Prenatal exposure to influenza H3N2 induced altered social and locomotion behaviour (Miller et al., 2013) and ASD-associated anatomical changes in offspring (Short et al., 2010).

1.9.2 *Escherichia coli*

Maternal urinary tract infection influenced the development of rat offspring. Pregnant rats were infected with *E. coli* in the urinary tract. The offspring of the infected rats had restricted fetal growth and impaired cognitive function (Cronise & Kelly, 2001).

1.9.3 *Listeria monocytogenes*

When *L. monocytogenes* was used to infect pregnant mice intravenously on E14, there was a reduction in the protective TH-1 cytokines produced in the maternal compartment. The immune response influenced the pregnancy outcome and increased fetal resorption was recorded in the study (Abram & Schlu, 2003).

1.9.4 Porphyromonas gingivalis

Periodontal infection was reported to have a negative effect on pregnancy outcome. Pregnant mice were subcutaneously infected with *P. gingivalis* on E7.5, which induced an upsurge of TNF-α and reduction of IL-10 in the fetal compartment. The maternal inflammatory responses restricted fetal growth and increased fetal resorption (Lin et al., 2003).

1.9.5 Campylobacter rectus

Fetal resorption and growth restriction was linked to maternal exposure to *C. rectus* on E7.5. Prenatal exposure to *C. rectus* also increased pup mortality and induced neuroinflammation in the brain of infected mice offspring (Offenbacher et al., 2005).

1.9.6 Plasmodium berghei

Pregnant mice were exposed to *P. berghei*, a malaria causing parasite. The behavioral of pups born was altered, offspring had social deficits, learning difficulties, poor memory and depression-like moods that persisted to adulthood. Impaired learning skills were correlated to reduced biogenic levels in the brain (McDonald et al., 2015).

1.9.7 Zika Virus

ZIKV gestational exposure in guinea pigs did not affect fetal growth and development. ZIKV was not transmitted to the offspring but a strong immune response against ZIKV was detected in both the mothers and offspring (Bierle et al., 2017).

1.9.8 Staphylococcus aureus enterotoxins

S. aureus enterotoxins A and B (SEA and SEB) were used to induce MIA in pregnant mice and immune dysregulation was reported in pups born to SEA (elevated IL-4, IFN-γ, IL-2, IL-6, TNF-α of and IL-17) and SEB (elevated IL-2, IFN-γ and IL-6) infected mothers (Glass *et al.*, 2018). The behavioral impairments in the pups were attributed to the immune dysregulation emanating from prenatal exposure to the bacterial enterotoxins.

1.9.9 Cytomegalovirus

Gestational exposure to CMV on E13 induced congenital CMV infection and fetal growth restriction. The offspring born to CMV infected mice presented with neuroinflammation, neuronal loss and impaired neuronal function particularly in the olfactory bulb region of the brain (Lazarini et al., 2018).

1.10 ASD behaviour phenotyping

The behaviours of rodents relate to those of humans and so measuring the core behaviours displayed in neurodevelopmental disorders is key in understanding the symptoms and mechanisms involved in the disorders. The core symptoms of ASD are reduced sociability, communication challenges and repetitive patterns. All three can be measured in rodents using tests that have been developed (Chaliha et al., 2020; Chang et al., 2017; Crawley, 2004).

1.10.1 Three-chamber social interaction test.

The behavioural test was developed by Jacqueline Crawley (Crawley, 2004). It measures the preference of rodent to a social stimulus compared to an object, and the degree of social novelty preference when presented with a familiar rodent as well as a non-familiar rodent (Chang et al., 2017). The test is conducted in a box made up of three chambers that are connected to each

other by doors in the central chamber. The test mouse has free choice to choose a compartment to enter during two different sessions. The first session, the subject is presented with an object in one chamber and a rodent in the other chamber, the last session the subject is presented with a familiar rodent in one chamber and an unfamiliar rodent in the other chamber (Figure 6). The sessions can be recorded, and time spent in each chamber can be scored by an observer blinded to the treatments given to subjects. (Chang et al., 2017; Silverman et al., 2010).

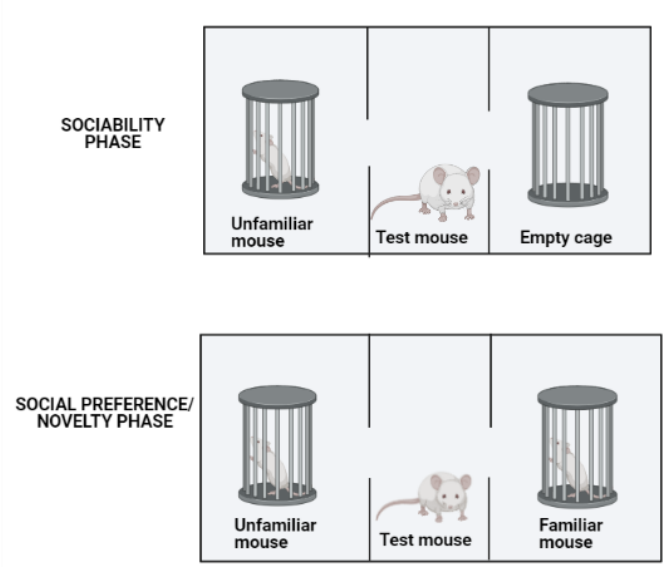

Figure 1. 6: Three-chamber social interaction test set up showing first session assessing sociability and second assessing social preference/ novelty.

1.10.2 Repetitive Behaviours

When a normal behavior is displayed for a longer period, it is a repetitive behavior. Self-grooming, circling and jumping are some of the behaviours used to measure the degree of repetitive patterns in ASD models (Chaliha et al., 2020). The normal self-grooming time is less than 10 seconds long in rodents. These tests are usually done under another behavioural assay such as the three-chamber social interaction test. During the habituation phase, the time spent grooming can be scored from the video recordings (Lawande et al., 2020).

The marble burrowing test is another test that measures repetitive patterns in rodents. A rodent is presented with marbles that are resting on bedding in a cage and the number of marbles

buried over 10 minutes indicates the frequency of digging, which is a repetitive behaviour (Thomas et al., 2009).

1.10.3 Communication

Rodents communicate through emission of ultrasonic vocalisations that can be recorded using special microphones designed to pick the frequencies in ultrasonic vocalisations. In ASD individuals, impaired communication is shown through unresponsiveness to speech such as name calling, delays in responding to conversations and failure to use expressive languages like communicative gestures (Hrabovska & Salyha, 2016; Silverman et al., 2010) Ultrasonic vocalisations from rodents are compared to speech in humans. When separated from a mother, a pup emits ultrasonic vocalisations for the mother to retrieve it (Chang et al., 2017).

1.11 Study rationale

TB disease during pregnancy has become increasingly common in Africa due to the HIV/AIDS epidemic in the region (WHO, 2020). The exact prevalence of TB in pregnant women remains unknown globally, however, more than 200 000 pregnant women were estimated to have TB globally in 2011. At least 70% of these cases occurred in Africa and South East Asia (Sugarman et al., 2014). Epidemiological studies have established that prenatal exposure to viral, bacterial and parasitic infection increases the risk of impaired fetal development resulting in neurodevelopmental disorders (NDDs) that persist postnatal (Atladottir et al, 2010; Patterson, 2011; Sørensen et al., 2009; Zerbo et al., 2016). The timing of infection during pregnancy has an impact on severity and diversity of NDDs (Hornig et al., 2018; Horvath & Bjørke-monsen, 2015). Recently, hospitalized pregnant women with bacterial infections in the second and third trimester were associated with the highest risk of delivering an autistic child (Zerbo et al., 2016). According to a 2015 Swedish study, 1% of the children born between 1984 and 2007 were diagnosed with ASD and 3.7% of them were born to mothers who had gestational infections (Lee et al., 2015). Similarly, frequent exposure to gestational infections increased ASD risk more than 2 fold in Norwegian infants (Hornig et al., 2018). Maternal infections of influenza and rubella have been reported to increase the risk of brain dysfunction in children (Desmond, 1969; Doyle et al., 2013). These clinical reports reveal that maternal infections in pregnant women contribute to the aetiology of ASD.

Most MIA studies have induced infections using lipopolysaccharide (LPS) bacterial extract and the viral mimic (Poly I:C) to study neurodevelopmental disorders. Borna Disease Virus

(BDV), Influenza virus, *Campylobacter rectus, Staphylococcus epidermidis, Staphylococcus aureus* enterotoxins and *Plasmodium berghei* have been used to induce MIA in neurodevelopmental studies in rodents (Offenbacher et al., 2005; Glass et al., 2018; Hallensleben et al., 1998; Kronforst et al., 2012; McDonald et al., 2015). Maternal respiratory infections occurring in the second trimester increased the risk of impaired brain development by 0.91% in people born between 1960 and 1967 (Brown et al., 2000). The specific infectious agents were not identified in this study, however, with the high prevalence of TB in Oakland, California (Davy-Mendez et al., 2019), *Mtb* was most likely among the pathogens that caused maternal respiratory infections. TB in pregnancy is associated with increased risks of distress, low birth weight, infant mortality and prematurity (Jana et al., 1994). A recent South African cohort study reported 65% prematurity, 59% low birth weight and 12% infant mortality among 75 children born to *Mtb* infected mothers (Bekker et al., 2016). However, research has not tapped into possible neurodevelopmental effects of maternal *Mtb* exposure in the infants born. Therefore, this study aims to investigate the effects of prenatal *Mtb* exposure on offspring developmental patterns and ASD-associated phenotype.

1.12 Aim of the study

To determine whether early life exposure to *Mtb* infection induces Autism-like phenotype in a mouse model.

1.13 Study objectives

- To subject pregnant Balb/c mice to *Mtb* infection and VPA treatment individually and in combination and determine effects on pregnancy health and offspring development.
- To determine maternal and congenital Mtb infection through culturing lung tissues of the mothers and pups in all groups in the study.
- To determine the integrity of the offspring's BBB using Evans Blue dye evasion method.
- To evaluate pathological changes associated with neuroinflammation in the PFC and CER using IBA-1 and GFAP markers of microglia and astrocytes using immunohistochemistry.
- To assess the ASD associated neurobehavioural parameters (social behaviors and restricted repetitive patterns) in the pups using the three-chamber social interaction test.
- To evaluate synaptic function through measuring gene expression of neurexins and neuroligins in the cerebellum of the pups using Real-Time Quantitative Reverse Transcription PCR (RT-qPCR).

- To determine systemic immune regulation through measuring plasmatic Th17 cytokines (Il-1β, IL-6, TNF-α, IL-10, TGF-β and IL-17) using the Bioplex platform.

1.14 Brief overview of methodology and study design

To accomplish the study objectives, established laboratory methods and protocols were strictly adhered to as illustrated in chapter 2, 3 and 4 of this thesis. Animal experimental work was conducted with approval of the UKZN Animal Research Ethics Committee (AREC 077/018M) and following the National Institute of Health for care and use of laboratory animals, South Africa.

1.15 Potential benefit of this research

The research is a multidisciplinary research that will contribute to one of the highly prevalent infectious diseases in Africa and non-communicable diseases such as Autism. Through findings from this research, we add onto the existing literature on maternal infections and their association with neurodevelopmental disorders. Targeted interventions aimed at *Mtb* infections in pregnancy can be developed to ameliorate impaired neurodevelopmental outcomes in the foetus.

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This chapter is a manuscript entitled **'**Gestational *Mycobacterium tuberculosis* exposure affects pregnancy and leads to developmental delays in infected mice offspring' that has been submitted to a DHET accredited and Scopus indexed journal. *Experimental Physiology*

CHAPTER TWO

Gestational *Mycobacterium tuberculosis* exposure affects pregnancy and leads to developmental delays in infected mice offspring

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Running Title: Prenatal *Mtb* exposure impairs offspring development

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2 Abstract

Maternal infections during pregnancy are a significant risk for neurodevelopmental disorders. *Mycobacterium tuberculosis* (*Mtb*) is a major respiratory pathogen common in pregnant women because pregnancy-associated immune changes favour *Mtb* infection establishment. Although it is known that some pharmacological drugs and maternal infections in pregnancy can affect offspring development, it is not known whether gestational *Mtb* affects offspring development. The current study infected pregnant Balb/c mice with *Mtb* H37Rv and Valproic acid (VPA) individually and in combination. Maternal effects were measured by pregnancy success, lung infection and litter size. The pups' development was measured by fetal resorption, eye-opening pattern between postnatal day (PND) 12 and 16 and body weight between PND 7 and 28. Pregnancy survival and fetal development was severely affected by *Mtb*, causing 40% fetal resorption than the saline group. The *Mtb* infection significantly reduced the litter size compared to the saline. *Mtb* delayed eye-opening on PND 14 and 15. There were no congenital *Mtb* infections, but offspring development was significantly affected. Overall our data demonstarte that prenatal *Mtb* exposure threatens fetal survival and delays offspring development. These findings implicate a potential role of gestational *Mtb* infections in offspring development.

2.1 Introduction

Environmental risk factors such as pathogenic infections contribute immensely to the impaired development of children. Maternal infections of human Cytomegalovirus (HCMV), influenza, Herpes, Rubella, *Toxoplasma gondii* contribute to neurodevelopmental disorders (Parner, 2010; Patterson, 2011; Sørensen et al., 2009; Zerbo et al., 2016). Much attention (S. H. Fatemi et al., 1999; S. Hossein Fatemi et al., 2009; Miller et al., 2013; L. Shi et al., 2005) has been awarded to influenza in pregnancy and its association with impaired neurodevelopment leading to delays in physical development and altered behavioural patterns observed in ASD patients.

Developmental disorders are a challenge in the health care system due to their complex etiology. Autism Spectrum Disorder (ASD) is a group of developmental disorders that affect social and communication domains. These symptoms of ASD present at about 3 years of age. Children also show delays in the onset of speech, walking and other motor skills (E.-J. Yang et al., 2016). Developmental delays were present in 2.2% of ASD diagnosed children (Ventola et al., 2007). In sub-Saharan Africa, developmental disabilities increased by 71.3% between

1990 and 2006, and about 94.9% of children below 5 years of age are living with developmental disabilities in resource-deprived economies like Africa (Olusanya et al., 2018).

VPA is an anticonvulsant and mood stabiliser that is used to treat epilepsy and depression disorders (Deckmann et al., 2018). Treatment of pregnant women with VPA is associated with higher incidence of ASD and physical deformities in children (Bambini-junior et al., 2014; Roullet et al., 2013). Experimental models have extensively shown that prenatal exposure of with VPA on E12.5 induces ASD phenotype including delayed physical development in the offspring (Pelsőczi et al., 2020; R. Ruhela et al., 2017; Schneider & Przewłocki, 2005). The VPA model is a well-established tool to study developmental disabilities, owing to its ability to recapitulate human symptoms of ASD.

Tuberculosis (TB) is a global health problem with a high prevalence in Africa. About 27% of TB incidence cases among females were reported in Africa and of these, an estimated 32.1% were in their reproductive years (WHO, 2019). In South Africa, TB affects 1 in every 200 people and 32.1% of these are women in their reproductive years. Clinical studies (Brown et al., 2000; Lee et al., 2015) supported by experimental animal models (Bauman. D. M & and Paul, 2014; Careaga et al., 2017; Schwartzer et al., 2013; C. Yang, 2013) have regarded maternal infections during pregnancy as a factor that increases the risk of neurodevelopmental disorders. Maternal respiratory infections occurring in the second trimester led to impaired brain development of children in Oakland, California (Brown et al., 2000). The specific infectious agents were unidentified in the study, however, *Mtb* was most likely among the pathogens that caused maternal respiratory infections in the pregnant women (Brown et al., 2000). Tuberculosis is common in pregnant women and can cause spontaneous abortions, low maternal weight gain, preterm labour, and diminishing children's birth weight (Awodele et al., 2012; Ormerod, 2001). Previously, we observed immune dysregulation accompanied by a "leaky" blood-brain barrier, synaptic defects, increased repetitive patterns and reduced sociability in *Mtb*-induced MIA offspring (Manjeese et al., 2021a, 2021b). What is not known is whether *Mtb* infections in pregnancy also influence the developmental patterns of offspring. In this study, we aimed to examine the effects of prenatal exposure to *Mtb* on pregnancy, offspring development and ASD-like behaviours.

2.2 Materials and Methods

2.2.1 Animals

Twenty Balb/c mice were acquired from Africa Health Research Institute (AHRI). All animals were raised in a Biosafety level 3 laboratory (BSL3) and all procedures were conducted in accordance with (University of Kwazulu Natal Animal Research Ethics) UKZN AREC and ARRIVE guidelines. The animals were kept in individual cages under 12-hour light/dark cycle and a constant supply of food and water.

2.2.3 Mating

Eight weeks old mice were group-housed (5 per group), and bedding from the male cages was added to the female cages to induce estrous. The mice were group-housed for 5 days to synchronise their estrous cycles. After 5 days of group housing, mice were mated on a ratio of 1:1 (male: female) overnight. Vaginal plugs were checked early morning and males were immediately removed upon confirmation of vaginal plug presence and this marked embryonic day 0.5 (E0.5). In our pilot study, adminstration of 500 mg/ kg of VPA and *Mtb* together induced complete fetal resorption hence for this study we lowered the VPA dose to 350 mg/kg to increase fetal survival rate in the VPA+*Mtb* group. The VPA group was the positive control of the study and the VPA+*Mtb* group was created to assess the the effects of dual exposure to two ‘’environmental insults’’ on pregnancy and offspring development. The weights of the pregnant mice were recorded weekly to minimise handling and disturbance of pregnancy. Twenty pregnant 8 weeks old Balb/c mice were randomly assigned to 4 groups (5 per group) and treatments administered on embryonic day 12.5(E12.5) as shown in Table 2.1 and Fig 2. 1A. Each animal raised her own litter in a BSL3 laboratory during the study. Litters were culled to 12 per group and no more than 4 animals per litter (2 per sex).

Table 2. 1: Groups and treatments in the study

Group	Treatment and dosage	Route of administration
Saline	0.2 ml of Saline	Single i.p
VPA	0.2 ml of 500 mg/kg valproic acid	Single i.p
VPA+*Mtb*	0.2 ml of 350 mg/kg valproic acid + 10ml of $1x10^8$ CFU (OD_{600}=1)	Single i.p + aerosol infection
Mtb	10ml of $1x10^8$ CFU (OD_{600}=1)	Single aerosol infection

* OD- optical density, CFU- colony forming OD- optical density, i.p- intraperitoneal

2.2.4 *Mtb* infection

Ten mice from *Mtb* and VPA + *Mtb* group were placed in a Glas-col infection chamber (Terre Haute, IN, USA) with 10ml of $1x10^8$ CFU (OD_{600}=1) *Mtb* H37Rv inoculum expelled into the nebulizer using a syringe. The Glas-col machine cycle was set to the following conditions: preheat (15 minutes), nebulisation (30 minutes), cloud decay (30 minutes), decontamination (15 minutes) and cool down (10 minutes). The mice were carefully removed from the machine using forceps and placed into cages with bedding, food and water.

2.2.5 Culturing lungs

Lung culturing was performed to confirm *Mtb* infection of the mothers and assess vertical transmission. Half the lung (left side) from each mouse (mother and pups) was homogenised using a Mag analyser, and 10-fold serial dilutions were prepared from the lung homogenate and plated onto 7H11 agar in triplicate and incubated at 37˚C for 4 weeks before CFU counts.

2.2.6 Postnatal growth and maturation

The postnatal growth and maturation tests of pups were performed according to methods by Favre et al., 2013. Eye-opening was observed once daily between postnatal day 12 (PND 12) and 16 and scored as follows: both eyes closed – 0, 1 eye open – 1, both eyes open – 2. The animal cages were opened under a biosafety cabinet to perform the test in the morning.

2.2.7 Developmental weights

The weights of the pups were recorded once a week from PND7 to PND28. Birth weights were not measured because the biosafety cabinet's temperature was too cold to expose the neonates.

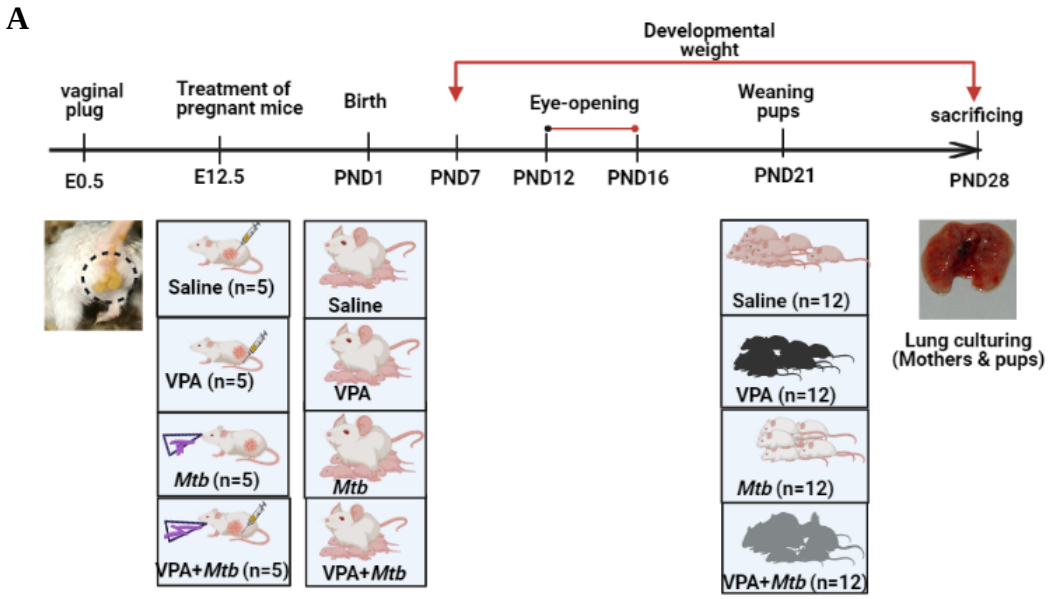
2.2.8 Statistical Analysis

Unless otherwise specified data is presented as mean ± SEM and *p* value <0.05 was considered significant. Data analysis was performed using Kruskal wallis test for for non-parametrically distributed data (Fetal resorption), one or two way ANOVA for parametrically distributed data followed by posthoc tests for multiple comparisons using either Bonferroni or Dunnett's tests (as appropriate) with GraphPad Prism version 7 (GraphPad software, USA).

2.3 Results

2.3.1 *Mtb* compromises maternal lung integrity in Balb/c mice

The health and pathological consequences on maternal lungs following the different treatments in our study is shown in Fig 2.1 B-E. There was no growth of Mtb in the lungs from VPA or saline exposed subjects as expected while the *Mtb* and VPA+*Mtb* group lungs showed a decrease in lung integrity, which is consistent with TB pathology. The offspring lungs were all healthy. Colony counts obtained from culturing all the maternal lungs were converted to $\log_{10}(x+1)$, where x represents the total number of viable bacilli counted per lung. We observed increased bacterial burden in maternal lungs from *Mtb* and VPA+*Mtb* lungs as expected, and no colonies grew on the Saline and VPA agar plates (Table 2.2) meaning that there was no cross-contamination in our treatment groups.



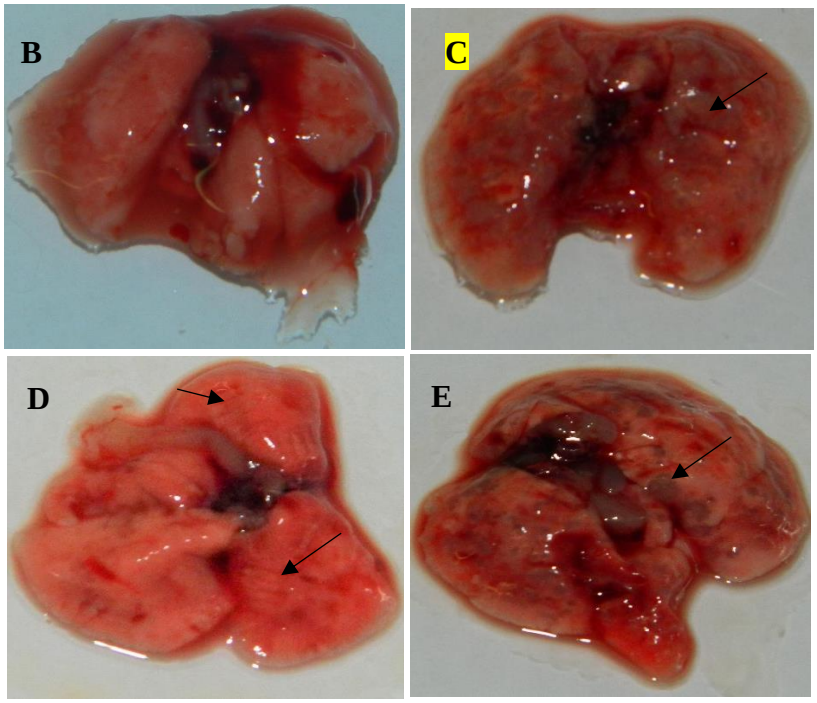


Figure 2. 1: **A** graphical representation of the study design; **B-E:** Representative images of maternal lung tissue of Balb/c mice 4 weeks post-delivery, $n = 5$ (B- Saline; C- VPA+*Mtb*; D-VPA; E- *Mtb*). The lungs were visually examined for pathological changes in comparison with the non-infected Saline group. The lungs from the negative control (Saline) group were healthy, but those from *Mtb* (E) and VPA+*Mtb* (C) groups showed granulomas (represented by arrows) at mid-gestation. The lungs from VPA (D) treated mice had drug-induced injuries (represented by arrows).

The bacterial load was determined through culturing maternal lungs 4 weeks post-delivery on MH711 agar, and CFUs were calculated (Table 2.2). Data represented as Mean ± SEM. There was no growth of *Mtb* in the lungs from VPA and Saline as expected.

Table 2. 2: Bacterial load in maternal lungs.

Group	CFU
Saline	**-**
VPA	**-**
TB	**$1.92 \pm 2.19 \times 10^{8***}$**
VPA+TB	**$1.91 \pm 2.19 \times 10^{8***}$**

*** denotes $p<0.0001$ when compared to the non-infected control group (saline).

2.3.2 *Mtb* exposure induces fetal resorption and reduces litter size in pregnant mice.

Twenty mice were confirmed pregnant by the presence of a vaginal plug and 40% body weight gain from E0.5 to E12.5. There was 40% fetal resorption in the *Mtb*, VPA and VPA+*Mtb* treated pregnant mice and there was no fetal resorption observed in the Saline treated group. There was no statistical significance ($p>0.05$) in fetal resorption rate between Saline and all the groups, but generally, a high fetal resorption rate was observed with all the treated groups (Fig. 2.2B). Only 60% of the pregnant mice from VPA, VPA+*Mtb* and *Mtb* groups delivered pups (Fig. 2.2A). One-way ANOVA showed that there was a treatment effect on the number of pups born ($p<0.005$, Table A1). The litter (the number of pups born) size was reduced in these pregnancies compared to Saline treated group. The number of pups born to VPA and VPA+*Mtb* exposed mothers were significantly lower than the Saline group, 3.6 ± 1.50 ($p<0.0015$) and 3.4 ± 1.69 ($p<0.0012$), respectively. The *Mtb* group had the smallest litter size (2.6 ± 1.08) with the highest statistical significance ($p<0.0001$) compared to the Saline group (Fig. 2.2A). There was no statistical significance between VPA and *Mtb* groups ($p>0.05$).

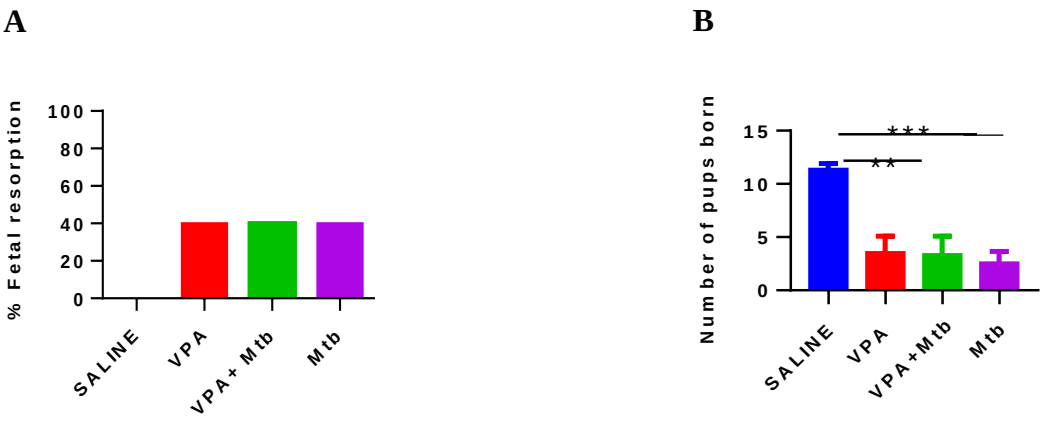


Figure 2. 2: Fetal resorption and litter size (A) Graph showing fetal resorption rates in all the groups, *n*=5 in each group, kruskal wallis statistic= 2.74 and *p*>0.05. Data represented as a percentage of pregnant mice that did not deliver any pups. Fetal resorption rate did not significantly differ between VPA, *Mtb* and VPA+*Mtb* and there was no fetal resorption in the negative control (Saline). (B) Graph showing the number of pups born from all the groups. Saline -11.4 ± 1.81, VPA- 3.6±1.81 (*p*<0.001), *Mtb*- 2.6 ± 1.81 (*p*<0.0001) and VPA+*Mtb*- 3.4 ± 1.81 (*p*<0.001) pups. *Mtb* reduced litter size the most. There was no statistical significance in *p*-value between positive control (VPA) and *Mtb* (p>0.05). ** denotes p<0.001 and *** *p*<0.0001 vs Saline. One-way ANOVA, detailed statistics information is available in Appendix Table A1 and A2

2.3.3 Prenatal *Mtb* exposure delays eye-opening in Balb/c mice.

The development of pups was measured by eye-opening status from PND 12 to 16. Prenatal exposure to *Mtb* delays maturation in both males (Fig 2.3A) and females (Fig 2.3B), but females are the most affected, *Mtb* female pups began opening their eyes on PND 16 and males on PND 15 (*p*<0.001 vs Saline). The female VPA+*Mtb* and Saline groups opened eyes on PND 13 and VPA on PND 15. It is interesting to note that a "double hit" of VPA and *Mtb* did not result in the most significant delay in eye-opening and there was no statistical significance in eye-opening trend between VPA+*Mtb* and Saline groups. When males and females were combined (Fig 2.3C) there was an overall delay in eye-opening of all the treated groups (VPA, *Mtb*, VPA+*Mtb*) when compared to the negative control group (Saline). Eye-opening scores were significantly low in all the treated groups (VPA, *Mtb* and VPA+*Mtb*) compared to the Saline group on PND 14 (*p*<0.001). Mtb group scored the least (0) while VPA and VPA+*Mtb*

scored higher, showing that animals in the VPA+*Mtb* group had at least 1 eye had opened by PND 14.

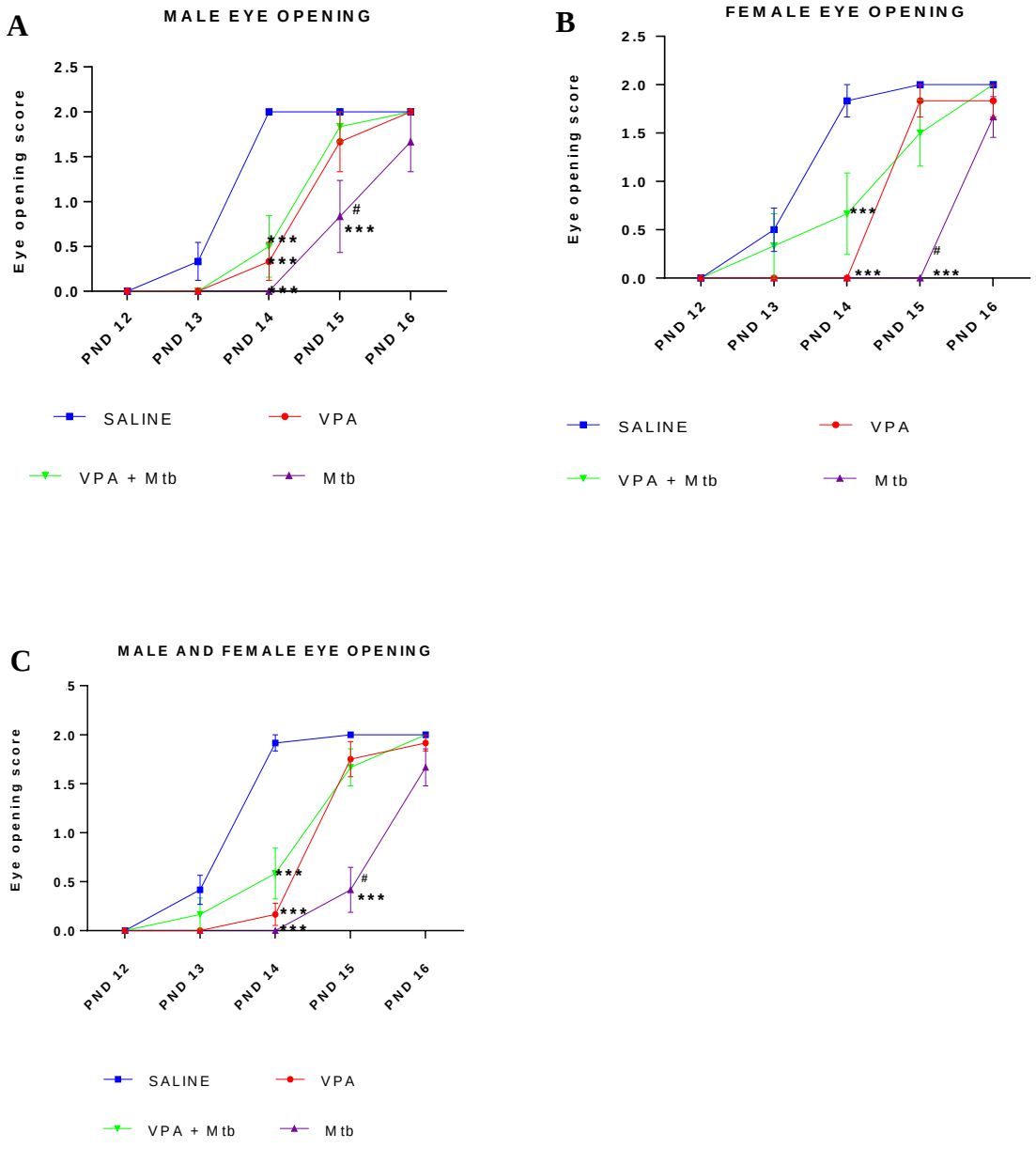


Figure 2. 3: Eye opening patterns in offspring, A- Male eye-opening: The graphs shows the eye-opening trends in males (*n*=6) between postnatal day (PND) 12 and 16. One eye open was scored 1, both eyes open-2 and both eyes closed- 0. Compared to Saline there was 2 days delay in eye-opening of *Mtb* pups, 1 day delay in VPA pups and 1 day delay in VPA+*Mtb* pups. Compared to VPA there was a significant 2 day delay in eye-opening of *Mtb* pups. *** denotes

$p<0.001$ vs Saline and $^{\#}$denotes $p<0.001$ vs VPA. **B- Female eye-opening:** The graph shows eye-opening trends in females ($n=6$) between PND12 and 16. One eye open was scored 1, both eyes open-2 and both eyes closed- 0. Compared to Saline there was a significant 3 day delay in eye-opening of *Mtb* pups, 2 days delay in VPA pups and no delay in VPA+*Mtb* pups. Compared to VPA there was 3 days delay in eye-opening of *Mtb* pups. *** denotes $p<0.0001$ when compared to Saline and $^{\#}p<0.0001$ when compared to VPA. **C- Male and Female eye opening:** The graph shows the combined male and female eye-opening trends between postnatal day (PND) 12 and 16. One eye open scored 1, both eyes open-2 and both eyes closed-0 (n=12). Compared to Saline there was 2 days delay in eye-opening of *Mtb* pups, 1 day in VPA pups and no delay in VPA+*Mtb* pups. Compared to VPA there was 1 day delay in eye-opening of *Mtb* pups. ***denotes $p<0.0001$ when compared to Saline, $^{\#}p<0.0001$ when compared to VPA. Two-way ANOVA, detailed statistics information is available in Appendix Table A3, A4 and A5.

2.3.4 Prenatal *Mtb* exposure diminishes offspring developmental weight.

We measured the body weight of the pups on PND 7, 14, 21 and 28. Two-way ANOVA showed that there was a treatment effect on developmental weight between PND7 and PND28 ($p<0.001$) .Fig. 2.4 shows a general trend where VPA exposed pups had the lowest body mass which persisted throughout the study. *Mtb* exposed pups weighed significantly less (17.22±0.581; $p<0.05$) than Saline treated pups on PND 28. Compared to VPA exposed pups, *Mtb* exposed pups' weight was significantly higher on PND 21 (11.11±0.581) and PND 28 ($p<0.001$). The VPA+*Mtb* pups' weight was significantly lower (17.13±0.581; $p<0.05$) than Saline exposed pups on PND 28.

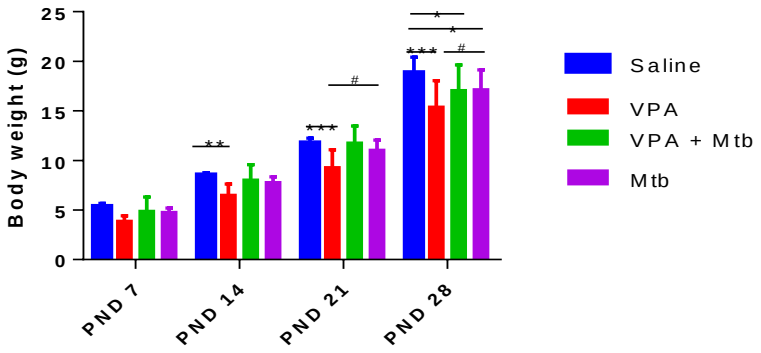


Figure 2. 4: Body weight of pups (*n*=12) was measured in grams (g) on different postnatal days (PND). There was a general trend of body weight increase between PND 7 and PND 28 in all the groups. Compared to Saline, *Mtb* and VPA+*Mtb* pups' weight was significantly lower (17.13±0.581 and 17.22±0.581 respectively). Compared to VPA, *Mtb* pups' body weight was significantly increased on PND 21 and PND 28. Compared to VPA, *Mtb* pups weight was significantly higher (11.11±0.581). *Mtb* and VPA+*Mtb* pups had similar weights throughout the study. ***** denotes $p<0.05$, ****** $p<0.001$ and ******* $p<0.0001$ vs Saline and $^{\#}$ $p<0.001$ vs VPA on marked postnatal days. Two-way ANOVA, detailed statistics information is available in Appendix Table A6.

2.4 Discussion

Delays in developmental milestones such as sitting, walking and speech are parameters used to measure postnatal development in children. Gestational infections can influence pregnancy outcomes by restricting fetal growth and altering developmental programming leading to developmental disorders. Bacteria causing respiratory infections are implicated in the etiology of developmental disorders. TB is caused by *Mtb* which chiefly affects the lungs. The impact of TB on pregnancy is determined by disease severity and the timing of infection in pregnant women.

In this study, *Mtb* infection was not vertically transmitted, but we observed adverse effects on maternal health and pregnancy outcome. We found that gestational *Mtb* infection had severe impacts on pregnancy success and reduced the fetus' survival rate. Fetal growth and development requires an anti-inflammatory immune state mediated via T Helper 2 (TH-2) pathway (Hussein et al., 2011; Mor et al., 2017; Mor & Cardenas, 2010; Morelli et al., 2015).

Mtb infection elicits T Helper 1 (TH-1) response and this threatens fetal survival resulting in a higher risk of abortion (Lyandova and Panteleev, 2015; Morelli et al., 2015).

This is the first study to document the effects of gestational *Mtb* infection on pregnancy outcome, growth and development of Balb/c mice offspring to the best of our knowledge. We report that Mtb infection reduced the litter size in Balb/c mice. Similarly, gestational exposure to bacterial endotoxin lipopolysaccharide (LPS) caused fetal loss and the surviving offspring were growth restricted (Renaud et al., 2011). Our VPA treated mice's results complement previously documented data of litter size reduction following VPA gestational exposure (Favre et al., 2013; Komariah et al., 2017). We found no significant difference in litter size between the positive control (VPA treated) and *Mtb* infected mothers (Fig 2.2B).

The lung tissue is the main site of *Mtb* infection. A high bacterial load *(Mtb)*was present in the maternal lungs from the *Mtb* and VPA+*Mtb* groups. The growth of *Mtb* elicits inflammatory host responses that cause extensive lung damage. The *Mtb* infected lungs presented with granulomatous lesions (Fig 2.1C and E), which are an *Mtb* infection mediated immune response that avoids disseminating bacteria to other tissues (Gupta et al., 2016; Ravimohan et al., 2018; R. Shi & Sugawar, 2013). VPA is a drug known to affect lungs by causing eosinophilic pleural effusions (Wendy Bullington et al., 2007) and alveolar hemorrhaging in humans (Choi et al., 2011). VPA compromised the integrity of the maternal lung tissue as shown by the presence of interstitial lesions. The VPA effects on the maternal lungs are possibly a direct consequence of VPA toxicity and drug-induced inflammation.

Eye-opening and weight gain are typical developmental milestones in rodent models of ASD. We observed 2 days delay in eye-opening in the *Mtb* group than the Saline group. Similarly, eye-opening was delayed in mice exposed to bacterial endotoxin lipopolysaccharide (LPS) (Abu-taweel et al., 2013). The delay in eye-opening could be due to disturbed optic nerve myelination and possible reduction in amacrine cells in response to *Mtb* induced maternal inflammation (Loeliger et al., 2007). This study's eye-opening pattern suggests that maternal *Mtb* infection affects development more than VPA since the *Mtb* group took a day longer to open eyes than the VPA group. However, it is interesting to note that delayed development is somehow rescued when a lower VPA dose and *Mtb* are administered together. The maternal inflammatory response to *Mtb* saves VPA-induced developmental delays. Findings from

Hrubec *et al*. (2012) support this result; in their study, the maternal immune stimulation by the cytokine IFN-γ prevented neural tube defects in VPA exposed mice (Hrubec et al., 2012).

Eye-opening delays are more pronounced in females than males following *in utero Mtb* exposure. It took *Mtb* exposed females a day longer than their male counterparts to open eyes, suggesting that a male neuroprotective mechanism could be modulating the gender specific difference in eye-opening. When the *Mtb* group was compared to VPA, there was a 1-day delay in eye-opening regardless of gender. However, the delay trend remained pronounced in females suggesting that *Mtb* exposure affects eye-opening more than VPA regardless of gender.

Body weight is a common developmental milestone in mice (Yang et al., 2016). Our results with VPA treatment are consistent with previous findings that reported reduced body weight in mice exposed to VPA *in utero* (Kolozsi et al., 2009; R. K. Ruhela et al., 2019; Schneider & Przewłocki, 2005; Podgorac et al., 2016). *Mtb* on its own and in combination with a lower VPA dose does not influence body weight from neonatal to juvenile age (PND 7 to PND 21), suggesting that a lower VPA dose has no effect on *Mtb*-induced MIA developmental weight. The mechanism behind this is unclear at this point of our study. All the treatments (*Mtb*, VPA+*Mtb* and VPA) affected body weight gain on PND 28. The change in weight gain on PND 28 could have been influenced by weaning. Weaning exerts nutritional and physiological stress on the pups. The change in diet can cause digestive problems that influence feeding efficiency in the offspring (Campbell et al., 2013). A classic study by Rodier and colleagues (1996) observed damage of swallowing-associated neurons leading to inadequate food intake and dimished body weight in offspring exposed to VPA in utero (Rodier et al., 1996). Weaning is also associated with an upregulation of pro-inflammatory cytokines that regulate growth and this can also affect the developmental weight of weaned pups. We previously reported elevated plasmatic IL-6 and IL-17A levels in *Mtb* treated offspring and this immune dysregulation could have influenced the reduction in body weight observed on PND 28 (Manjeese et al., 2021a).

2.5 Conclusion

This study demonstrates that gestational *Mtb* infection threatens fetal survival in early pregnancy and interferes with fetal development. Although *Mtb* infection is not vertically transmitted to the fetus, normal development is affected. Altered developmental patterns persist postnatal as reflected by diminished body weight and delays in eye-opening. The maternal

immune response to *Mtb* infection triggers eye-opening delays, which are more pronounced in females than males.

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2.7 Statement of Ethics

Ethical approval for this study was obtained from University of KwaZulu Natal Animal Research Ethics Committee (Reference: AREC/076/018M)

2.8 Conflict of Interest Statement

The authors have no conflicting interests.

2.9 Funding Sources

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2.10 Author Contributions

All authors contributed equally and discussed the results in the final manuscript.

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Appendix

Table A1: number of pups born

ANOVA	DF	F(DFn,DFd)	*P* value
Treatment (between columns)	3	F (3, 16) = 10.4	0.0005
Residual (within columns)	16		
Total	19		

Table A2: Fetal resorption

ANOVA	DF	F(DFn,DFd)	*P* value
Treatment (between columns)	3	F (3, 16) = 0.8889	P=0.4680
Residual (within columns)	16		
Total	19		

Table A3: Male Eye-opening pattern

ANOVA	DF	F(DFn,DFd)	*P* value
Interaction	12	F (12, 80) = 4.348	P<0.0001
Time	4	F (4, 80) = 85.53	P<0.0001
Column Factor	3	F (3, 20) = 36.56	P<0.0001
Subjects (matching)	20	F (20, 80) = 0.3874	P=0.9904
Residual	80		

Table A4: Female Eye-opening

ANOVA	DF	F(DFn,DFd)	*P* value
Interaction	12	F (12, 80) = 7.455	P<0.0001
Time	4	F (4, 80) = 79.8	P<0.0001
Column Factor	3	F (3, 20) = 33.31	P<0.0001
Subjects (matching)	20	F (20, 80) = 0.7297	P=0.7843
Residual	80		

Table A5: Male and Female Eye-opening combined

ANOVA	DF	F(DFn,DFd)	*P* value
Interaction	12	F (12, 176) = 10.64	P<0.0001
Time	4	F (4, 176) = 163.5	P<0.0001
Column Factor	3	F (3, 44) = 73.11	P<0.0001
Subjects (matching)	44	F (44, 176) = 0.5057	P=0.9955
Residual	176		

Table A6: Developmental weights

ANOVA	DF	F(DFn,DFd)	*P* value
Interaction	9	F (9, 132) = 2.552	P=0.0098
Time	3	F (3, 132) = 1571	P<0.0001
Column Factor	3	F (3, 44) = 7.828	P=0.0003
Subjects (matching)	44	F (44, 132) = 6.412	P<0.0001
Residual	132		

This chapter is a manuscript entitled **‘**M*ycobacterium tuberculosis* causes a leaky blood-brain barrier and neuroinflammation in the prefrontal cortex and cerebellum regions of infected mice offspring’ that is published in a DHET accredited and Scopus indexed journal. *International Journal of Developmental Neuroscience.*

INTERNATIONAL JOURNAL OF
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RESEARCH ARTICLE

Mycobacterium tuberculosis causes a leaky blood-brain barrier and neuroinflammation in the prefrontal cortex and cerebellum regions of infected mice offspring

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ABSTRACT

The maternal system's exposure to pathogens influences fetal brain development through the influx of maternal cytokines and activation of the fetal immune status to a persistent inflammatory state characterised by glia cell activation. Neuroinflammation influences the blood-brain barrier's (BBB) permeability allowing peripheral immune cell trafficking into the brain. *Mycobacterium tuberculosis* (*Mtb*) is a pathogen that causes

CHAPTER THREE

M*ycobacterium tuberculosis* causes a leaky blood-brain barrier and neuroinflammation in the prefrontal cortex and cerebellum regions of infected mice offspring.

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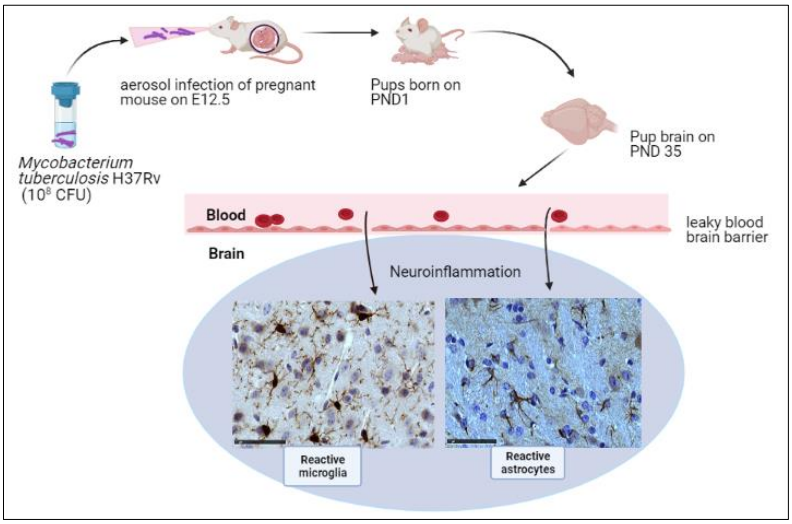
Keywords: maternal immune activation, *Mycobacterium tuberculosis*, neuroinflammation, blood brain barrier, microglia, astrocytes

3 Abstract

Maternal immune activation (MIA) can alter the fetal immune status to a persistent inflammatory state characterised by glia cell activation. Immune dysregulation influences the blood-brain barrier (BBB) permeability. The maternal system's exposure to pathogens influences fetal brain development. *Mycobacterium tuberculosis* (*Mtb*) is a global pathogen that causes Tuberculosis, a global pandemic responsible for health and economic burdens. Although it is known that maternal infections increase the risk of neurodevelopmental disorders, it is not known whether gestational *Mtb* infections contribute to impaired fetal neurodevelopment. Here we infect pregnant Balb/c mice with *Mtb* H37Rv and Valproic acid (VPA) individually and in combination. Neuroinflammation was measured by assessing microglia and astrocyte population in the prefrontal cortex (PFC) and cerebellum (CER) of pups. *Mtb* infection increased the microglia population and caused morphological changes to a reactive phenotype in the PFC. Also, the astrocyte population was significantly increased in the PFC of *Mtb* pups ($p<0.05$). The BBB permeability was determined by measuring the Evans Blue (EB) dye concentation in the PFC and cerebellum 1 hour post receiving intravenous EB dye injection. We found that prenatal *Mtb* exposure significantly increased the BBB permeability in the PFC (1.14 ± 0.05 ng/mg of tissue; $p<0.0001$) and cerebellum of pups (1.26 ± 0.05 ng/mg of tissue; $p<0.001$) vs Saline. Overall, our data demonstrate that prenatal exposure to *Mtb* compromises the BBB integrity and causes neuroinflammation in offspring. These findings implicate a potential role of gestational *Mtb* infections in the aetiology of neurodevelopmental disorders.



Graphical Abstract

3.1 Introduction

Maternal infections in pregnancy are now known to contribute to the aetiology of neurodevelopmental disorders. Clinical and experimental reports indicate an association of prenatal exposure to Herpes simplex virus, influenza virus, HIV, *Plasmodium berghei*, *Taxoplasma gondii* and *Staphylococcus aureus* with an increased risk of neurodevelopmental disorders (Dinel et al., 2014; Doyle et al., 2013; Glass et al., 2018; Hornig et al., 2017; Hornig et al., 2018; McDonald et al., 2015; Zerbo et al., 2016).

Microglia are CNS resident macrophages that become activated to respond to injury and infection and in that state, they exhibit an amoeboid appearance with enlarged cell bodies that have thicker and shorter cellular processes while in their resting state they are ramified with long processes (Bilbo et al., 2018; Monnet-Tschudi et al., 2011). They also remove CNS debris and "prune" synapses to remove extraneous and damaged synapses in the brain (Petrelli et al., 2016; Prins et al., 2018). Astrocytes are star-shaped with cytoplasmic processes (end-feet) that are in proximity with endothelial cells in the BBB allowing crosstalk between the CNS and circulatory system (Erickson and Banks, 2018). During CNS development, astrocytes assist in cellular migration and regulation of synaptic activities through their metabolic interactions with neurons. Astrocyte reactivity is characterised by increased expression of glial fibrillary acidic protein (GFAP), the release of inflammatory mediators and thick cellular processes (Monnet-Tschudi et al., 2011). The activities of astrocytes and microglia are interdependent with both contributing to neuronal and immune functions hence neuroinflammation influences synaptic functions. Overactivation of glial cells leads to chronic neuroinflammation witnessed in neurodevelopmental disorders (Morgan et al., 2010; Vargas et al, 2005).

The BBB is a selectively permeable cellular interface isolating the CNS from the peripheral circulatory system. It is made up of a single layer of brain endothelial cells, astrocyte end-feet, pericytes and microglia that regulate trafficking of immune cells and solutes from the periphery into the brain through tight junctions (TJs) (Morris et al., 2018). Decreased expression of TJs and secretion of endothelial disrupting factors by pericytes allow infiltration of immune cells into the CNS where they promote neuroinflammation (Liebner et al., 2018; Erickson and Banks, 2018).

The MIA model is used to study the effects of gestational maternal infections on fetal brain development. The most common MIA models involve the injection of pregnant rodents with

synthetic viral RNA Polyriboinosinic: polyribocytidilic (Poly I:C) and bacterial endotoxin lipopolysaccharide (LPS) to induce inflammation (Solek et al., 2018). The offspring of MIA models show neuroinflammation as indicated by postnatal microglia and astrocyte activation (de Souza et al., 2015; Zhu et al., 2014). *Mtb* is a global pathogen that causes Tuberculosis and like other infectious pathogens, it induces inflammation which may create a hostile environment to a developing fetus during pregnancy. While *Mtb* infections are common in pregnant women, they are difficult to diagnose because the immunological changes induced by pregnancy conceal Tuberculosis symptoms (Loto and Awowole, 2012; Mathad and Gupta, 2012). Children born to *Mtb* infected mothers are at an increased risk of having low birth weight, infant mortality and prematurity (Bekker et al., 2016; Jana, 1994). What is not known is whether *Mtb* infections in pregnancy also influence brain development causing reactive changes observed in other MIA models. In this study, we aimed to examine the effects of prenatal exposure to *Mtb* on the BBB, microglia and astrocytes in the PFC and cerebellum brain regions which are implicated in ASD pathology.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Ethical approval for this study was obtained from the University of KwaZulu Natal Animal Research Ethics Committee (Reference: AREC/076/018M). Balb/c mice at 8 weeks of age were obtained from Africa Health Research Institute (AHRI), South Africa. Females were group-housed (5 per group) for 5 days to synchronise their estrous cycle, bedding from the male cages was added to the female cages to induce the estrous phase. The mice were mated on a ratio of 1:1 (male: female) overnight. Vaginal plugs were checked early morning and males were immediately removed upon confirmation of vaginal plug presence and this marked embryonic day 0.5 (E0.5). Twenty pregnant mice were randomly assigned to 4 groups (5 per group) and treatments administered on E12.5 as shown in Table 3.1. In our pilot study, adminstration of 500mg/ kg of valproic acid (VPA) and *Mtb* together induced complete fetal resorption and fetal survival rate only increased when the VPA dose was lowered to 350 mg/kg. Based on our pilot study findings, we used 350mg/kg of VPA in the VPA+*Mtb*. Only 3 animals per group had successful pregnancies hence litters were culled to 12 per group and no more than 4 animals per litter (2 per sex). All animals were raised in a Biosafety level 3 laboratory (BSL3) and all procedures were conducted following (University of Kwazulu Natal Animal Research Ethics) UKZN AREC and ARRIVE guidelines. Each animal raised her litter under

12-hour light/dark cycle and a constant supply of food and water. Litters were weaned at postnatal day 21 (PND 21), at which males and females were separated but grouped according to treatments. The minimum required number of animals were randomly picked from all the litters for immunohistochemistry and BBB experiments.

Table 3.1: Groups and treatments in the study

Group	Treatment and dosage	Route of administration
Saline	0.2 ml of Saline	i.p injection
VPA	0.2 ml of 500 mg/kg valproic acid	i.p injection
VPA + *Mtb*	0.2 ml of 350 mg/kg valproic acid + 10ml of $1x10^8$ CFU (OD_{600}=1)	i.p injection + aerosol infection
Mtb	10ml of $1x10^8$ CFU (OD_{600}=1)	Aerosol infection

*OD- optical density, CFU- colony forming units, i.p- intraperitoneal

3.2.2 *Mtb* infection

Ten pregnant mice from *Mtb* and VPA + *Mtb* group were placed in a Glas-col infection chamber (Terre Haute, IN, USA) with 10ml of $1x10^8$ CFU (OD_{600}=1) *Mtb* H37Rv inoculum expelled into the nebulizer using a syringe. The Glas-col machine cycle was set to the following conditions:

preheat	15 minutes
nebulisation	30 minutes
cloud decay	30 minutes
decontamination	15 minutes
cool down	10 minutes

The mice were carefully removed from the machine using forceps and placed into cages with bedding, food and water.

3.2.3 Determining the BBB permeability

The experiment was performed as previously described (Goldim et al., 2019). Briefly, 5 weeks old mice were sedated using 10% ketamine hydrochloride (80 mg/kg) and 2% xylazine hydrochloride (10 mg/kg) administered intraperitonially. Using a 1 ml syringe, 3 ml/kg of 2% (w/v) EB dye solution (Sigma Aldrich) in normal saline was gently administered to a sedated mouse in a biosafety cabinet. The animal was placed on a heating pad (Elektra comfort, South Africa) with cotton for 1 hour to avoid hypothermia. An hour following EB dye injection, the animal was perfused using normal saline to remove intravascular blood containing EB dye. After all the blood had been removed, the animal was decapitated using a guillotine. The whole brain was removed from the skull and stored at -80°C until analysis.

3.2.4 Evaluating EB dye in brain tissue

All brain tissues were thawed and weighed using an analytical balance (Accuris scientific, USA). The prefrontal cortex and cerebellum regions of the brain were dissected and individually homogenised in 50 % trichloroacetic acid solution using a motor and pestle. The homogenate was centrifuged for 20 minutes at 10 000 x g and the supernatant was collected. The supernatant was diluted 3 fold with ethanol to provide an optical path for spectroscopy measurement. Absorbance was measured at 620 nm wavelength and EB dye tissue content was quantified from a linear standard curve derived from known amounts of the dye, and it was expressed as ng/mg of tissue (Goldim et al., 2019; Wang and Lai, 2014).

3.2.5 Immunohistochemistry

Three offspring per group (1 from each litter) at postnatal day (PND) 35/ 5 weeks old were deeply anaesthetised with xylazine/ketamine and transcardially perfused with PBS followed by 4% formalin. Brains were removed from the skull and post-fixed in 10% neutral buffered formalin and processed into paraffin-embedded blocks. Coronal 10 μm thick sections, 1 in 5 series for 4 sections of the PFC and cerebellum regions were sectioned using a microtome (Leica Biosystems, USA). The slides were soaked in xylene to remove paraffin and rehydrated through an ethanol gradient, followed by PBS. The sections were pre-treated using heat mediated antigen retrieval, blocked with 5% goat serum and incubated with either mouse monoclonal anti-GFAP (ab10062) or anti-Iba-1 (ab153696) antibodies (Abcam) at 1/500 dilution for 24 hours at 4 °C, the sections were washed with PBS and incubated with an anti-mouse HRP conjugated polymer system for 1 hour at room temperature. Dab (3,3' diaminobenzidine) substrate kit (Cell signalling, USA) was used as the chromogen and sections were counterstained with haematoxylin. Images of the sections were captured using a digital slide scanner (Nano-zoomer, Japan) (Zulu et al., 2018).

Z-stack Iba-1 and GFAP stained PFC and cerebellar sections were taken at X40 objective. The images were processed for cell counting in imagej software as previously described (Zhu et al., 2014). Briefly, 8-bit images were converted to maximal projection images using FFT bandpass filter and unsharp mask functions to remove small fragments while maintaining the larger features. A constant capture field of 200 x 100 x 10 μm in the PFC and cerebellum was used for cell counting and counterstained nuclei were removed using the Threshold colour plug-in (imagej) which left only the $GFAP^+$ or $Iba1^+$ areas that were quantified using the cell counter

function in ImageJ. The capture field was randomly applied to 4 areas in the PFC and cerebellum, the total number of $GFAP^+$/ $Iba1^+$ cells was counted and the average of the 4 capture fields was taken and used to calculate the average of $GFAP^+$/ $Iba1^+$ cells in the 5 sections per animal. The total number of cells was expressed as the number of $GFAP^+$/ $Iba1^+$ cells in 2 x 10^5 μm^3 of brain tissue.

3.3 Statistics

Data is presented as mean ± SEM and *p*-value <0.05 was considered significant. Shapiro and Wilk tests for normality were applied to determine data distribution. Data analysis was performed using one-way Analysis of variance (ANOVA) followed by posthoc tests for multiple comparisons using either Bonferroni or Dunnett's tests (as appropriate) with GraphPad Prism version 7 (GraphPad Software, USA).

3.4 RESULTS

3.4.1 Prenatal exposure to *Mtb* compromises BBB integrity in the PFC and Cerebellum

Following EB dye injection, animals immediately changed skin colour (Figure 3.1A) and the brain was macroscopically blue in regions that had a disrupted BBB (Figure 3.1B). Our results show that the BBB was damaged in the PFC and cerebellum regions. The optical density was measured at 610 nm and values were converted to ng dye/mg brain tissue using a standard curve of EB dye in ethanol. The EB dye concentration (Figure 3.1C) in the PFC of *Mtb* (1.14±0.05; $p<0.0001$) and VPA+*Mtb* (0.82±0.0001; $p<0.05$) pups was significantly increased compared to the saline group. Prenatal exposure to *Mtb* significantly increased EB dye concentration in the cerebellum (1.26±0.05; $p<0.05$) but a dual exposure to VPA and *Mtb* did not exacerbate the permeability to EB dye (Figure 3.1D), the effects of *Mtb* were rescued (0.778±0.05; $p<0.0001$). *Mtb* pups showed an increased concentration of EB dye in both PFC (1.14±0.05; $p<0.0001$) and cerebellum (1.26±0.05; $p<0.0001$) regions compared to the positive control (VPA group).

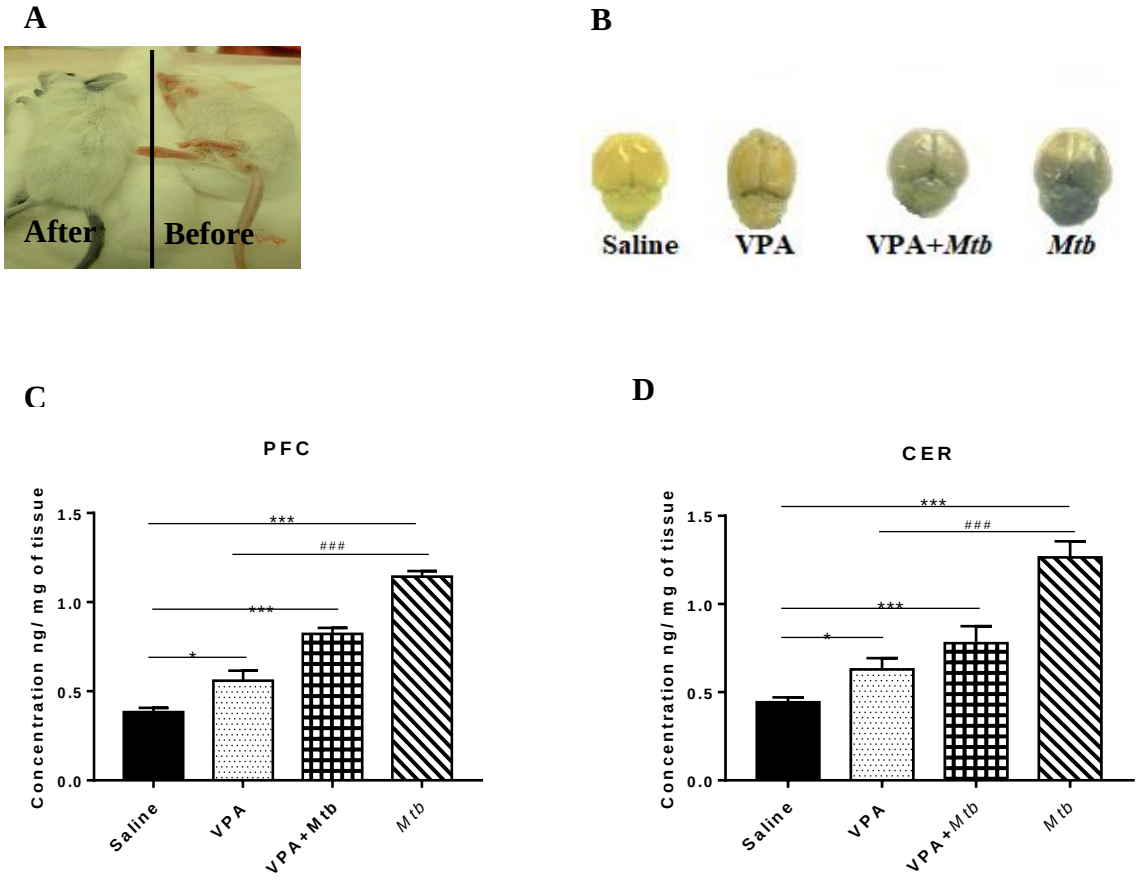
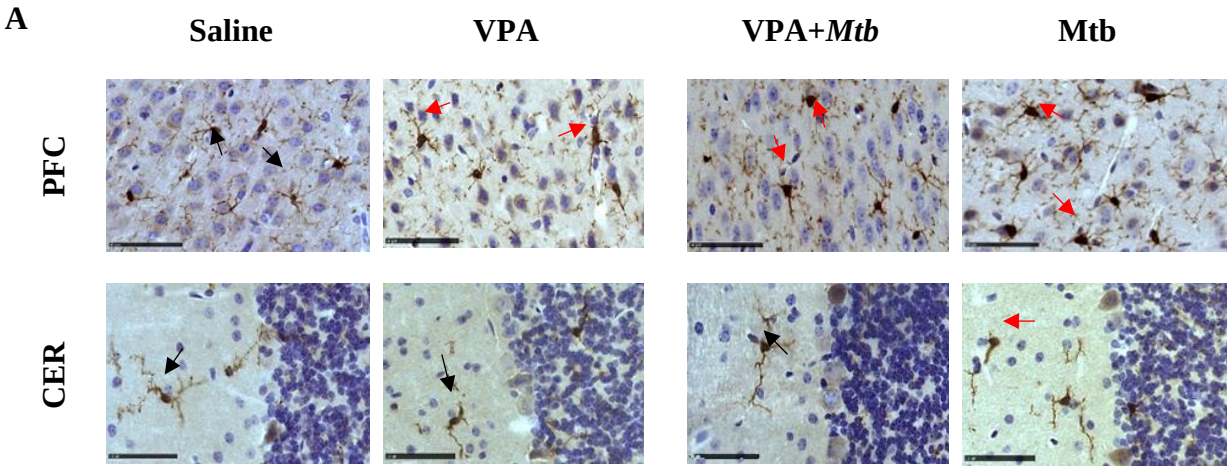


Figure 3. 1: Representative image of sedated mice before and after EB dye intravenous injection, **B** representative images of brain tissue 1-hour post EB dye injection, the brain regions with a disrupted BBB can be identified macroscopically by a blue colour, **C** graph shows EB dye concentration in the PFC where intra uterine exposure to VPA, VPA+*Mtb* and *Mtb* significantly increased the BBB permeability allowing for the penetration of EB dye from the periphery, $p < 0.05$ in the VPA group, $p < 0.0001$ in both VPA+*Mtb* and *Mtb* groups vs Saline. When compared to VPA (positive control) there was a significant increase in EB dye concentration found in the PFC, $p < 0.0001$ in *Mtb* group, **D** graph shows the concentration of EB dye found in the cerebellum region of the brain. Prenatal exposure to VPA, VPA+*Mtb* and *Mtb* significantly increased the concentration of EB dye that infiltrated the cerebellum, $p < 0.05$ for the VPA group and $p < 0.0001$ for VPA+*Mtb and Mtb* groups vs Saline group. EB dye concentration was significantly higher in the cerebellum of *Mtb* group than VPA group ($p < 0.0001$). All data is represented as mean ± SEM for 4 animals per group, *$p < 0.5$, **$p < 0.001$ and *** $p < 0.0001$ compared to Saline. $^{###}p < 0.0001$ compared to VPA. Optical density was

measured at 610 nm and values were converted to ng dye/mg brain tissue using a standard curve of EB dye in ethanol.

3.4.2 Prenatal *Mtb* exposure increases astrocyte and microglia population in the PFC and cerebellum of offspring

We used immunohistochemistry to examine microglia and astrocytes in the PFC and cerebellum brain regions. As shown in Figure 3.2, *Mtb* significantly increased the number of PFC Iba1$^+$ cells (p=0.0157) and no change was observed in the cerebellum (p=0.350) compared to saline. Combining VPA and *Mtb* did not cause significant changes in the number of Iba1+ cells in the PFC (p=0.458) and cerebellum (p=0.946) compared to saline. We did not observe any changes in the Iba1$^+$ population in the PFC and cerebellum of *Mtb* and VPA+*Mtb* when compared to the positive control (p>0.05). Iba1$^+$ cells in the *Mtb* group showed enlarged cell bodies with less branching compared to saline. We also counted the number of GFAP+ cells and as shown in Figure 3.3, we found that when compared to saline, *Mtb* increased the GFAP$^+$ cell population in the PFC (p=0.0169) and not the cerebellum (p=0.0538). There were no changes in both brain regions when compared to VPA (p>0.05). Taken together, these trends suggest that *Mtb* infection of mothers caused glial activation state in the offspring which was more pronounced in the PFC region.



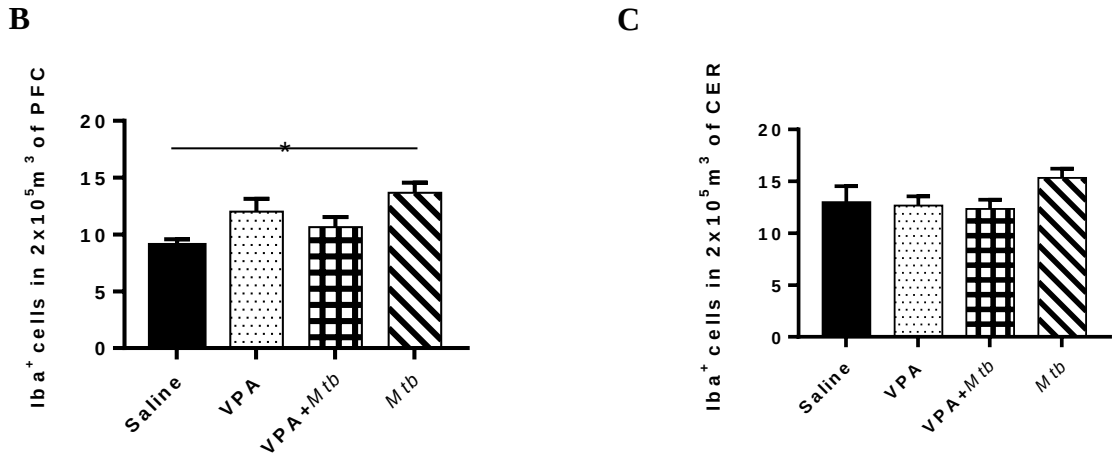
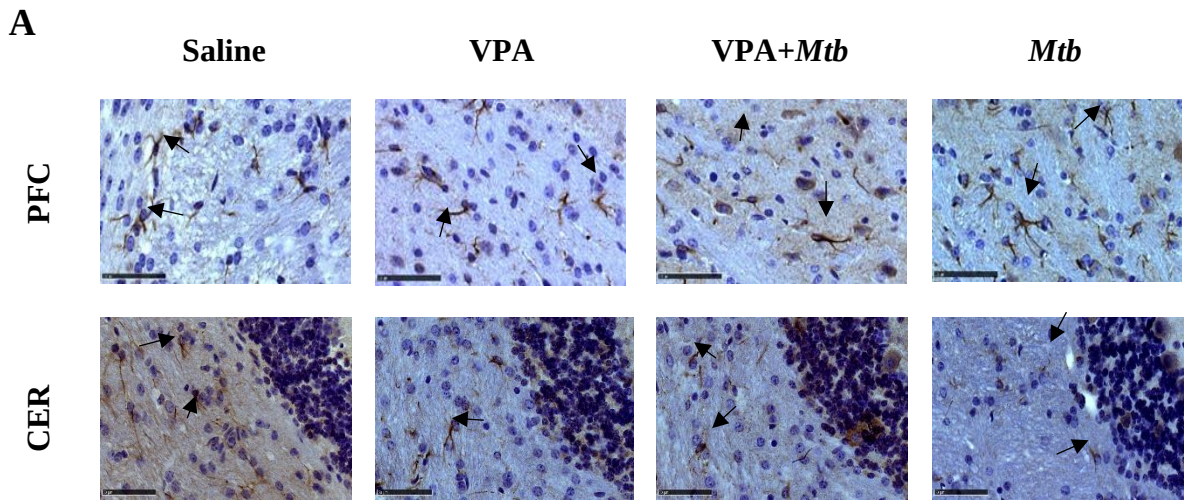


Figure 3. 2: Representative photomicrographs of Iba1$^+$ PFC coronal sections at X63 objective lens. Iba1$^+$ cells are brown with processes extending from the cell body. Resting microglia (black arrows) can be seen especially in the Saline group with a ramified appearance, while *Mtb* group shows a less ramified morphology with enlarged cell bodies (red arrows). Scale bar is 50 μm in all images. 2B: Graph shows the number of Iba1$^+$ cells in the PFC, *Mtb* offspring showed significantly increased Iba$^+$ cells compared to saline offspring ($p < 0.05$). * denotes $p < 0.05$ vs saline. 2C: Graph shows the number of Iba1$^+$ cells in the CER.



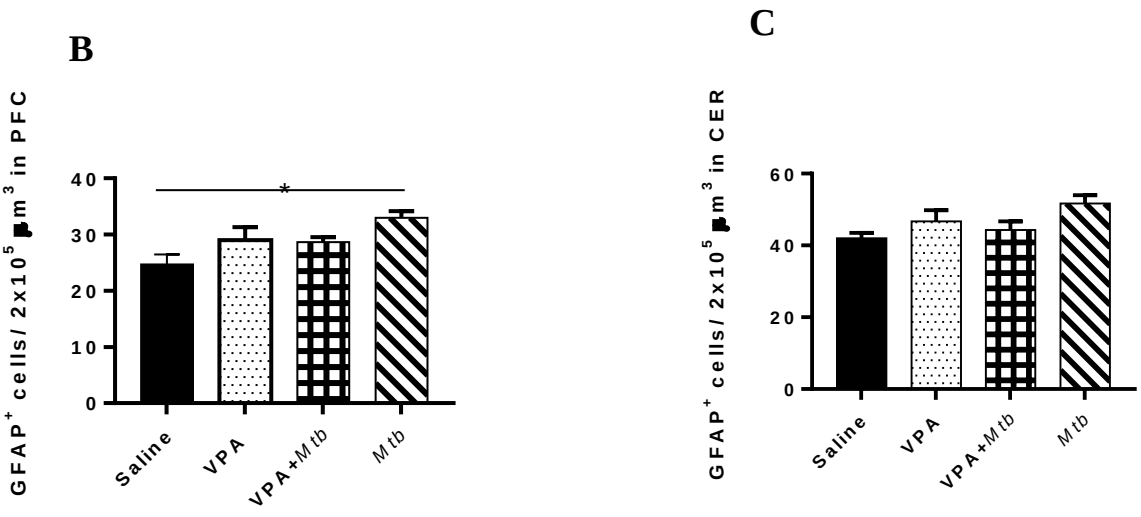


Figure 3. 3: Representative photomicrographs of GFAP$^+$ CER coronal sections and GFAP$^+$ cell populations

Representative images of GFAP$^+$ in the coronal PFC and CER sections at X63 objective. GFAP$^+$ cells are star-shaped and stained brown (black arrows) in the PFC and cerebellum. The scale bar is 50 µm in all images. Graph B shows the number of GFAP$^+$ cells in the PFC and C shows the number of GFAP$^+$ cells in the CER. There was no change in GFAP$^+$ cell numbers in the CER. GFAP$^+$ population was significantly increased in the PFC when compared to Saline ($p<0.05$) and no significant change when compared to VPA. Data is represented as mean ± SEM for 3 animals per group, *$p<0.5$ vs Saline.

3.5 DISCUSSION

The major findings of this study are that prenatal *Mtb* exposure significantly increased the BBB's permeability in the PFC and CER, and it also increased microglia and astrocytes population in the PFC. To the best of our knowledge, this is the first report demonstrating an association of *Mtb* infection with a developing brain in an Autism animal model. Prenatal VPA exposure is a widely used experimental model of ASD as it recapitulates the symptoms observed in humans. The PFC and CER are commonly studied in ASD because their functions are directly linked to ASD symptoms. The PFC controls executive functions of social behaviours, decision making and communication while the CER controls motor skills and higher-order cognition (Donovan & Basson, 2016; Fatemi et al., 2012; Varghese et al., 2017).

In this study, we found that a single exposure to *Mtb* on E 12.5 of pregnancy compromised the integrity of the BBB of offspring. Previously, we observed plasmatic cytokine dysregulation accompanied by synaptic defects, increased repetitive patterns and reduced socialbility in *Mtb*-induced MIA offspring (Manjeese et al., 2021). A suggested mechanism is that *Mtb* triggered maternal immune responses that evoked fetal immune response that influenced the BBB permeability (Coisne & Engelhardt, 2011; Rosenberg, 2009). A classic study demonstrated that inflammatory mediators indeed alter the BBB's function reinforcing the hypothesis that a provoked maternal immune system can influence the BBB function of a developing brain (Saija et al., 1995). Our results also agree with experimental (Kumar & Sharma, 2016) and clinical (Fiorentino et al., 2016) models that reported a compromised BBB associated with ASD pathology.

Our findings also show that prenatal exposure to *Mtb* increases the population of $GFAP^+$ cells/ astrocytes in the PFC region. Related studies reported increased GFAP expression in the CNS of offspring exposed in utero (de Souza et al., 2015; Hao et al., 2010; O'Loughlin et al., 2017). An increase in $GFAP^+$ cell number reflects increased astrocyte reactivity and activation which is a neuropathological feature in ASD subjects (Vargas et al., 2005). An increase in the astrocyte population can lead to increased pro-inflammatory cytokines which can be toxic to neurons causing synaptic dysfunction (Eftekharian et al., 2018). Furthermore, astrocytes assist in the maintenance of the BBB hence dysfunction of astrocytes can influence the BBB's permeability. Further studies are needed to explore cytokine levels in the PFC and CER brain regions of mice exposed to *Mtb* in utero.

Prenatal exposure to *Mtb* increased the microglia population and altered the phenotype to a less ramified and ameboid morphology especially in the PFC of developing brains. Previously, prenatal exposure to Poly I:C (Duchatel et al., 2018; Li et al., 2014) and LPS increased microglia activation in the cortex (Cieslik et al., 2020), PFC and CER (Bronzuoli et al., 2018) regions of the brain. Microglia activation was also reported in the PFC and CER of Autistic brains (Morgan et al., 2010; Vargas et al., 2005). Inflammation triggers microglia morphologic response to a reactive state in which microglia soma are enlarged and processes are retracted (Gumusoglu & Stevens, 2019; Miryam & Goar, 2016). Previously (Mattei et al., 2017), MIA altered the transcriptome of phagocytosis-related genes indicating that indeed maternal

infections influence microglia activity causing chronic neuroinflammation in a developing brain. Future studies should explore the interaction of *Mtb* and immune-related genes.

It is interesting to note that dual prenatal exposure to VPA and *Mtb* rescued microglia activation and reduced the BBB's permeability (Figure 1C and 1D). The mechanism behind the reduced brain injury is not clear but our findings suggest a possible VPA induced antimicrobial effect on *Mtb* that suppresses the Mtb-induced MIA effects on a developing brain. Previously, Rao and co-workers (2018) demonstrated that VPA had antimicrobial effects against *Mtb* indicated by a reduction in *Mtb* colony forming units (CFU). The authors suggested that VPA can achieve this through inhibition of Rv1151c, a deacetylase enzyme encoded by *Mtb.* This disturbs enzyme activity in *Mtb's* central metabolism subsequently disrupting *Mtb* growth (Rao et al., 2018). It is therefore possible that the reduction in neuro-anatomical changes could be a result of VPA antimicrobial effects on *Mtb* that suppress maternal immune activation driven neuro-immune changes in the VPA+*Mtb* group. Another possible mechanism could be an indirect rescue mechanism against VPA-induced effects on the BBB. In a similar study, MIA offered an indirect immune protective effect against chemical induced teratogenesis in mice (Holladay et al., 2000).

3.6 CONCLUSION

Our findings demonstrate that *Mtb*-induced MIA compromises BBB integrity and causes cellular abnormalities in offspring. There is increased PFC specific astrocyte and microglia activation. Microglia and astrocyte dysfunction can affect synapse formation and neuronal networks. Furthermore, microglia and astrocytes are intimately involved in phagocytosis hence our study supports the notion that glial dysfunction and neurodevelopment underlie the symptoms of developmental disorders. These findings highlight the potential of *Mtb* infections in pregnancy to harm a developing brain and as such this may contribute to the understanding of the aetiology of ASD.

3.7 CONFLICT OF INTEREST

The authors have no conflicting interests.

3.8 ACKNOWLEDGEMENTS

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3.10 AUTHOR CONTRIBUTIONS

W. Manjeese carried out the experiments and wrote the manuscript, T. Mpofana, N. E. Mvubu and A. J. C Steyn conceived and designed the study. All authors discussed the results and contributed to the final manuscript.

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Article

Mycobacterium tuberculosis-Induced Maternal Immune Activation Promotes Autism-Like Phenotype in Infected Mice Offspring

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Abstract: The maternal system's exposure to pathogens during pregnancy influences fetal brain development causing a persistent inflammation characterized by elevated pro-inflammatory cytokine levels in offspring. *Mycobacterium tuberculosis* (*Mtb*) is a global pathogen that causes tuberculosis, a pandemic responsible for health and economic burdens. Although it is known that maternal infections increase the risk of autism spectrum disorder (ASD), it is not known whether *Mtb* infection is sufficient to induce ASD associated behaviors, immune dysregulation and altered expression of synaptic regulatory genes. The current study infected pregnant Balb/c mice with *Mtb* H37Rv and valproic acid (VPA) individually and in combination. Plasma cytokine profiles were measured in offspring using the Bio-plex Th17 pro mouse cytokine panel. *Mtb* infection increased plasma interleukin (IL)-6 and IL-17A, while tumor necrosis factor alpha (TNF-α), interferon (IFN)-γ and IL-1β were reduced when compared with saline. *Mtb*-induced maternal immune activation (MIA) offspring displayed increased grooming behavior. The study also revealed dysregulation in gene expression of synaptic molecules in the cerebellum. MIA rescued the VPA-induced effects on self-grooming and social interaction behaviors. Our finding therefore highlights a potential role of *Mtb* as a MIA agent that can potentially contribute to ASD.

Keywords: maternal immune activation; *Mycobacterium tuberculosis*; cytokines; social behaviors; Autism; synaptic genes; restrictive repetitive patterns

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CHAPTER FOUR

Mycobacterium tuberculosis- induced maternal immune activation promotes autism-like phenotye in infected mice offspring

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4 Abstract

The maternal system's exposure to pathogens during pregnancy influences fetal brain development causing a persistent inflammation characterized by elevated pro-inflammatory cytokine levels in offspring. *Mycobacterium tuberculosis* (*Mtb*) is a global pathogen that causes tuberculosis, a pandemic responsible for health and economic burdens. Although it is known that maternal infections increase the risk of autism spectrum disorder (ASD), it is not known whether *Mtb* infection is sufficient to induce ASD associated behaviors, immune dysregulation and altered expression of synaptic regulatory genes. In the present study, pregnant Balb/c mice were infected with *Mtb* H37Rv and valproic acid (VPA) individually and in combination. Plasma cytokine profiles were measured in offspring using the Bio-plex Th17 pro mouse cytokine panel. *Mtb* infection increased plasma interleukin (IL)-6 and IL-17A, while tumor necrosis factor alpha (TNF-α), interferon (IFN)-γ and IL-1β were reduced when compared with saline. Offspring from mothers with *Mtb*-induced maternal immune activation (MIA) displayed increased grooming behavior. The study also revealed dysregulation in gene expression of synaptic molecules in the cerebellum. MIA rescued the VPA-induced effects on self-grooming and social interaction behaviors. Our finding therefore highlights a potential role of *Mtb* as a MIA agent that can potentially contribute to ASD.

Keywords: maternal immune activation; *Mycobacterium tuberculosis*; cytokines; social behaviors; Autism; synaptic genes; restrictive repetitive patterns

4.1 Introduction

Autism spectrum disorder (ASD) is a group of neurodevelopmental disorders (NDDs) caused by a complex interaction between genes and prenatal environmental factors. It is characterized by repetitive behaviors, communication deficits and lack of social interaction skills [1]. Maternal immune activation (MIA) caused by infections in pregnancy are an important environmental risk factor that can influence the developing brain. Second trimester respiratory infections and bacterial infections increase the risk of NDDs such as ASD [2, 3]. The maternal system strikes a balance between immune tolerance of the growing fetus and immune response against invading pathogens [4]. Maternal immune response to infection triggers production of inflammatory cytokines which can traverse the fetal–placental barrier inducing fetal inflammation that disrupts brain development. This inflammation persists through postnatal life as evidenced by elevated pro-inflammatory cytokines in the CNS and serum/plasma of ASD patients [5, 6]. Maternal infections of Poly I:C, LPS, influenza, *C. rectus* and *L. monocytogenes* alter cytokine levels in the placenta, amniotic fluid, fetal brain and plasma [4, 7-10]. Cytokines mediate neuro-immune communication, and they also regulate neurogenesis and synaptic plasticity; hence, an imbalance in cytokine levels during development can be detrimental to the developing brain.

Several genes have been implicated in ASD and they are known to converge on biological pathways that are involved in the regulation of neuronal activity [11, 12]. These genes include SH3 and multiple ankyrin repeat domains 3 (*SHANK3*), neurexins (*NRXN*s) and neuroligins (*NLGNs*) which encode proteins involved in synaptic transmission and plasticity [13]. Neurexins are pre-synaptic cell adhesion molecules encoded by *NRXN*1, 2 and 3 genes, while *NLGN*s are post-synaptic cell adhesion molecules encoded by *NLGN*1, 2, 3 and 4 [13, 14]. *SHANK3*, *NRXN1*, *NRXN2*, NLGN1 and *NLGN2* are candidate NDD susceptibility genes. Mutations and deletions in these genes are implicated in the pathophysiology of ASD [15-17]. *NLGN1* and *NLGN2* are expressed on excitatory and inhibitory synapses, respectively, while *NLGN3* and 4 are found in both synapses. Changes in expression patterns of *NRXNs* and *NLGNs* affect the synapse excitation/inhibitory (E/I) balance leading to altered information processing.

Valproic acid (VPA) is an anticonvulsant and mood stabilizer that is used to treat epilepsy and depression disorders [18]. Treating pregnant women with VPA is associated with higher incidence of ASD and physical deformities in children [19, 20]. It has been extensively shown

that gestational VPA exposure of mice on E12.5 induces ASD-traits in offspring, which include social deficits, repetitive behaviors, communication challenges and neuronal damage [21-24]. The VPA model is a well-established tool for studying ASD research, owing to its ability to recapitulate human symptoms of ASD.

In this study we used *Mycobacterium tuberculosis (Mtb)* H37Rv to induce MIA in pregnant mice. *Mtb* is a global pathogen that is common in pregnant women, and children born to them are at an increased risk of having low birth weight, infant mortality and prematurity [25, 26]. *Mtb* causes tuberculosis, a highly prevalent disease in Africa that causes social and economic challenges. Conversely, neurodevelopmental disorders are becoming increasingly common even though they are poorly understood and underdiagnosed in Africa [27]. No study has evaluated maternal *Mtb* infection and its potential influence on ASD-associated genes and on the immune systems of infected mice's offspring. This study evaluates *Mtb* infection and its possible link to ASD-like phenotype in the offspring of infected mice.

4.2 Materials and Methods

4.2.1. Animals

Balb/c mice at 8 weeks of age were obtained from Africa Health Research Institute (AHRI), South Africa. The study followed the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines, and animals were handled according to the principles of National Institutes of Animal Care and Use of Laboratory Animals of the National Academy of Sciences. Females were group-housed (5 per group) for 5 days to synchronize their estrous cycle. Bedding from the male cages was added to the female cages to induce the estrous phase. The mice were mated on a ratio of 1:1 (male to female) overnight. Vaginal plugs were checked early morning, males were immediately removed upon confirmation of vaginal plug presence and this confirmation marked embryonic day 0.5 (E0.5). In our pilot study, administration of 500 mg/kg of VPA and *Mtb* together induced complete fetal resorption; hence, for this study we lowered the VPA dose to 350 mg/kg to increase fetal survival rate in the VPA + *Mtb* group. The VPA group was the positive control of the study, and the VPA + *Mtb* group was created to assess the effects of dual exposure to two ''environmental insults''. Twenty pregnant mice were randomly assigned to 4 groups (5 per group), and the treatments were administered on E12.5, as shown in Table 4.1 and Figure 4.1a. Only 3 animals per group had successful pregnancies; hence, litters were culled to 12 per group and no more than 4 animals per litter (2 per sex). All animals were raised in a biosafety level 3 laboratory (BSL3). Each animal raised

her litter under 12 h light/dark cycle and a constant supply of food and water. Litters were weaned at postnatal day (PND) 21, at which time males and females were separated but grouped according to treatments. Five animals were randomly picked from all the litters for cytokine and gene expression profiles.

Table 4. 1: Groups and treatments in the study.

Group	Treatment and Dosage	Route of Administration
Saline	0.2 mL of saline	Single i.p.
VPA	0.2 mL of 500 mg/kg valproic acid	Single i.p.
VPA + *Mtb*	0.2 mL of 350 mg/kg valproic acid + 10mL of 1×10^8 CFU ($OD_{600} = 1$)	Single i.p. + aerosol infection
Mtb	10 mL of 1×10^8 CFU ($OD_{600} = 1$)	Single aerosol infection

OD—optical density, CFU—colony forming units, i.p.—intraperitoneal.

4.2.3 *Mtb* Infection

Ten pregnant mice from the *Mtb* and VPA + *Mtb* groups were placed in a Glas-col infection chamber (Terre Haute, IN, USA) with 10 mL of 1×10^8 CFU ($OD_{600} = 1$) *Mtb* H37Rv inoculum expelled into the nebulizer using a syringe. The Glas-col machine cycle was set to the following conditions: preheat (15 min), nebulization (30 min), cloud decay (30 min), decontamination (15 min), cool down (10 min). The mice were carefully removed from the machine using forceps and placed into cages with bedding, food and water.

4.2.4 Social Interaction Test

This test was performed on PND 35 as previously described [28]. All procedures were conducted in a biosafety level 3 cabinet. Briefly, a 40 × 15 cm transparent Perspex box divided in three compartments with the following measurements: sides (15 × 15 cm); central (10 × 15 cm). The central compartment was connected to the side compartments by 7.5 cm sliding doors. A camera (Microsoft Lifecam Studio, Beijing, China) was placed above the apparatus to record all the areas of the three-chamber box during the test. The test was conducted in 3 phases: (i) Habituation phase—each mouse was allowed acclimatize and freely explore the chambers for 5 min; (ii) Social stimulus phase—an unfamiliar sex and age matched mouse was placed in a

cylinder in 1 of the side chambers; the other side chamber contained an identical cylinder that was empty; and (iii) Social novelty phase—A novel sex- and age-matched mouse was placed in a cylinder in 1 of the side chambers while the other side chamber housed the familiar mouse that was encountered during the social stimulus phase (Figure 4.1d,e). During each phase, the test animal was placed in the center compartment and doors were opened to test the animal's preferred compartment. The total amount of time spent in each compartment was analyzed and scored using BORIS software.

4.2.5 Self-Grooming/Repetitive Behaviors

To assess behaviors in the repetitive domain, the total time spent grooming (scratching and licking) was observed during the habituation phase of the three-chamber social interaction test. Five-minute videos were recorded, and the cumulative time spent grooming during the sessions were later scored by an investigator blinded to the treatments using Behavioral Observation Research Interactive Software (BORIS, v 7.7.4. Viale Mattioli 25, TO, Italy).

4.2.6 Blood Collection

On PND 35, balb/c mice were humanely sacrificed using halothane overdose; thereafter, cardiac puncture was performed to collect blood into EDTA-coated 1 mL tubes. The tubes were left standing for 30 min before centrifuging at 10,000 rpm at room temperature for 10 min. Plasma samples were aliquoted and flash frozen on dry ice then stored at −80 °C until analyzed.

4.2.7 Brain Tissue Collection

On PND 35, five animals per group were slightly anaesthetized and decapitated using a guillotine. The brain tissue was carefully removed from the skull on ice, and the whole cerebellum was dissected and immediately frozen on dry ice then stored at −80 °C until analysis.

4.2.8 Relative mRNA Expression in the Cerebellum (quantitative PCR)

Total mRNA was extracted from whole cerebellar tissues according to manufacturer's instructions using the Zymo Research Quick-RNA Miniprep kit (Zymo Research, Irvine, CA, USA). Briefly, cerebellar tissues were mechanically homogenized in RNA lysis buffer, and the homogenate was centrifuged at 10,000× g for 30 s. The flow through was diluted (1:1) with ethanol and centrifuged on a Zymo-Spin IIICG column which was then treated with DNase 1 to digest genomic DNA. After recommended washing steps and centrifugation, RNA was eluted in 50 μL DNAse/RNAse free water. A nanodrop spectrophotometer (Thermo scientific

Nanodrop 1000, Waltham, MA, USA) was used to assess the purity and concentration of the extracted RNA. A total of 1 μg of RNA was reverse transcribed into cDNA according to the manufacturer's protocol using the iScript cDNA synthesis kit (Bio-Rad Laboratory Pty, Ltd., Hercules, CA, USA). All primers were designed using Primer-Blast designing program (http://www.ncbi.nlm.nih.gov/tools/primer-blast, accessed on 25 September 2019), then validated and optimized prior to the experiment. Real-time polymerase chain reaction (qPCR) conditions used were as follows: initial denaturation at 95 °C for 3 min followed by 40 cycles of polymerase activation and denaturation at 95 °C for 15 s, annealing/extension and plate read at 58 °C with a single fluorescent measurement. The housekeeping gene used in the analysis was glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the fluorescent dye utilized in the analysis was SYBR Green 1 (SSO Advanced Universal SYBR GR. SUPRMIX, Bio-Rad Laboratory, Hercules, CA, USA). The oligonucleotide primer sequences for the cerebellar synaptic genes investigated are shown in Table 4.2:

Table 4. 2: Oligonucleotide primer sequences.

Genes	Forward Primer	Reverse Primer	Amplicon Size
NRXN1	AGGTTCCGTGTGTCACTTGC	TCCTGTGTGTGTCTGGGGAT	452
NRXN2	GCAGGGATTGGACACGCTAT	GAACTGTGACTGCCTACCCC	464
NLGN1	GGGGATGAGGTTCCCTATGT	GGTTGGGTTTGGTATGGATG	190
NLGN2	TTTCCGTCCTCCCCATCCAAT	TAGGAGCCGCCGTGTAGAAA	923
SHANK3	GGCCATTTCAACAGAAGCCC	TGCGCCTTCGATCTCATGG	119
GAPDH	CCCTTAAGAGGGATGCTGCC	ACTGTGCCGTTGAATTTGCC	118

4.2.9 Cytokine Analysis

Cytokine analysis was conducted using a Bio-plex Pro™ Mouse Th17 Cytokine magnetic bead-based kit (Bio-Rad Laboratory, Hercules, CA, USA. Lot# 64313813). The kit was used to measure plasma levels of interleukin 6 (IL-6), interferon gamma (IFN-γ), interleukin 10 (IL-10), interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α) and interleukin 17A (IL-17A). All steps were conducted according to the manufacturer's instructions. Briefly, samples were diluted (1:4) in diluent provided by manufacturer. Antibody coupled beads were

incubated with 50 µL of diluted plasma. After the recommended washes, biotinylated detection antibody was added to the beads and incubated. Streptavidin-PE was added to the beads, a Bioplex 200 System (Biorad) was used to measure fluorescent signals, and data were analyzed using Bio-plex Manager 5 Software. A 5-parameter model was used to calculate cytokine concentrations. Unknown concentrations were calculated based on a standard curve of known concentrations provided by the manufacturer. Cytokine concentrations below the limit of detection (LOD) were calculated as LOD/2 for statistics. Cytokine concentrations are expressed as pg/mL.

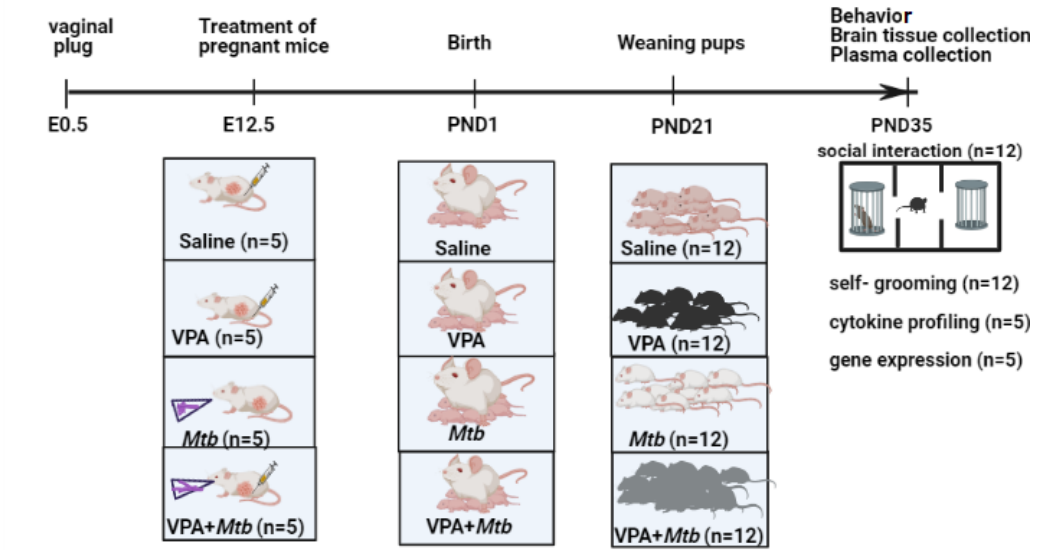
4.3 Statistical Analysis

Results were analyzed using GraphPad Prism software (version 7). Shapiro and Wilk tests for normality were applied to determine data distribution. The data were analyzed by one-way or two-way ANOVA followed by a Dunnett's or Bonferroni multiple comparison post hoc test as appropriate to determine differences between the groups which were considered statistically significant when $p < 0.05$. Results are presented as the mean + standard error of the mean (mean + SEM) for $n = 12$ in behavioral tests and $n = 5$ for cytokine and gene expression analyses.

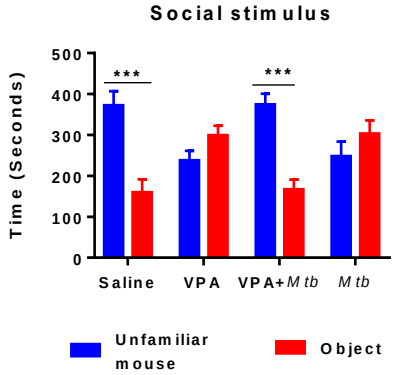
4.4 Results

4.4.1 The Effect of Prenatal *Mtb* Infection on Offspring Social Behaviors

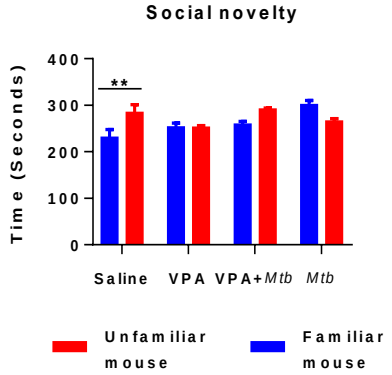
The three-chamber social interaction test measured the sociability of animals in the social stimulus phase, where propensity to spend time with another mouse was compared with time spent with an object over a 10 min period. Although not statistically significant ($p > 0.05$), *Mtb* offspring spent more time with an object than a mouse (Figure 4.1b). Interestingly, mice born to VPA + *Mtb* treated mothers showed a preference for the unfamiliar mouse over the object ($p > 0.0001$), which indicated normal social behavior. We measured social novelty as the preference between a familiar mouse (previously encountered animal) and unfamiliar mouse. Mice are social animals that normally would show a preference for novel social experiences. In this phase, two-way ANOVA showed that there was no treatment effect on the choice between familiar and unfamiliar mouse; however, *Mtb* offspring were inclined to interact with the familiar mouse more than with the unfamiliar mouse (Figure 4.1c). Although not statistically significant ($p > 0.05$), mice born to VPA + *Mtb* treated mothers preferred the unfamiliar mouse to the familiar mouse.


vaginal plug
Treatment of pregnant mice
Birth
Weaning pups
Behavior
Brain tissue collection
Plasma collection
E0.5
E12.5
PND1
PND21
PND35
social interaction (n=12)
Saline (n=5)
VPA (n=5)
Mtb (n=5)
VPA+Mtb (n=5)
Saline
VPA
Mtb
VPA+Mtb
Saline (n=12)
VPA (n=12)
Mtb (n=12)
VPA+Mtb (n=12)
self- grooming (n=12)
cytokine profiling (n=5)
gene expression (n=5)

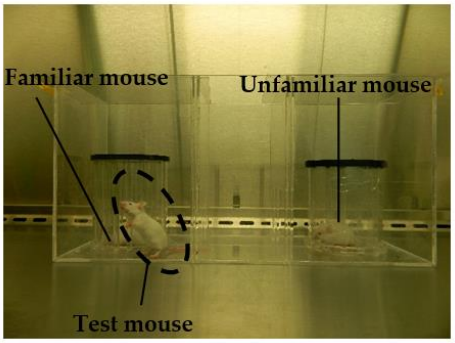
(a)


Social stimulus
Time (Seconds)
500
400
300
200
100
0
***
***
Saline
VPA
VPA+Mtb
Mtb
Unfamiliar mouse
Object

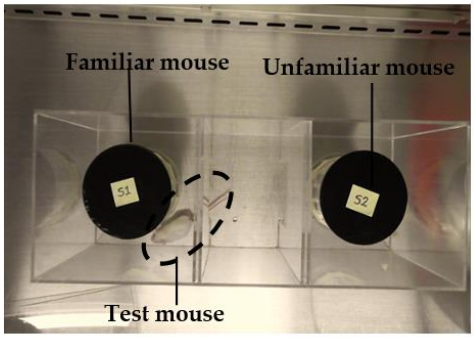
(b)


Social novelty
Time (Seconds)
400
300
200
100
0
**
Saline
VPA
VPA+Mtb
Mtb
Unfamiliar mouse
Familiar mouse

(c)


Familiar mouse
Unfamiliar mouse
Test mouse

(d)


Familiar mouse
Unfamiliar mouse
Test mouse

(e)

Figure 4. 1 (**a**) Study design; (**b**) Social stimulus—Graph shows the average time spent with a familiar mouse compared with object. Unlike the saline offspring ($p < 0.0001$), *Mycobacterium tuberculosis (Mtb)* offspring spent more time with an object than a mouse. Valproic acid (VPA) + *Mtb* offspring spent significantly more time ($p < 0.0001$) with an unfamiliar mouse than the object. *** denotes $p< 0.0001$ (two-way ANOVA); (**c**) Social novelty—Graph shows the average time (±SEM) spent with an unfamiliar mouse compared with a familiar mouse in a 10 min phase. Unlike the saline offspring ($p < 0.001$), *Mtb* and VPA+*Mtb* offspring failed to choose the unfamiliar mouse. ** denotes $p < 0.001$ ($n = 12$ per group, two-way ANOVA); detailed statistics information is available in Appendix B, Tables A3 and A4; (**d**) Representative images of the side-view and (**e**) aerial view of social novelty phase during the three-chamber social interaction test.

4.4.2 *Mtb* Infection Increases Self-Grooming Behavior of offspring

The duration of whole-body grooming was monitored and recorded. An unusually long duration of a grooming pattern of the whole body was observed in this study. There was a significant increase ($p < 0.0001$ vs. saline; $p < 0.001$ vs. VPA) in grooming behaviors of the *Mtb* offspring (Figure 4.2). Interestingly, *Mtb* on its own was sufficient to increase self-grooming ($p < 0.001$ vs. VPA + *Mtb*) and dual exposure to VPA and *Mtb* did not increase the grooming behavior but restored it to the negative control (saline) frequency, suggesting a possible rescue mechanism of the grooming behavior.

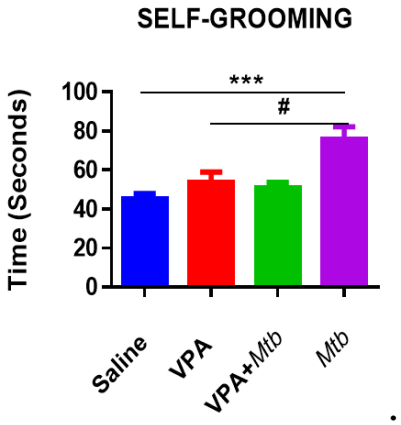
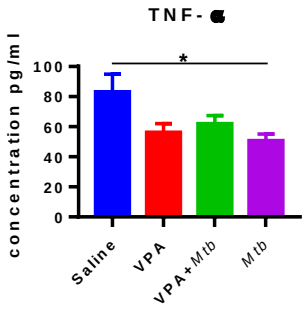


Figure 4. 2: Graph showing total time spent while animals groomed themselves over 5 min ($n = 12$ per group). Prenatal exposure to *Mtb* significantly increased grooming when compared with saline and VPA, $p < 0.0001$ and $p < 0.001$, respectively. *** $p < 0.0001$ vs. saline and #

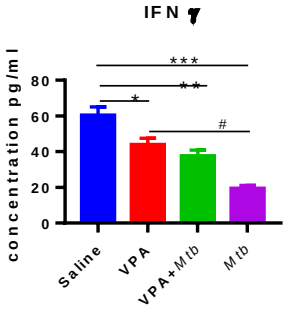
$p < 0.001$ (one-way ANOVA). Detailed statistics information is available in Appendix B, Table A5.

4.4.3 Prenatal *Mtb* Infection Causes Immune Dysregulation in Offspring

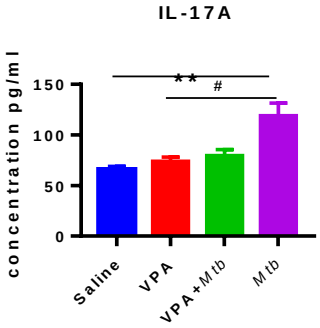
The expression and subsequent production of IL-6, IL-1β, IFN-γ, IL-17A, TNF-α and IL-10 was measured in the plasma of MIA offspring. We found that IL-6 and IL-17A were significantly increased in mice that were prenatally exposed to *Mtb* infection ($p < 0.001$; $p < 0.05$ vs. saline respectively, Figure 4.3). Offspring from *Mtb* infected mice had significantly reduced TNF-α, IFN-γ and IL-1β ($p < 0.05$; $p < 0.0001$; $p < 0.001$, respectively) compared with saline exposed offspring. It was also observed that IFN-γ was reduced ($p < 0.001$) and IL-17A significantly elevated ($p < 0.001$) in the plasma of *Mtb* pups compared with VPA pups (positive control). *Mtb* infection had no effect on circulating IL-10 levels in the offspring. The VPA + *Mtb* offspring had significantly reduced plasma IFN-γ and IL-1β levels ($p < 0.001$) compared with saline offspring. *Mtb* on its own was sufficient to induce the elevation of IL-17A ($p < 0.05$) and IL-16 ($p < 0.0001$) compared with VPA and *Mtb* combined (VPA + *Mtb*) in the plasma of offspring (Appendix A). Taken together, there was a persistent dysregulation and imbalance in pro-inflammatory and anti-inflammatory cytokines in the plasma of MIA offspring.



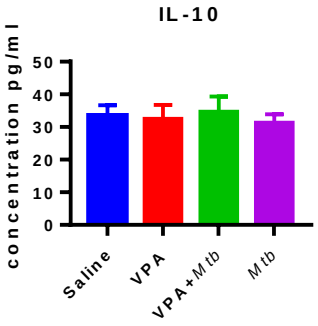
(**a**)



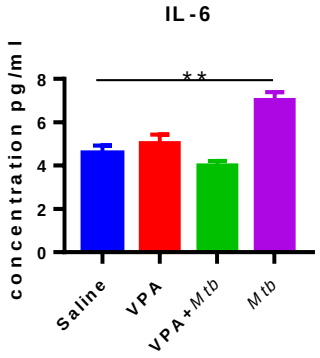
(**b**)



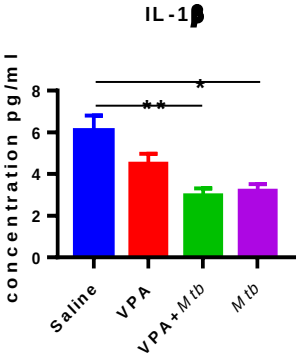
(**c**)



(**d**)



(**e**)



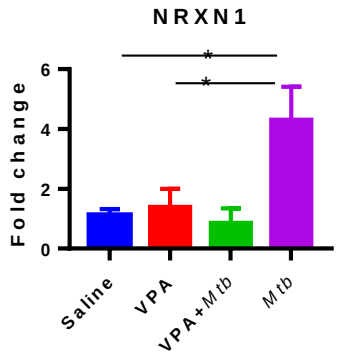
(**f**)

Figure 4. 3: Graph showing concentrations of plasma cytokines at postnatal day (PND) 35 for **(a)** TNF-α **(b)** IFNγ **(c)** IL-17A **(d)** IL-10 **(e)** IL-6 **(f)** IL-1β. IL-6 and IL-17A plasma cytokine concentrations are heightened in *Mtb* offspring and restored to negative control levels in VPA + *Mtb* offspring. IL-1β, IFN-γ and TNF-α productions are reduced: # $p < 0.001$ vs. VPA, * $p < 0.05$, ** $p < 0.001$ and *** $p < 0.0001$ vs. saline; $n = 5$ animals per group (one-way ANOVA). Detailed statistics are available in Appendix B, Table A6.

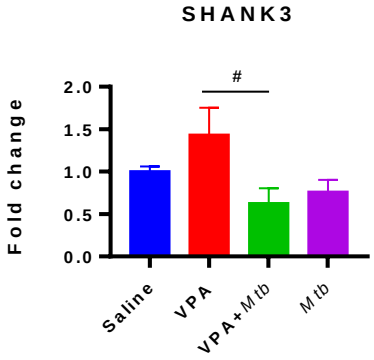
4.4.4 *Mtb* Infection Dysregulates Gene Expression of Synaptic Molecules in Offspring Cerebellum

The expression of *NRXN1*, *SHANK3*, *NLGN1*, *NRXN2* and *NLGN2* genes was measured relative to the *GAPDH* gene (Figure 4.4). *NRXN1* and *NLGN1* genes were upregulated ($p < 0.05$ vs. saline) in the cerebellum of mice exposed to *Mtb* in utero. Exposure to VPA+*Mtb* upregulated *NRXN2* expression ($p < 0.05$ vs. saline) and downregulated *SHANK3* and *NLGN1*

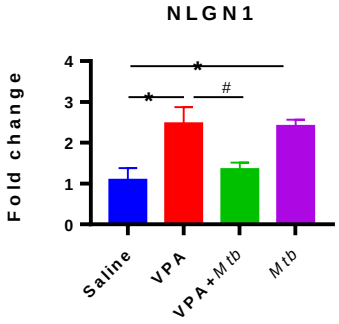
expression ($p < 0.05$ vs. VPA). *Mtb* caused a 2-fold increase in *NRXN1* expression ($p < 0.05$) compared with the positive control (VPA), but it had no effect on the expression of *NRXN2* and *NLGN2* in the cerebellum of infected mice offspring. *Mtb* without VPA significantly upregulated NRXN1 ($p < 0.001$) and NLGN1 ($p < 0.05$) expression compared with VPA+*Mtb* (Appendix B, Table A2).



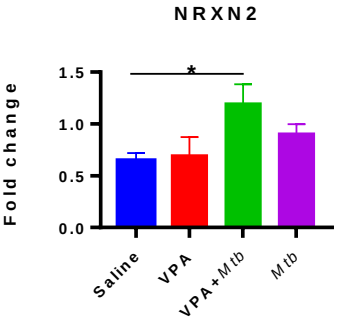
(**a**)



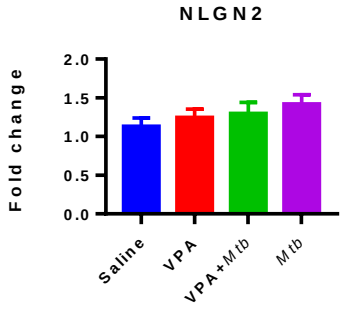
(**b**)



(**c**)



(**d**)



(**e**)

Figure 4. 4: Graphs showing relative fold change in gene expression of **(a)** *NRXN1*, **(b)** NLGN1, **(c)** *NRXN2*, **(d)** *NLGN2* and **(e)** *SHANK3*. *NRXN1* and *NLGN1* are highly expressed in *Mtb* pups ($p < 0.05$) and *NRXN1*, *SHANK3*, *NLGN1* gene expression levels are restored in VPA+*Mtb* offspring. # $p < 0.05$ vs. VPA and * $p < 0.05$ vs. saline, $n = 5$ animals per group (one-way ANOVA). Detailed statistics are available in Appendix B, Table A7.

4.5 Discussion

Peripheral immune dysfunction is a key feature in ASD and MIA offspring. MIA increases the levels of pro-inflammatory cytokines (IL-6, TNF-α and IL-1β) that induce inflammation in the growing fetus; failure of negative feedback control leads to chronic inflammation that persists into the postnatal period. To the best of our knowledge, this is the first report that associates *Mtb* infection with ASD-like phenotype. Our results show an increase in plasma IL-6 and IL-17A following *Mtb*-induced MIA. IL-6 is a key mediator of nervous and immune system cross talk, owing to its ability to cross the blood–brain barrier (BBB). IL-6 levels are elevated in ASD patients [6]. IL-6 is involved in TH17 differentiation, leading to production of IL-17; hence, it is expected that an increase in IL-6 is accompanied by an increase in IL-17A as well. *Mtb* infected mice offspring also showed decreased social skills as revealed by their preference for an object to a mouse (Figure 4.1b). Furthermore, these animals failed to choose between a familiar mouse and an unfamiliar mouse. Consistent with our findings are impaired social behaviors in Poly I:C-induced MIA offspring [29, 30]. Recent findings indicate that IL-17A is a mediator of MIA that reduces social interaction and induces repetitive behaviors in MIA offspring [4, 31], suggesting that the elevated plasma IL-17A levels in *Mtb*-induced MIA offspring most probably evoked repetitive behaviors and a lack of social skills in our study. Additionally, elevated plasma IL-6 levels are associated with ASD-like behaviors. Injecting

pregnant mice with IL-6 was sufficient to induce ASD-like behaviors, which were rescued by anti-IL-6 antibodies [32]. Taken together, circulating IL-6 and IL-17A somehow influence ASD-like behaviors in *Mtb*-induced MIA offspring.

Furthermore, our results indicate a significant reduction in plasma IFN-γ, IL-1β and TNF-α levels in *Mtb* offspring. This contradicts studies [33-35] that reported heightened levels of these pro-inflammatory cytokines in MIA offspring. This could be a result of differing immune response to Poly I:C, LPS and *Mtb*. It is also possible that *Mtb* infection is insufficient to evoke a full spectrum of ASD-associated immune changes that persists into the postnatal life of the offspring. The reduced production of pro-inflammatory cytokines could be an effect of the anti-inflammatory role of IL-6 [36, 37] and IL-17A; hence, they can suppress the release of pro-inflammatory cytokines IL-1β and TNF-α [38] while elevating IL-10 to counter inflammation in the immune system. However, there was no change in IL-10 as expected, suggesting a failure of anti-inflammatory regulatory activity. This reduced production in IL-10 is supported by previous reports that did not find a change in IL-10 levels in ASD patients [39, 40] and MIA offspring [31].

Finally, we assessed the cerebellar expression profile of genes encoding synaptic molecules that are implicated in ASD pathophysiology. The cerebellum is involved in sociability, emotions and motor coordination; hence, the cerebellum is thought to be involved in developmental disorders characterized by altered social patterns and repetitive behaviors [41]. Our results show that gestational *Mtb* infection does not affect the cerebellar expression of *NLGN2*, *NRXN2* and *SHANK3* but upregulates the expression of *NRXN1* and *NLGN1* genes in offspring. Previous findings indicate that increased expression of *NRXN*s reduces GABAergic neurotransmission [42], while overexpression of NLGN1 increases glutamatergic neurotransmission [43], suggesting an excitation/inhibition imbalance in the synapse that can influence behavior. Increased repetitive behaviors were also observed in other MIA offspring [4, 29]. The mechanism underlying repetitive behaviors is not clear; however, they are thought to arise from a combination of genetic and environmental factors affecting the cerebellum development [44, 45].

It is interesting and noteworthy that pups born to mothers treated with VPA and *Mtb* (VPA + *Mtb*) did not present systemic inflammation and behavioral deficits; more so, altered cerebellar *NRXN1* expression, social stimulus and self-grooming behaviors were restored to normal levels

(saline). Our results are consistent with previous studies in which birth defects were reduced and altered gene expression profiles restored by MIA in teratogen-exposed rodents [46, 47]. Although the mechanism behind the rescue mechanism of MIA in rodents exposed to teratogens is not well understood, it has been suggested that MIA can protect against teratogenic effects of VPA through the activity of maternal cytokines that normalize proliferation events and reduce developmental disorders [48, 49].

4.6 Conclusions

In conclusion, this study provides new evidence that *Mtb* infection in pregnancy is sufficient to influence brain development such that offspring exhibit increased self-grooming, enhanced systemic inflammation, altered gene expression at synapses and impaired social interaction, which reflect ASD core features. Overall, our study provides new insights and roles of a global pathogen in the MIA pathway implicated in the etiology of ASD. Future studies should focus on measuring maternal cytokines during pregnancy as well as in the brain tissue of *Mtb*-induced MIA offspring.

4.7 Author Contributions

Conceptualization, W.M. and T.M.; methodology, N.E.M., T.M. and W.M.; validation, N.E.M., A.J.C.S. and T.M.; formal analysis, W.M.; resources, A.J.C.S., N.E.M. and T.M.; writing—original draft preparation, W.M.; writing—review and editing, A.J.C.S., N.E.M. and T.M.; funding acquisition, A.J.C.S, N.E.M. and T.M. All authors have read and agreed to the published version of the manuscript.

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4.9 Institutional Review Board Statement

The study was approved by the Animal Research Ethics Committee of University of KwaZulu Natal, South Africa (ethics registration number AREC/076/018M).

4.10 Informed Consent Statement

Not applicable

4.11 Data Availability Statement

Data will be made available on request

4.12 Acknowledgments

We would like to acknowledge the Africa Health Research Institute (AHRI) for providing access to BSL3 animal facilities and the Discipline of Microbiology, College of Agriculture and Engineering University of KwaZulu-Natal, for providing access to the molecular laboratory. The authors also thank the Africa Health Research Institute's staff (K. Thambu, K. Beepraj, P. Ramkalawon and K. Khuluse) for the technical assistance.

4.13 Conflicts of Interest

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Appendix A

Table 1. Plasma cytokine concentrations

Cytokine	*Mtb* Concentration (pg/mL)	VPA+*Mtb* Concentration (pg/mL)
TNF-α	50.7 + 11.21 *	61.9 + 11.21
IFN-γ	19.6 + 4.78 ***	37.7 + 4.78
IL-17A	119 + 10.35 [a,]#	79.7 + 10.35
Il-10	31.3 + 5.35	34.6 + 5.35
Il-6	7.03 + 0.481 [b,]**	4.00 + 0.481
Il-1β	3.20 + 0.701 *	2.98 + 0.701 **

[a] $p < 0.05$; [b] $p < 0.0001$ vs. VPA+*Mtb*; * $p < 0.5$; ** $p < 0.001$; *** $p < 0.0001$ vs. saline; # $p < 0.05$.

Table 2. Gene expression profiles of synaptic molecules.

Gene	*Mtb*	VPA+*Mtb*

	Fold Change	Fold Change
NRXN1	4.31 + 0.971 *,[a]	0.855 + 0.971 #
SHANK3	0.764 + 0.278	0.629 + 0.278 #
NLGN1	2.42 + 0.376 *,[b]	1.35 + 0.376
NRXN2	0.908 + 0.197	1.19 + 0.197 *
NLGN2	1.42 + 0.168	1.30 + 0.168

[b] $p < 0.05$, [a] $p < 0.001$ vs. VPA+*Mtb*; * $p < 0.05$ vs. saline, # $p < 0.05$ vs. VPA.

Appendix B

Table A3. ANOVA results for Social stimulus.

ANOVA	**DF**	**F (DFn,DFd)**	***p* Value**
Interaction	3	F (3, 88) = 14.12	$p < 0.0001$
Row FActor	3	F (3, 88) = 0.04163	$p = 0.9886$
Column Factor	1	F (1, 88) = 13.61	$p = 0.0004$
Residual	88		

Table A4. ANOVA results for Social novelty.

ANOVA	**DF**	**F (DFn,DFd)**	***p* Value**
Interaction	3	F (3, 88) = 7.405	$p = 0.0002$
Row FActor	3	F (3, 88) = 4.065	$p = 0.0094$
Column Factor	1	F (1, 88) = 3.146	$P = 0{,}0796$

Table A5. ANOVA results for Self-grooming.

ANOVA	**DF**	**F (DFn,DFd)**	***p* Value**
Treatment (between columns)	3	F (3, 44) = 11.29	$p < 0.0001$

Table A6. ANOVA results for Cytokine concentration

CYTOKINES	**DF**	**F (DFn,DFd)**	***p* Value**
TNF-α	3	F (3, 16) = 3.727	$p = 0.0332$
IFN-γ	3	F (3, 16) = 24.97	$p < 0.0001$
IL-17A	3	F (3, 16) = 10.47	$p = 0.0005$
IL-10	3	F (3, 16) = 0.1383	$p = 0.9356$
Il-6	3	F (3, 16) = 15.01	$p < 0.0001$
Il-1β	3	F (3, 16) = 8.232	$p = 0.0015$

Table A7. ANOVA results for Gene expression

GENE	**DF**	**F (DFn,DFd)**	***p* Value**
NRXN1	3	F (3, 16) = 5.51	$p = 0.0086$
SHANK3	3	F (3, 16) = 3.258	$p = 0.0492$
NLGN1	3	F (3, 16) = 7.215	$p = 0.0028$
NRXN2	3	F (3, 16) = 3.29	$p = 0.0478$
NLGN2	3	F (3, 16) = 1.013	$p = 0.4127$

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CHAPTER FIVE
SYNTHESIS

Tuberculosis (TB) is a global pandemic with a high prevalence in Africa. South Africa is a high TB burden country with one in every 200 South Africans having TB and adult women account for 37% of TB cases in the country [1]. While both sexes and all ages can contract TB, the consequences are detrimental for pregnant women. TB in pregnant women is life threatening to the unborn child and if the child survives, the development is often affected [2]. Moreover, the brain development may also be impaired as maternal infections in pregnancy disturb brain development leading to neurodevelopmental diseases like Autism Spectrum Disorder (ASD). However, the molecular mechanisms leading from maternal infections in pregnancy to ASD pathology remain unclear. It is well established in the literature that pre-natal exposure to infection is a risk factor for NDDs like ASD (Chapter 1). While many studies have made association between gestational respiratory infections and NDDs, none has focused on *Mycobacterium tuberculosis Mtb* and its possible contribution to NDDs. We observed behavioural, molecular and cellular alterations associated with ASD in mice following gestational *Mtb* exposure. This study provides evidence of the effects of *Mtb*-induced MIA on the developing brain in relation to ASD.

Developmental delay is a major hallmark of most NDDs. Delayed physical development and maturation is evident in ASD, therefore the developmental milestones are an attractive target in understanding ASD pathogenesis. Eye-opening and weight gain are typical developmental milestones in rodent models of ASD, hence, eye-opening pattern (PND12-16) and body weight (PND7-28) were monitored. In our study, *Mtb* infection was not vertically transmitted but we observed adverse effects on offspring health as demonstrated by reduced developmental weight and delayed eye-opening patterns in the *Mtb* offspring compared to Saline offspring (negative control). Eye opening is important for glutamatergic synapse maturation [3] hence *Mtb* appears to affect neurodevelopmental processes. Along with increases in fetal resorption, there was reduction in litter size and pregnancy success in the *Mtb* group. This suggests that *Mtb* infection in pregnancy restricts fetal growth and delays development in early life. These findings corroborate the results of Murray et al. (2019), who similarly reported reduced body weight in poly I:C-induced MIA offspring [4]. It is interesting to note that delayed eye-opening is somehow rescued when a mild VPA dose and *Mtb* are administered together. This suggest a VPA induced antimicrobial effect on Mtb-induced MIA effects on eye opening patterns.

Another mechanism could be the maternal inflammatory response to *Mtb* that rescues VPA-induced developmental delays.

Experimental studies have linked maternal immune activation (MIA) in the pathogenesis of ASD with neuroinflammatory events in the developing brain and long-lasting changes in immune system activity, MIA is therefore believed to induce changes that are involved in brain malformation and behavioural alterations in offspring. Altered BBB permeability is involved in events leading to neuroinflammation in ASD patients [5]. To assess the influence of *Mtb*-induced MIA on the BBB's function and neuroinflammation, the concentration of Evans blue dye, expression of IBA1 (microglia marker) and GFAP (astrocyte marker) in the PFC and cerebellum regions of offspring was measured. Our data demonstrated that prenatal *Mtb* exposure increases the BBB permeability in the PFC and cerebellum of pups. Astrocytes are essential for the development and maintenance of the BBB. Under inflammatory conditions, reactive astrocytes secrete vascular endothelial growth factor (VEGF-A), which activates eNOS signalling in EC and downregulates the expression of tight junction proteins (occludin and claudin-5) leading to increased permeability of the BBB [6]. Furthermore, reactive astrocytes secrete inflammatory cytokines such as IL-6, IL-1β and TNF-α, which can activate microglia [7]. The activated microglia also contribute to the dysfunction of the BBB using different pathways such as secretion of inflammatory cytokines (TNF-α and IL-1β), leading to downregulation of tight junction proteins that form the neurovascular unit in the BBB [8]. Another possible mechanism is through phagocytosis of astrocytic end feet by activated microglia [9]. Reactive microglia were found colocalised with a glycocalyx marker (Ulex Europaeus Agglutinin 1) in CNS string vessels, suggesting that activated microglia are involved in the pathologic events leading to damage of blood vessels in the CNS [10]. It is therefore not surprising that *Mtb*-induced MIA offspring presented reactive astrocytes and microglia coupled with a disrupted BBB. Dual prenatal exposure to a mild dose of VPA and *Mtb* increased the BBB permeability in the PFC and cerebellum suggesting that teratogenic effects of VPA and *Mtb* induced inflammation work synergistically to promote BBB dysfunction. The dual mechanism involved in the BBB disruption is not clear, however, our data is supported by reports from a similar MIA model that demonstrated increased BBB permeability following exposure to LPS and VPA *in utero* [11].

Accumulating evidence has under scored the significance of neuroinflammation in the pathology of NDDs [12, 13]. The role of *Mtb*-induced MIA in neuroinflammation was

demonstrated by increased microglia and astrocyte reactivity in the PFC and cerebellum. Sustained microglia cell activation during development affects glia-cell dependant processes like synaptic pruning, leading to dysfunction in synapse formation and activity [14]. Under inflammatory conditions, microglia morphological changes are accompanied by increased synthesis of pro-inflammatory cytokines. Cyclooxygenase-2 (COX-2) is expressed in response to inflammation to promote increased activation of MAPK (P38 mitogen activated protein kinase) subsequently increasing proinflammatory cytokines [15]. IL-6 and TNF-α expression were reportedly upregulated along with microglia activation in the brain of LPS-induced MIA offspring [16]. The present study did not measure cytokine expression in the brain, however, the neuroinflammation data suggests a possible elevation in pro-inflammatory cytokines in the brain. Dual exposure to VPA and *Mtb* did not exacerbate microgliosis in the PFC and cerebellum, rather *Mtb* rescued the VPA-induced effects on microglia reactivity (Figure 3.2B) suggesting that inflammation rescues teratogenic effects of VPA on microglia in a developing brain. Holladay *et al* (2002) suggested that a maternal immunoregulatory response that alleviates teratogenic effects on the developing foetus may be responsible for this mechanism [17].

Based on the neuroinflammation findings in this study and evidence of systemic immune dysregulation in MIA offspring [18, 19] and ASD patients [13, 20, 21], we sought to determine plasma cytokine profiles and ASD-linked behavioural patterns in *Mtb*-induced MIA offspring. ASD children that were born to mothers with MIA history displayed social deficits [22]. Our results showed that *Mtb*-induced MIA offspring had impaired social skills and these animals showed preference for an object to a mouse. More so, the *Mtb*-induced MIA offspring failed to choose the unfamiliar mouse over the familiar mouse, however, when a mild dose of VPA was co-administered with *Mtb*, the offspring showed normal social behaviours. Besides affecting social behaviour, *Mtb*-induced MIA impaired self-grooming behaviour indicating increased repetitive patterns. *Mtb* infection increased plasma IL-6 and IL-17A and reduced TNF-α, IFN-γ and IL-1β (Figure 4.3). The immune dysregulation in *Mtb* offspring might be the reason for the impaired behaviours. Cytokines regulate brain development hence an imbalance disturbs fetal brain function. Increased IL-6 and IL-17 levels were associated with repetitive behaviours and lack of social skills [20, 23-25]. Circulating IL-6 can cross the BBB and trigger inflammation in the brain thus affecting connectivity and neurotransmitter function, subsequently leading to altered behavioural patterns. Previously, elevation of IL-6 caused

impaired synapse formation and an imbalance in neuronal excitation/ inhibition that led to ASD behaviours [26]. Cytokines influence glutamate activity by increasing its release and reducing re-uptake of glutamate leading to excitotoxicity [27]. The elevated IL-6 and IL-17A levels and neuroinflammation in this study are most likely the effectors of the repetitive and social patterns observed.

To understand the role of brain molecular changes in ASD-phenotype, we examined the cerebellar expression of synaptic cell adhesion molecules. Our findings demonstrated an upregulation of *NRXN1* and *NLGN1* genes in offspring exposed to *Mtb* in utero. Consistent with the role of neurexins and neuroligins in synaptic function, overexpression of *NLGN1* increased the number of synapses [28] while overexpression of *NRXN1* was associated with increased hyperactivity and repetitive patterns [29]. NLGNS and NRXNS modulate excitation/ inhibition in synapses, therefore altered expression in the NRLGN/NRXN complex disturbs the synaptic plasticity leading to impaired social behaviours and repetitive patterns. Prenatal exposure to combined *Mtb* and VPA restored cerebellar *NRXN1* gene expression levels to normal.

In conclusion, our findings suggest that *Mtb* infection in pregnancy affects fetal health and development, which persists in postnatal life. *Mtb*-induced MIA induces fetal resorption and the surviving offspring health is also affected as revealed by delayed physical development and maturation. These altered developmental milestones are accompanied by peripheral inflammation and neuroinflammation, which are consistent with ASD pathophysiology. IL-6 crosses the BBB and induces microglia and astrocyte activation. Activated glia cells increase oxidative stress in the brain. The “leaky” BBB in the pre-frontal cortex (PFC) and cerebellum emanating from immune dysregulation implies that activated glia cells secrete pro-inflammatory cytokines that increase reactive oxygen species (ROS) leading to oxidative stress that causes neuronal damage [30]. Immune dysregulation also affects gene expression patterns of the NRXN/NLGN complex leading to synaptic defects in the cerebellum of offspring. Furthermore, the synaptic defects coupled with immune dysregulation in *Mtb* offspring could be responsible for the repetitive patterns (self-grooming) and reduced social interaction skills displayed. The restoration in *NRXN1* expression, social interaction, delayed eye-opening rescue and reduced astrocyte damage, may be a feedback response to *Mtb* induced inflammation in the presence of VPA (VPA+*Mtb*). Some maternal cytokines produced in response to infection can cross the placenta to counter the teratogen mediated effects in the

developing foetus [31, 32]. Another mechanism could be the antimicrobial effect of VPA on *Mtb* that supresses the *Mtb*-induced MIA effects on the fetal brain. VPA can hinder *Mtb* growth through inhibition of *Mtb* encoded deacetylase enzyme (Rv115c) involved in its central metabolism [33].

To the best of our knowledge, this is the first study to provide insight into the possible association of *Mtb* infection in pregnancy with impaired neurodevelopment outcome in offspring. We may therefore conclude that the evidence produced in this study lays the groundwork for further investigations into the potential impact of gestational *Mtb* infections on ASD in children born to infected mothers, especially in regions where TB is endemic.

Recommendations and Future studies

The following recommendations are made to further understand the effects of prenatal *Mtb* exposure on offspring development and ASD:

1. To evaluate an association between prenatal *Mtb* infection and neuronal damage in offspring
2. To evaluate if anti-inflammatory agents can mitigate the *Mtb*-induced MIA effects on neurodevelopment and associated behaviours.
3. To evaluate the gut microbiota in *Mtb*-induced MIA offspring, a novel target in ASD pathophysiology.
4. To evaluate the immune profiles of *Mtb* infected pregnant mice and assess the expression of maternal auto-antibodies in the fetal brain.
5. To evaluate if *Mtb*-induced MIA primes the fetal brain for a second environmental insult in adolescence.

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APPENDICES

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

15 April 2019

Ms Wadzanai Manjeese (218074724)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Ms Manjeese,

Protocol reference number: AREC/076/018M
Project title: The effect of maternal Tuberculosis infection on neurodevelopment and Autism-like behaviour in a mouse model

Full Approval – Research Application

With regards to your revised app lcation received on 28 November 2018 and response received on 12 April 2019 to our letter of 13 December 2018. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 15 April 2020.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

Dr **Sanil D Singh, PhD**
Deputy Chair: Animal Research Ethics Committee

/ms

cc Supervisor: Dr Thabisile Mpofana
Cc Acting Academic Leader Research: Dr Brenda de Gama
Cc Registrar: Mr Simon Mokoena
Cc School Administrator: Ms Prathna Dudhrajh

Animal Research Ethics Committee (AREC)
Ms Mariette Snyman (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8350 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

BIOMEDICAL RESOURCE UNIT

July 31, 2018

Dear Prof Islam
Chair: Animal Research Ethics Committee
c/o School of Life Sciences

RE: ATTENDANCE OF LAS COURSE

This letter certifies that Miss Wadzanai Manjeese , 218074724 have attended the Laboratory Animal Course that was hosted by the Biomedical Resource Unit.

The course was held on the 21 – 22 June 2018 and entailed the following:

Introduction to laboratory animal sciences. Bioethics and Animal experimentation. Animal Research Methodology. Experimental design, environmental enrichment and occupational safety.

The course was completed satisfactorily and may be allowed to initiate research after the relevant practical procedures was done to a level of competency that was signed off by the veterinarian in charge.

Kind Regards

[redacted]

Dr L A Bester
Acting: BRU Manger

Miss Ritta Radebe, Room 201, 2nd Floor U- Block ,Tel 031 260 7671,Fax 031 260 7730E-mail radeber@ukzn.ac.za

REDUCE, REFINE AND REPLACE

K-rith Tower Building
Level 6
University of Kwa-zulu Natal
Nelson R Mandela Medical School
719 Umbilo Road
Durban
4000

25/10/2018

UKZN Animal Ethics Committee

Re: BSL3 Training and Practice/ Animal Unit Training

To whom it may concern

This letter serves to confirm that Ms Wadzanai Manjese and Shasthri Maharaj, have completed their BSL3 training and practice as per AHRI's compliance policies. They have also demonstrated competence in dissection procedures as required by Animal unit policies.

They are both cleared to work in AHRI's BSL3 labs.

Should you require any further information please do not hesitate to contact me

Yours sincerely

[redacted]

Mel van Eeden
Quality Compliance & Safety Manager
031 260 4947

[redacted]

Kershnee Thambu
Animal Research Core Supervisor
031 260 4933

UNIVERSITY OF KWAZULU-NATAL

Biomedical Resource Unit

July 24, 2018

Dear Prof Islam
Chair: Animal Research Ethics Committee
c/o School of Life Sciences

RE: COMPETENCE TRAINING ON RATS ONLY

This letter confirms that Miss Wadzanai Manjeese , 218074724 has undergone an evaluation for invasive procedures on the 30 May 2018 and shows competence regarding the following:

a. IP Administration
b. Oral gavage
c. Subcutaneous procedures

Kind Regards

[redacted]

Dr SD Singh BVSc. (Mumbai) MS (Illinois) LAS (Utrecht) CVE (Pretoria)
HOD: Biomedical Resource Unit
Veterinarian

To Reduce Replace and Refine Animal Research