Evaluating the Role of Maternal Separation Stress on The Development of Autistic-Like Behaviour in Sprague-Dawley Rats

BY

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TITLE PAGE

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DECLARATION

I, Chanel Heeralall, declare as follows:

That the work described in this thesis has not been submitted to UKZN or other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

That my contribution to the project was as follows:

All research theory, animal work, laboratory analysis and the compiling of data, as well as the writing of this dissertation was done by myself.

That the contributions of others to the project were as follows:

Dr Thabisile Mpofana, my supervisor and Dr Lihle Qulu my co-supervisor, oversaw all my work through this project.

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ACRONYMS

AREC - Animal Research Ethics Committee

AS - Asperger syndrome

ASD - Autism Spectrum Disorders

CDC - Centres for Disease Control and Prevention

DI - Discrimination Index

ELISA - Enzyme-Linked Immunosorbent Assay

HPA - Hypothalamic-Pituitary-Adrenocortical

GABA - Gamma Aminobutyric Acid

GABBR1 - Gamma-Aminobutyric Acid B Receptor 1

GABRB3 - Gamma-Aminobutyric Acid Type A Receptor Subunit Beta-3

GND - Gestational Day

LBW - Low Birth Weight

MS - Maternal separation

PDD - Pervasive developmental disorders

PDD-NOS - Nonspecific Pervasive Development

PFC - Prefrontal Cortex

PND - Post-Natal Day

RT PCR - Real Time Polymerase Chain Reaction

SERT - Serotonin Transporter

TF - Time spent exploring the Familiar object

TN - Time spent exploring the Novel object

VPA - Valproic Acid

STUDY OUTLINE

This master's dissertation is presented in manuscript format, with two studies and five chapters; Chapter 1: background/literature review, Chapter 2: manuscript 1, Chapter 3: manuscript 2, Chapter 4: synthesis and conclusions. Chapter 5: appendices. The abstract states the aims and main findings of this study. Chapter one is a detailed literature review which discusses what is known about autism, the gaps that may exist in the knowledge of autism and how the current study aims to fill in those gaps. Chapter two contains the first manuscript which focuses on the effects of prenatal exposure to valproic acid. This manuscript will be submitted to the International Journal of Developmental Neuroscience and is written in accordance to the journal guidelines. Chapter three contains the second manuscript which focuses on the effects of maternal separation stress with prenatal exposure to valproic acid. Chapter four is a synthesis which identifies the link between the two studies and concludes the overall findings. Chapter five contains additional appendices including ethical approval, protocols used in this study and conference output.

ABSTRACT

Autism Spectrum Disorders (ASD) are a category of neurodevelopmental disorders that have become more prevalent, causing much concern. Autistic individuals display symptoms ranging from repetitive behaviours to communication deficiency and impaired executive function. There are numerous risk factors for autism including exposure to particular medications, like valproic acid (VPA), and certain environments. VPA has been shown to increase the risk of autism in unborn children. Furthermore, early stressful events like maternal deprivation are known to have a part in causing neurodevelopmental disorders. Therefore, in this study we used VPA to induce autism and aimed at investigating how VPA alters the major neurotransmitter system pathways, which results in autistic-like behaviour. Furthermore, we conducted maternal separation to investigate the effect of stress together with VPA.

This study used forty-eight Sprague-Dawley rat pups which were divided in to control (saline-0.9%, 3.3 ml/kg), VPA (VPA-500 mg/kg) and stressed groups. Maternal separation stress was conducted for 12 days. The novel-object recognition test and open field test were conducted to assess the pup's behaviour. PCR was conducted to measure the expression of GABA receptors in the hippocampus and cerebellum. The concentrations of glutamate, serotonin transporter and corticosterone were assessed in the prefrontal cortex, hippocampus, amygdala and blood.

Exposure to VPA resulted in decreased glutamate concentrations in the hippocampus and prefrontal cortex whilst it caused an increase in hippocampal GABBR1, GABRB3 and cerebellum GABRB3, but a decrease in the cerebellum GABBR1. These alterations were accompanied by repetitive and hyperactive behaviour, seen in the open field test whilst memory deficits were observed in the novel object recognition test. Exposure to maternal separation stress and VPA caused a dysfunction in the stress response which lead to a decrease in corticosterone concentration. Maternal separation stress and VPA exposure also decreased the serotonin transporter concentration in the amygdala and prefrontal cortex. These alterations were accompanied by anxiety-like behaviours, seen in the open field test where we observed a decrease in the time spent in the centre and the total excretion.

This study shows that exposure to VPA alters neurotransmitter balance thus resulting in an autistic-like phenotype. Furthermore, exposure to maternal separation stress and VPA causes a desensitization in the stress response.

Key words: Autism, valproic acid, maternal separation stress, neurotransmitters, behaviour

CHAPTER ONE

Chapter one consists of a brief background on the overall topic, states the research objectives/aims and includes a literature review which covers the knowledge known about autism as well as identifies the gaps we aim to fill.

Introduction

Autism is a neurodevelopmental disorder characterized by restricted, repetitive behaviours, decreased social cooperation and communication deficiency (Lord *et al.*, 2000, Frith and Happé, 2005). These impairments vary greatly hence the term Autism Spectrum (ASD) was developed to acknowledge this diversity (Faras *et al.*, 2010). The autism spectrum ranges from Asperger's syndrome, Kanner's syndrome, childhood disintegrative disorder, nonspecific pervasive developmental disorder and Rett's syndrome (Hrabovska and Salyha, 2016). ASD commonly develops during infancy or in the first years of life (Lord *et al.*, 2000).

Dysfunction in the major neurotransmitter systems, like the GABAergic, glutamatergic and serotonergic systems, are linked to the pathogenesis of ASD (Trottier *et al.*, 1999, Kwong *et al.*, 2000, Cetin *et al.*, 2015). Gamma aminobutyric acid (GABA) is essential in many processes like stages of maturation and early developmental stages of cell migration (Ben-Ari *et al.*, 2012). Whilst glutamate is a principal excitatory neurotransmitter (Cetin et al., 2015). Serotonin plays an important role in many processes, including emotional states plus sexual behaviour, and is essential for the development of social skills (Buhot *et al.*, 2000, Whitaker-Azmitia, 2001, Brummelte *et al.*, 2017). Therefore, these systems have important roles in many neurodevelopmental disorders, including ASD (Coghlan *et al.*, 2012, Cetin *et al.*, 2015).

The global prevalence of autism in the 1960's was around 4 per 10,000 (Frith and Happé, 2005). However, in 2018 it had increased to 1 in 59 children (Baio *et al.*, 2018). Evidence suggests that Autism Spectrum Disorders are caused by genetics and are one of the heritable disorders, however the risk of developing ASD's can be influenced by environmental factors (Frith and Happé, 2005, Faras *et al.*, 2010, Dietert *et al.*, 2011, Hrabovska and Salyha, 2016).

Direct exposure to particular drugs, stress, environmental chemicals and dietary factors are environmental risk factors for autism (Dietert *et al.*, 2011). In addition, exposure to pharmacological agents like valproic acid (VPA) and thalidomide are very current environmental risk factors (Dietert *et al.*, 2011). There are conditions that can increase the susceptibility of autism, through exposure during the prenatal, perinatal and neonatal periods (Baron-Cohen and Bolton, 1993, Guinchat *et al.*, 2012). During pregnancy stress and medication are considered to be risk factors of autism (Gardener *et al.*, 2009, Guinchat *et al.*, 2012).

During a stress event, the hypothalamic-pituitary-adrenocortical (HPA) axis is activated, as it is essential in controlling biological processes, like responding to stress (Corbett and Simon, 2014). Cortisol is the main stress hormone in humans which is released when the HPA axis is triggered (Corbett and Simon,

2014). Premature harmful experiences like disrupting the maternal-offspring bond results in life long consequences (Vetulani, 2013). Maternal separation is a form of early life stress that has been associated with the development of neurological disorders and therefore the maternal separation stress model is used to investigate the effects of premature trauma (Weiss *et al.*, 2011, Mpofana *et al.*, 2016).

There are several groups of autism animal models, including pre/postnatal exposure to particular environmental factors which replicates the main symptoms of autism (Hrabovska and Salyha, 2016). VPA is clinically used in treating epilepsy however, it's usage during pregnancy has become concerning as it has been linked with the risk of autism (Mabunga *et al.*, 2015). Hence, prenatal exposure to VPA is used in investigations to create autism animal models (Mabunga *et al.*, 2015).

Literature Review

Autism

Pervasive developmental disorders are a set of neurodevelopmental disorders characterized by impairments in reciprocal social interaction, communication and restricted repetitive interests or behaviours (Faras *et al.*, 2010). One of these neurodevelopmental disorders are known as autism (Faras *et al.*, 2010). Autism is characterized by impaired social reciprocity, deficits in communication and by abnormal repetitive, restricted behaviours and interests (Lord *et al.*, 2000, Frith and Happé, 2005). The display of these impairments varies in severity and range with age and ability, thus the concept of the autism spectrum was brought about to acknowledge this heterogeneity (Frith and Happé, 2005, Faras *et al.*, 2010). Autism spectrum disorders (ASD) is the term commonly used to describe this variation in impairments (Faras *et al.*, 2010). In the most severe cases, symptoms such as evading eye contact, little to no social interaction with peers, inability to understand and explain other's feelings and social cues, neglect in developing peer relationships, absence of seeking to share enjoyment, lack in reciprocating emotion, as well as awkward and even complete absence of speech (Arruda, 2017). Dysfunction in the neurotransmitter systems is considered to be the reason for ASD, through affecting synaptogenesis, differentiation, neuronal cell migration and lastly the developmental processes of the brain (Kwong *et al.*, 2000, Cetin *et al.*, 2015). The GABAergic, glutamatergic and serotonergic neurotransmitter systems are the main systems associated with the pathogenesis of ASDs (Trottier *et al.*, 1999). Studies have observed a reduction in GABAB and GABAA receptor subunits in several brain regions which substantiates the extensive dysfunction of the GABAergic system in patients with ASD (Rolf *et al.*, 1993, Fatemi *et al.*, 2009b, Oblak *et al.*, 2010). Changes in the expression of GAD65/67 and GABA receptor subunits have been documented to result in alterations in the excitatory/inhibitory equilibrium in the brain which cause many cognitive impairments linked with autism (Fatemi *et al.*, 2010). Several studies confirm the theory of "decreased GABAergic transmission in ASD patients" (Fatemi *et al*., 2009b, Oblak *et al*., 2010). Gamma-aminobutyric acid receptor subunit beta-3 (GABRB3) and Gamma-

aminobutyric acid type B receptor subunit 1 (GABBR1) deficiencies in autism has been shown to cause impairments in memory, exploratory and social behaviour (DeLorey *et al.*, 2008, Fatemi *et al.*, 2009b, Fatemi *et al.*, 2010). Alterations in the glutamatergic system is crucial as disruptions impair cognitive functions like memory and have been associated with repetitive and restricted behaviours (Carlsson, 1998, Jamain *et al.*, 2002, Koppers, 2010, Cetin *et al.*, 2015, Horder *et al.*, 2018). ASD patients experience dysfunctions in the serotonergic systems (Cetin *et al.*, 2015). Serotonin is essential in the development of social skills during early developmental periods and inadequate stimulation of serotonin in the premature stages of life can result in unpreventable abnormalities in serotonin metabolism (Brummelte *et al.*, 2017, Cetin *et al.*, 2015). The autism disorder typically develops in infancy or at latest in the first three years of existence (Lord *et al.*, 2000). Parents become concerned once they notice differences in their child including the lack of utilizing words to communicate, addressing their parents by name, pointing to objects that they find interesting and not seeking the company of others when they feel happy (Lord *et al.*, 2000). In addition, Lord *et al.* (2000) documented that repetitive behaviours like specific hand movements as well as using the peripheral vision to look at wheels or lines can be observed in the preschool years of autistic children.

The Autism Spectrum

This heterogenous disorder includes Asperger's syndrome, Kanner's syndrome, childhood disintegrative disorder, nonspecific pervasive developmental disorder and Rett's syndrome (Hrabovska and Salyha, 2016). Patients with Asperger syndrome (AS) display similar symptoms to patients with autism including improper communication skills, restriction in interests and lack in social interaction (Barahona-Corrêa and Filipe, 2015). However, AS patients exhibit a wide variety of understated clinical characteristics which differentiate AS from autism (Barahona-Corrêa and Filipe, 2015). Complications in distinguishing AS from autism and problems with operationalising diagnostic criteria, eventually resulted in its merging in-to the combined category of ASD (Barahona-Corrêa and Filipe, 2015). Furthermore, according to Pearce (2005) communication impairment represents a diagnosis of autism and not Asperger's disorder. A psychiatrist named Leo Kanner, noted that in nearly all the cases the infant's behaviour was unusual from early infancy which he referred to as ''early infantile autism''(Kanner, 1943, Kanner, 1944, Pearce, 2005). Therefore, he proposed a probable genetic, inborn defect (Kanner, 1943, Kanner, 1944, Pearce, 2005). Whilst Rett's Syndrome and Childhood Disintegrative Disorder, commonly known as Heller's Syndrome, are identified by developmental regression with extreme autistic features (Barahona-Corrêa and Filipe, 2015). Furthermore, there is a category known as the nonspecific pervasive development (PDD-NOS), which is utilized in cases of pervasive impairment in communication and social interaction with the appearance of stereotyped behaviours or interests whenever specifications for a specific disorder are not met (Lord *et al.*, 2000).

Epidemiology

In the 1960s, when the first systematic studies on autism were conducted, the prevalence approximation was roughly 4 per 10,000 (Frith and Happé, 2005). However, in the modern world ASD's are global and the approximation, in the year 2005, for the entire autism spectrum was roughly 60 per 10,000 (Frith and Happé, 2005). This 15-fold elevation has caused the fear of an epidemic. For example, during 2011–2012 in the United States various ASD's were officially detected in approximately 2% of school children (Arruda, 2017). In addition, the statistics from the Centres for Disease Control and Prevention (CDC) s illustrates just how prevalent autism has become in the recent years, shown in Figure 1 (Baio *et al.*, 2018).

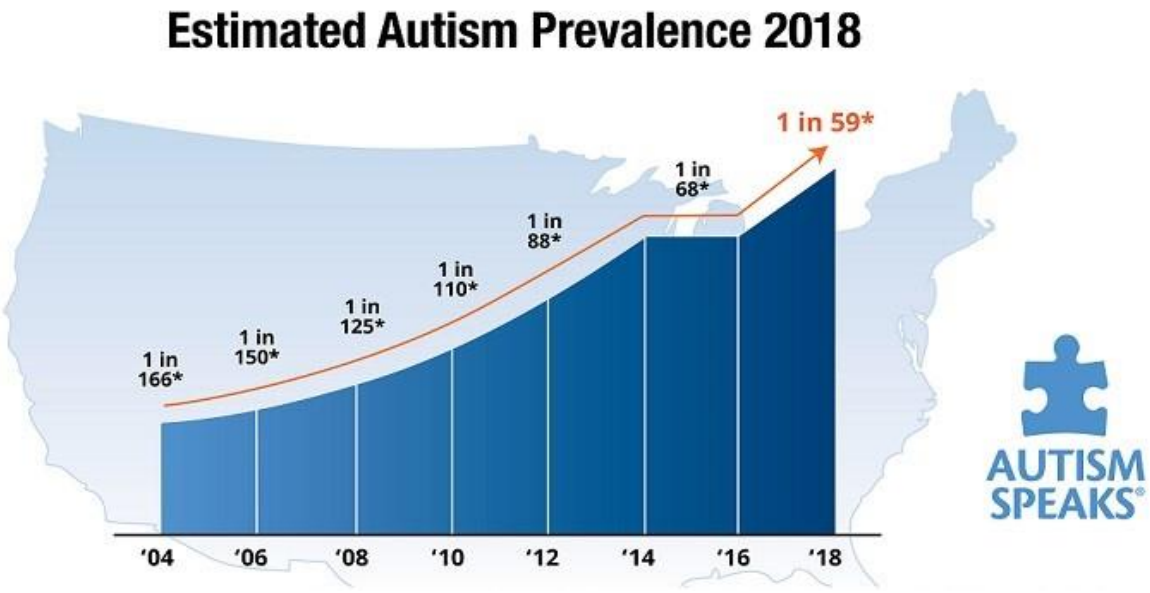

Figure 1: The estimated prevalence of autism in 2018 was according to the Centre's for Disease Control and Prevention (Autism Speaks, 2018)

According to the statistics of Autism South Africa, autism affects 1 in 110 children internationally whilst a staggering amount of 7665 autistic children are born in South Africa annually (Autism South Africa). This further indicates that 160 autistic children are born weekly and 23 autistic children daily, which essentially means that virtually one child with autism is born every hour (Autism South Africa).

However, according to Frith and Happé (2005) the diagnostic criteria have evolved substantially over the years and therefore only a small percentage of the present spectrum would have satisfied the 1960s criteria. In addition, the rise in the prevalence of identified autistic cases may be a result of the expansion of diagnostic criteria, diagnostic facilities or specialist provision as well as a significant increase in awareness (Frith and Happé, 2005).

Risk Factors

Currently there is no general agreement with regard to the causes or risk factors that result in the development of ASDs (Hrabovska and Salyha, 2016).

There is evidence which indicates that ASD is a result of genetics (figure 2), because it is associated with changes in the interaction of numerous genes as well as spontaneous mutations (Hrabovska and Salyha, 2016). Autism is familial and one of the most heritable developmental disorders, as the prevalence of autistic features and ASD is greater in siblings of autistic patients, and members of their families are 50 more times likely to have ASD than the general community (Frith and Happé, 2005, Faras *et al.*, 2010, Hrabovska and Salyha, 2016). Frith and Happé (2005) also observed a much higher concordance in identical twins compared to fraternal twins. Furthermore, interferences in brain development can be a result of defects in the genes that are responsible for such development and premature brain cell communication (Arruda, 2017). According to Autism South Africa and Faras *et al.* (2010) ASD is 4 - 5 times more prevalent in males than females.

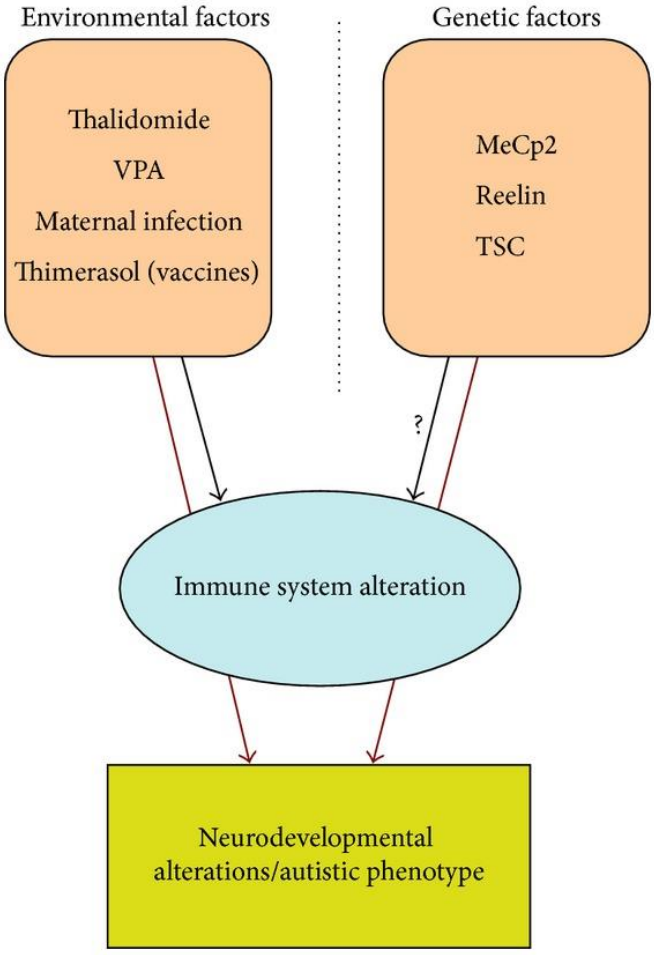

Figure 2 : The common environmental and genetic risk factors for autism (Gadad *et al.*, 2013)

Autism is a developmental disorder hence sensitive periods of developmental vulnerability occurs according to the period that the risk factor is exposed, namely: prenatal, perinatal and neonatal (Dietert *et al.*, 2011, Guinchat *et al.*, 2012).

Guinchat *et al.* (2012) recorded advanced paternal age, advanced maternal age and primiparous women to be familial risk factors for autism. It is possible that maternal age and autism maybe be associated as a result of an increase in the risk of chromosomal abnormalities in ova due to increased age or unstable trinucleotide repeats (Gardener *et al.*, 2009). Reichenberg *et al.* (2006) proposed that paternal age and autism maybe be associated as a result of de novo spontaneous mutations, imprinted genes which increase with the advance in the spermatogonia's age or confounding through sociocultural environmental components. A common risk factor for autism in literature is birth parity/ order, however this relationship is inconsistent as it may be as a result of possible effect modifications by sibship size (Gardener *et al.*, 2009).

Exposure to certain environmental factors pose a risk for autism, as shown in figure 2 above (Dietert *et al.*, 2011). The environment can potentially cause ASDs as there are indications that exposure to dangerous substances, have prenatal effects and have been found to be contributing factors in the development of autism (Dietert *et al.*, 2011, Hrabovska and Salyha, 2016, Arruda, 2017). However to Baron-Cohen and Bolton (1993) it appeared that there isn't an environmental trigger of ASD, but rather conditions that make an individual susceptible to autism.

Medication, stress and bleeding during pregnancy were identified as risk factors with regards to the pregnancy (Gardener *et al.*, 2009, Guinchat *et al.*, 2012). Neurotoxicants, like arsenic, lead, manganese plus mercury, are regarded as risk factors for autism as they disrupt neural development and an increase in their environmental concentrations in the recent years parallels the increased prevalence of autism (Dietert *et al.*, 2011). Exposure to particular pharmacological agents such as valproic acid (VPA) and thalidomide since the changing trends in the accessibility of certain drugs corresponds to the changing prevalence in autism (Dietert *et al.*, 2011). VPA is a mood stabilizer and anti-convulsant, however usage of this medication during pregnancy has been linked to the risk of autism as it is a human teratogen (Ornoy, 2009, Roullet *et al.*, 2013). Exposure to VPA has been documented to result in autistic-like behaviours in the progeny, including repetitive behaviours, deficits in communication and social behaviour (Roullet *et al.*, 2013). On the other hand, infections and vaccines have been extensively researched as being risk factors for autism as it is presumed that if the offspring is exposed to bacterial or viral infections in utero, then neurodevelopment is influenced which can result in adverse outcomes (Dietert *et al.*, 2011, Grabrucker, 2013). Getahun *et al.* (2017) documented that pregnancies involved with preeclampsia affects the placenta and as a result it undergoes morphological and histologic changes which impair placental function and this leads to foetal hypoxemia and chronic oxidative stress. Foetal

hypoxia, which is essentially oxygen deprivation during development, may be the root for the probable relationship between autism and gestational bleeding since hypoxia results in an increase in dopaminergic activity which is also evident in autistic individuals (Gillberg *et al.*, 1995, Gardener *et al.*, 2009). Exposure to prenatal stress has been linked to increased risk in autism and has been found to result in key symptoms of autism, like impaired social interaction and cognitive deficits (Kinney *et al.*, 2008). In addition, other pregnancy-related factors including prolonged labour, maternal hypertension, foetal distress, caesarean delivery, low Apgar score and cord complications are considered to be related to hypoxia and associated with autism risk (Gillberg *et al.*, 1995).

The main risk factors for autism in the perinatal period are a planned caesarean section, breech presentation and preterm birth, whilst birth defects, encephalopathy, hyperbilirubinemia, poor birth conditions like hypoxia/ low Apgar scores and low birth weight (LBW) are neonatal risk factors (Guinchat *et al*., 2012). Class *et al.* (2014) found that maternal bereavement stress postnatally is also linked to the increased risk of autism in the offspring.

Stress and The HPA Axis

Stress occurs in several forms which include psychological, infectious, physical and oxidative (Dietert *et al.*, 2011). Both perceived and actual threats can activate the primary stress system (Corbett and Simon, 2014). Stress is the physiological reaction in response to an occurrence by the primary stress systems, like the hypothalamic-pituitary-adrenocortical (HPA) axis, being activated (Corbett and Simon, 2014). The HPA axis is responsible for regulating numerous biological processes, with one of them being the response to severe psychological and physiological stress (Corbett and Simon, 2014).

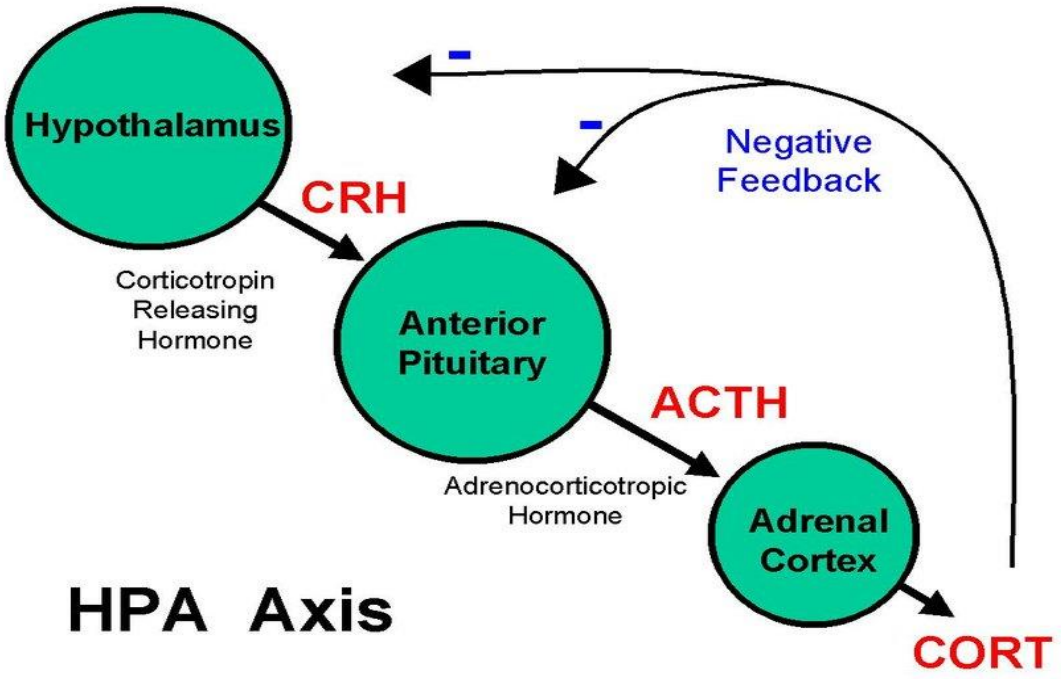

Figure 3: How the Hypothalamic-Pituitary-Adrenal (HPA) axis works in response to stress (Xiao, 2015)

When the HPA axis is activated (Figure 3) the adrenal cortices are stimulated to secrete glucocorticoids (corticosterone in rats and mice and cortisol in humans), which is crucial in adapting to stress (Aguilera, 2011, Corbett and Simon, 2014). These glucocorticoids establish necessary changes in the neuromodulators and metabolism to manage with challenges by responding to stressful situations and returning to homeostatic conditions quickly (Aguilera, 2011, Stephens and Wand, 2012).

The activation of the HPA axis during acute stress is compulsory for adaptation to stressful situations whilst in chronic stress exorbitant exposure to constant elevated levels of stress hormones result in pathological consequences (McEwen, 1998, Aguilera, 2011, Dunlavey, 2018). Prolonged exposure to stress hormones can result in the predisposition of immune, metabolic and psychological alterations hence it is essential for the stress response to end to prevent adverse effects as a result of altered levels of glucocorticoids and corticotropin releasing hormone (Aguilera, 2011). The effects of chronic stress are displayed in reduced immune health and neurodegenerative brain disease (Dunlavey, 2018). Activation of the HPA axis through chronic stress can lead to several forms of HPA axis dysregulation including: adrenal exhaustion, sensitized stress responses and chronic basal hypersecretion (Thomas and Lena, 2010, Herman *et al.*, 2011). Chronic stressors can cause a shift in the HPA axis from being over-responsive to under-responsive or non-responsive ("adrenal fatigue") and this adaptation of the HPA axis is believed to be a protective measure to survive, by preventing chronically increased cortisol levels from subduing immune function (Thomas and Lena, 2010). Furthermore, a state of hypo-cortisolism has

been observed in patients who experienced traumatic premature life events and chronic exposure to stressful environments (Thomas and Lena, 2010).

The first 2 weeks of life is crucial in mammals, as this is the period during which the stress system is developed and is therefore referred to as the stress-hyporesponsive period (Lai and Huang, 2011, Schmidt, 2019). The stress-hyporesponsive period is defined by the reduction in adrenocortical glucocorticoid secretion after stressful events (Schmidt, 2019). The development of the stress system is essential in moulding its function for adulthood which influences the vulnerability to disorders related to stress and resilience to stress in the future years (Schmidt, 2019). Early life stress during the period results in dysregulation of the HPA axis, modification in brain functioning and neurobehavior (Lai and Huang, 2011, Mpofana *et al.*, 2016). Stress and trauma has been documented as a risk factor for co-occurring plus exacerbating autism symptoms (Fuld, 2018). Chauhan *et al.* (2004) found that alterations in ceruloplasmin and transferrin regulation in autistic individuals resulted in irregular copper and iron metabolic processes which increased oxidative stress and therefore possibly played a role in autism. Ploeger *et al.* (2010), contended that the disturbance of early developmental events (20-40 gestational days in humans) maybe a route to infantile autism. Mueller and Bale (2008), documented that an early stress experience in prenatal development may cause patterns of male orientation in neurodevelopmental disorders. Jones *et al.* (2010) proposed that when prenatal stress is combined with maternal genotypic variations can result in the offspring having an autistic phenotype.

Maternal Separation Stress

New-born mammals are nurtured and protected by their mothers hence they remain within close range for some time (Sullivan *et al.*, 2011, Vetulani, 2013). The first few days of life are necessary as complex interactions occur which are associated with nursing and plays an essential part in the cognitive and emotional development of the children (Sullivan *et al.*, 2011, Vetulani, 2013). Detrimental early life events, including disturbances in the maternal-offspring bond, lead to long-lasting effects and result in acute disruptions (Nishi *et al.*, 2014). These effects evolve due to the continuous activation of stress mediators like catecholamines and glucocorticoids, known as the allostatic load (Vetulani, 2013). This allostatic load has an effect on the neurochemistry and behaviour throughout the mammal's lifetime and the outcomes of an uncontrolled load on an animal's behaviour can be regarded as models for diseases (Howell and Sanchez, 2011, Vetulani, 2013). Since near contact with the mother is significant, maternal separation in the early years leads to an intense reaction of despair and protest in the neonate which accelerates the allostatic load which then results in acute disruptions of neurohormonal and physiological functions which has crucial consequences for neurochemical characteristics and social behaviour in adulthood (Howell and Sanchez, 2011, Vetulani, 2013).

Maternal separation has been classified as a form of early life stress which has been reported to be responsible for altering the development of the neuronal circuitry which is linked to the development of neurodevelopmental disorders (Mpofana *et al.*, 2016). The maternal separation (MS) stress paradigm has been utilized in rodent models to examine the long-term effects of early life trauma (Weiss *et al.*, 2011). This model, which involves maternal deprivation for a particular duration in the first two weeks of birth, mimics neglect, early life deprivation and disturbs mother–infant interaction (Weiss *et al.*, 2011). This paradigm models early postnatal stress during the stress hypo-responsive period (Nishi *et al.*, 2013). Maternal separation paradigms can range from separation for 180–360 minutes to deprivation for 24 hours (Nylander and Roman, 2013). Maternal separation once a day for 3 hours or more during the first two weeks following birth has been shown to cause exaggerated responses to stress later in life by the HPA axis, more anxiety-like behaviour plus disruption in cognition and behaviour (Bondar *et al.*, 2018). Weiss *et al.* (2011) documented MS paradigms that cause changes in the HPA axis response to stressful environments and result in depressive/anxiety-like behaviour (Weiss *et al.*, 2011).

Animal Models of Autism

It is essential to research the causes and risk factors of ASD so that medication can be developed and strategies to manage the symptoms of these disorders can be introduced, this is the principal reason that animal models of autism are emerging (Hrabovska and Salyha, 2016). There are four groups of autism animal models, namely: (i) models that have neonatal impairments with abnormalities that are known to exist in the brain of individuals with autism (cerebellum, medial prefrontal cortex) (ii) models where animals that lack specific neuropeptide receptors (opioids, oxytocin, vasopressin receptors) (iii) genetic models, linked to the X-chromosome, which display autistic-like behaviours and alterations in the immune system (iv) models in which there is exposure to environmental factors which increase the probability of autism in humans (Thalidomide and Valproic Acid) (Gadad *et al.*, 2013, Hrabovska and Salyha, 2016).

Substances like 5-methoxytryptamine, thalidomide and valproic acid (VPA) are utilized to create autism models, seen above in group iv, as they can possibly cause epigenetic modifications (Gadad *et al.*, 2013, Mabunga *et al.*, 2015). According to Mabunga *et al.* (2015), the number of researchers using the prenatal VPA exposure animal model has increased in the last decade due to it its versatility and its powerful etiologic relevance.

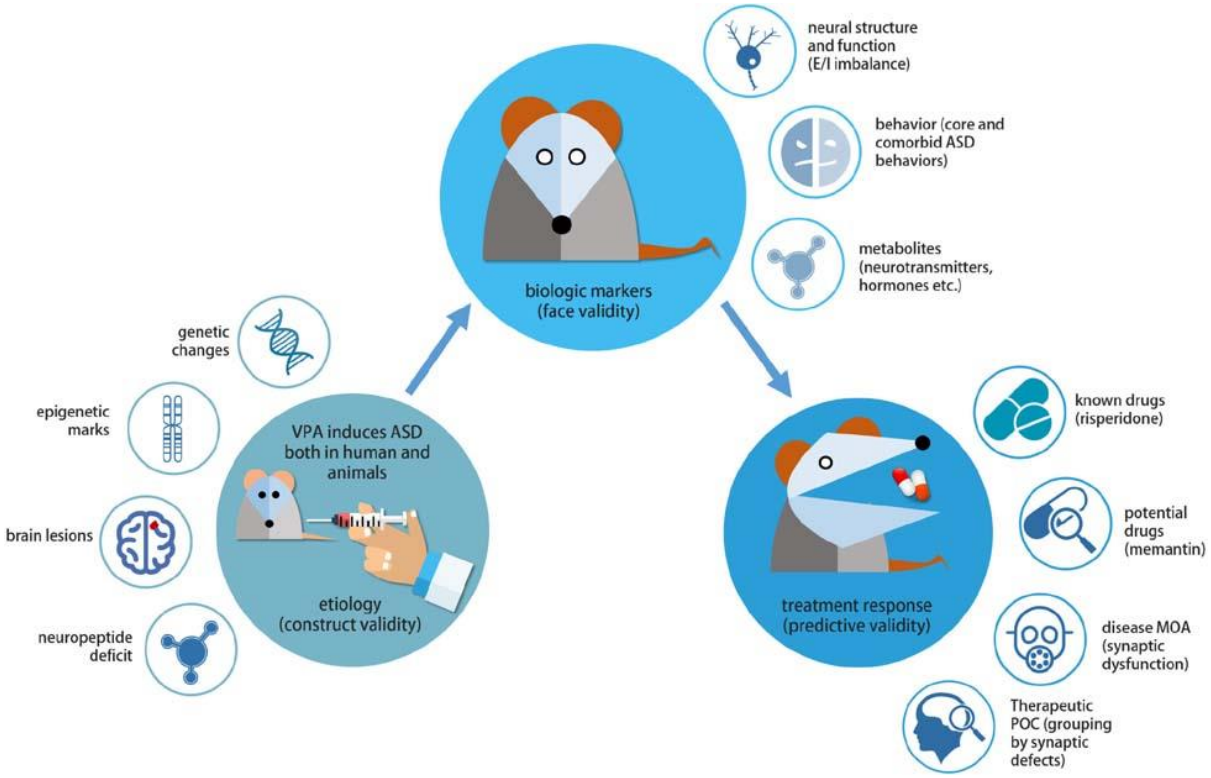

Figure 4: The validity of the VPA animal model (Mabunga *et al.*, 2015)

This usage of VPA is of concern because for many years, it was affiliated with several teratogenic effects (figure 4) and, more recently, increased risk of autism (Gottfried *et al.*, 2013, Mabunga *et al.*, 2015, Ornoy *et al.*, 2019). Thus clinical findings which have been reported, have resulted in a series of pre-clinical investigations utilizing VPA to induce autism-like responses in mice and rats (Mabunga *et al.*, 2015).

Purpose

Gray (1993) deduced from his study that autism has a unique stigma attached to it, due to the lack of public awareness and comprehension regarding autism, the typical physical appearance of children with autism and the disorderly nature of autistic symptoms. Particularly, the rural communities encounter significant challenges regarding availability of support services, treatment and diagnoses for those with ASD (Antezana *et al.*, 2017). This ultimately results in the possibility for delayed screening and diagnosis of ASD therefore producing lower functional and educational outcomes (Antezana *et al.*, 2017). The authors of this study hope their papers will be read by the community to bring about more awareness and to educate people on ASD.

Aims

The focus of this study was to:

- Investigate how VPA alters the major neurotransmitter system pathways, that result in autistic-like behaviour
- Determine the effects of early life stress on the VPA rat model of autism

Objectives

The research objectives for the purpose of this project were:

- To induce Autism in the offspring by injecting the pregnant rats with VPA
- To induce early postnatal stress through exposure of maternal separation
- To assess behavioural and neurochemical changes associated with VPA and Maternal separation exposure

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CHAPTER TWO

Manuscript 1

This manuscript will be submitted to the Journal of International Developmental Neuroscience.
The manuscript consists of a brief introduction highlighting autism, a detailed materials and methods section followed by the results and discussion sections. This manuscript was written in a submission format according to the authors guidelines for the journal. This paper focuses on how Valproic acid alters the neurotransmitter system to cause autistic-like behavior.

EVALUATING THE EFFECTS OF PRENATAL EXPOSURE TO VALPROIC ACID ON THE DEVELOPMENT OF AUTISTIC-LIKE BEHAVIOUR IN SPRAGUE-DAWLEY RATS

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ABSTRACT

Introduction: Autism is a neurodevelopmental disorder which is one of the crucial health care issues. This disorder is characterized by impaired social reciprocity, deficits in communication and by abnormal repetitive, restricted behaviours and interests which have been linked to alterations in the glutamatergic system and changes in the expression of GABA receptor subunits. Autism is primarily known as a genetic disorder, however there are numerous environmental risk factors that have been reported, including the exposure to certain drugs or medications such as the anti-convulsant Valproic acid, has been affiliated with several teratogenic effects and an increased risk in autism, when used during pregnancy. The aim of this study was to investigate how VPA alters the major neurotransmitter system pathways, to induce autistic-like behaviour.

Methods: This study used twenty-four rat pups which were divided in to control (saline-0.9%, 3.3 ml/kg) and experimental (VPA-500 mg/kg) groups. The novel-object recognition test and open field test were conducted to assess the pup's behaviour. A Glutamate assay and PCR was conducted to measure the glutamate concentration and expression of GABA receptors.

Results: Exposure to VPA caused a significant decrease in the glutamate concentration in the hippocampus and prefrontal cortex while it resulted in a significant increase in GABRB3 in the hippocampus and increasing tendency in the cerebellum. Furthermore, VPA caused an increasing tendency in hippocampal GABBR1, but a decreasing tendency in the cerebellum. These alterations were accompanied by repetitive behaviour, hyperactivity and memory deficits.

Conclusion: This study showed that valproic acid causes autistic-like behaviour by altering the glutamatergic and GABAergic neurotransmitter systems thus replicating the autistic-like phenotype.

Key Words: Pervasive developmental disorders, Autism, Valproic acid, glutamate,

GABA

INTRODUCTION

Pervasive developmental disorders (PDD) are amongst the most common neurodevelopment disorder globally (Faras *et al.*, 2010). One group of these neurodevelopmental disorders is well known as autism, autism forms one of the crucial health care issues (Faras *et al.*, 2010, Hrabovska and Salyha, 2016). Autism is characterized by impaired social reciprocity, deficits in communication and abnormal repetitive, restricted behaviours and interests (Lord *et al.*, 2000, Frith and Happé, 2005). The autism spectrum (ASD) acknowledges the variety in the display of impairments seen in autism (Frith and Happé, 2005, Faras *et al.*, 2010). Executive function issues are commonly observed throughout the spectrum (Barendse *et al.*, 2013). Severe cases of autism experience symptoms like evading eye contact, complete absence of speech and even hyperactivity as ASD commonly overlaps with Attention deficit hyperactivity disorder (Liao *et al.*, 2016, Arruda, 2017). Studies have shown that a deficiency of GABA receptors , including GABRB3/GABBR1, in autism can result in memory impairments plus changes in social and exploratory behaviour (DeLorey *et al.*, 2008, Fatemi *et al.*, 2009b, Fatemi *et al.*, 2010). Furthermore, changes in the glutamatergic system have been documented to impair cognition and play a role in repetitive behaviours (Carlsson, 1998, Jamain *et al.*, 2002, Koppers, 2010, Cetin *et al.*, 2015, Horder *et al.*, 2018). This heterogenous disorder typically develops in infancy or at latest in the first three years of existence and includes Asperger's syndrome, Kanner's syndrome, childhood disintegrative disorder, nonspecific pervasive developmental disorder and Rett's syndrome (Lord *et al.*, 2000, Hrabovska and Salyha, 2016).

There are several risk factors which can result in ASD's developing like genetics, as mutations, interaction of certain genes and inheritance has been reported as risks (Frith and Happé, 2005, Faras *et al.*, 2010, Hrabovska and Salyha, 2016). There are also environmental risk factors for autism which include medical procedures, stress, environmental chemicals and drugs (Dietert *et al.*, 2011). Drugs like thalidomide and valproic acid (VPA) have become easier to access, which correlates with the increased prevalence of autism (Dietert *et al.*, 2011).

Valproic acid is used in creating an autism model as it can result in epigenetic modifications (Gadad *et al.*, 2013, Mabunga *et al.*, 2015). VPA is clinically a medication for mood swings as well as epilepsy, however usage of this acid during childbearing years and early pregnancy has raised much concern because for many years, it was associated with several teratogenic effects and as of recent, increased risk for autism (Mabunga *et al.*, 2015). However, autism studies have not fully established how altered neurotransmitter pathways result in autistic-like behaviour as a result of VPA exposure.

The primary aim of this study is to investigate how VPA alters the major neurotransmitter system pathways, which result in autistic-like behaviour.

MATERIALS AND METHODS

Mating

Rats were obtained from the Biomedical Resource Unit of the University of Kwa-Zulu Natal and were kept in a humidity-and-temperature-controlled facility with a 12-hour light/dark cycle with water and food *ad* libitum. All the procedures performed were in accordance with the Animal Research Ethics Committee (AREC) regulations for animal experimentation, in South Africa (AREC/044/018M). 4 adult female rats were placed together for 5 days to synchronise their oestrous cycle. For mating, a ratio of 2 females: 1 male was used. Positive mating was indicated through the presence of sperm in the vaginal smears. The initial positive mating represented the gestational day 0 of the study (GND 0).

Valproic acid model

On gestational day 12.5 (GND 12.5), the pregnant mothers in the experimental group were injected intraperitoneally with a once off 500 mg/kg valproic acid (Sigma-Aldrich, SA) injection in 0.9% saline (100 mg/ml, pH 7.3), whilst the control group were injected with a once off saline injection (0.9%, 3.3 ml/kg). Rats were housed 2 per cage until birth.

Study Subjects

A total of 24 male and female Sprague-Dawley rat pups were obtained from the pregnant dams and used as test subjects in this study. The pups remained undisturbed, with the exception of routine cleaning of the cages once a week, with their mothers in clean cages that contained bedding, water and food. The pups were weaned on PND 21.

Behavioural Assessment

The Novel-object Recognition Test was performed on PND's 22-25 to assess the animal's behaviour when exposed to familiar and novel objects, to examine for memory alterations. Whilst the Open Field Test was performed on PND 26 to test for repetitive behaviour, explorative and motor function

Open field test

The open field test was used to assess the pup's explorative behaviour and motor function (Walsh and Cummins, 1976). Each rat was placed inside a 1m x 1m plexi-glass box, with 50 cm high sides. The box was demarcated with tape into 25 squares of equal size. The rat was placed in the centre square and allowed to move freely for 5-minutes, during which the time in the centre of the square, total number of lines crossed, rears on hind limbs, number of times of urination and defaecation was recorded. The box was cleaned with 70% ethanol after each rat.

Novel-object recognition test

The novel-object recognition test was used to assess the pup's response to a novel object in a 33x55x19.5 cm cage with a floor area of 1815^2. This test consisted of three phases namely, the habituation, familiarization, and test phase (Antunes and Biala, 2012).

The habituation phase: was conducted prior to the test on PND's 22 and 23 for 5-minutes per day in the testing apparatus whereby the pups freely explored the open-field area which lacked objects. The pups were then subjected to a 1-minute retention interval.

The familiarization phase: was thereafter conducted, on the same PND's as mentioned above, whereby a single pup was then placed for 10-minutes into the open-field area which contained 2 identical objects.

The test phase: on PND 24 comprised of an acquisition phase and a test phase, with a 1-hour break in between. In the acquisition phase, the pups were left for 5-minutes to explore the familiar objects that were placed near the 2 corners in the testing apparatus. 1-hour later, the animal was placed back into the testing apparatus, and presented with objects in the same positions as at acquisition, however 1 object was familiar whilst the other was a novel object. The pups were left to explore the objects for 10-minutes and the Discrimination Index (DI), which allows for the differentiation between the time spent exploring the familiar (TF) vs novel (TN) objects, was calculated using the following calculation [DI= (time spent with novel object -time spent with familiar object)/(time with novel object + time with familiar object)] (Antunes and Biala, 2012). This result can vary between +1 and −1, whereby a negative score indicates more time spent with the familiar object, a positive score indicates more time spent with the novel object, and a zero score indicates a null preference (Antunes and Biala, 2012).

Sacrifice and tissue collection

On PND 27 all the pups were euthanised by decapitation. Brain tissue was removed and placed in frozen saline in order to stop all biological processes including loss of proteins. This brain tissue was then dissected, to acquire and remove the hippocampus, cerebellum and prefrontal cortex.

Neurochemical analysis

Glutamate Assay

Glutamate levels were measured in the prefrontal cortex and hippocampi tissue samples using a Glutamate Assay Kit (ab83389) (Abcam Biotechnology, UK). Samples (20mg) were homogenized in assay buffer (200µl) through sonication and incubated on ice for 15 minutes and then centrifuged for 2 minutes at 4°C (15 000 rpm). The samples, standards and background control samples (50µl each) were pipetted into the respective wells in a 96 well plate. The Reaction mix (100µl) was then added in to each of the standard and sample wells whilst the Background Reaction mix (100µl) was added to each of the Background sample wells. This was then followed by mixing the wells and an incubation period of 30

minutes at 37°C. The Optical Density was then measured immediately using a micro-plate reader (SPECTROstar ^{Nano}, BMG Labtech, USA) set at 450nm.

Real Time Polymerase chain reaction (RT PCR)

RT PCR was conducted to assess the expression of Gamma-aminobutyric acid B Receptor 1 (GABBR1) and Gamma-aminobutyric acid type A receptor subunit beta-3 (GABRB3) in the hippocampus and cerebellum. All the primers were obtained from Inqaba Biotechnical Industries.

RNA Extraction

RNA was extracted from the tissue samples using of an RNA extraction kit (Zymo Research, United States). Samples were sonicated in RNA lysis buffer (500µl) then centrifuged at 12 000 rpm then the lysate was transferred to a Zymo-spin column and centrifuged at 8000 rpm for 30 seconds. Ethanol was added followed by centrifuging at 12 000 rpm for 1 minute. Thereafter, RNA prep buffer (400µl) was added and centrifuged for 1 minute at 12 000 rpm. Column was washed twice, first with 800µl and then with 400µl wash buffer. DNase/RNase free water (50µl) was added. The tube was centrifuged at 10 000 rpm for 30 seconds and RNA concentration and purity were determined by the Nanodrop.

CDNA synthesis

CDNA synthesis was performed using the iScript CDNA synthesis kit (Bio-Rad, South Africa). A reaction mix of 20µl was made following the manufacturers guidelines in the protocol. Thereafter, the complete reaction mix was put in to a thermal cycler with these conditions: priming (25°C for 5 minutes), reverse transcription (20 minutes at 46°C), RT inactivation for 1 minute (96°C) and hold (4°C).

RT PCR

The primers were made in to a stock solution according to Inqaba Biotechnical Industries and then further diluted to make working solutions. Each of the PCR tubes contained the following: 3µl nuclease free water, 10µl SYBR Green, 1µl reverse primer, 1µl forward primer and 5µl CDNA sample. GAPDH was used as the housekeeping gene. The Lightcyler 96 was programmed for denaturation (94°C, 5 mins), annealing (GABBR1-60°C), (GABRB3-56°C), (GAPDH-62°C) for 45 seconds and extension (72°C, 8 mins), for 45 cycles. PCR tubes were loaded and run. Data analysis was performed by: gene concentration/GAPDH concentration=final concentration

Receptor	Forward Primer Sequence	Reverse Primer Sequence
GABBR1	CAGCAAGTGTGACCCAGGGCAA	ATCCGGGCAGCCTCAGCTACAA
GABRB3	ATCGAGCTCCCACAGTTCTC	TCAATGAGAGTCGAGGGTAGG

Table 1: The Forward and Reverse primer sequences for GABBR1 and GABRB3

Statistical analysis

Graph Pad Prism Version 7 was used for statistical analysis. For all the tests performed, groups were compared using an Unpaired t test. P values of <0.05 were considered significant. All data sets are shown as the mean ±SEM.

RESULTS

The Open Field Test

The open field test was performed in the control and VPA groups to assess rearing and ambulation.

Rearing in the Open Field Test:

The VPA group had a significant increase in the number of rearing's when compared to the control NS group *(Control vs VPA; p<0,0001, Fig 1) which indicates a VPA effect.

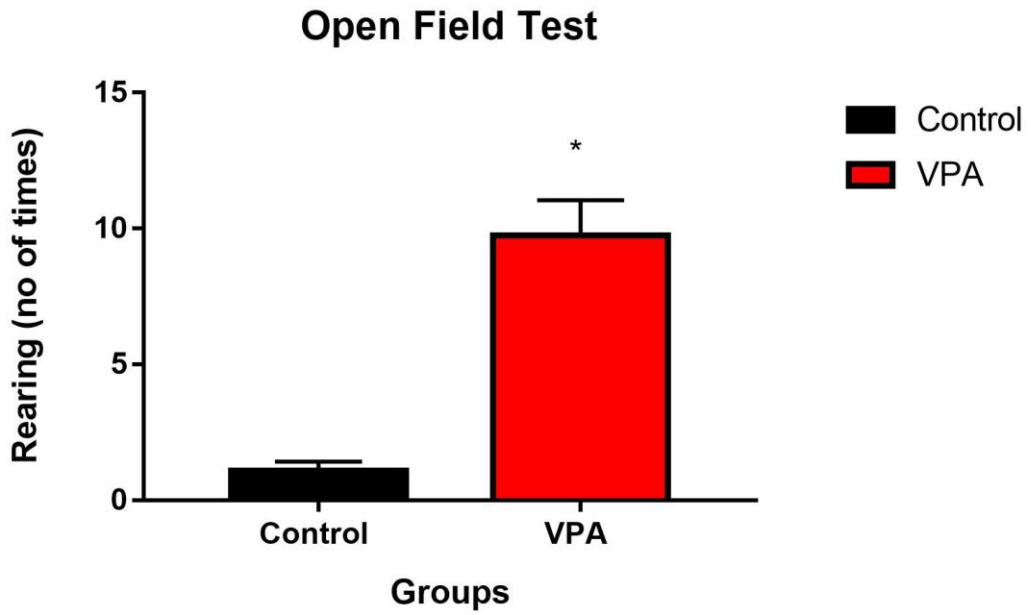

Figure 1: The frequency of rearing in the control and VPA group during the Open field test (n=12). *(Control vs VPA; p<0,0001)

Ambulation in the Open Field Test:

The VPA group significantly ambulated more when compared to the control group *(Control vs VPA; p<0,0001, Fig 2) which indicates a VPA effect.

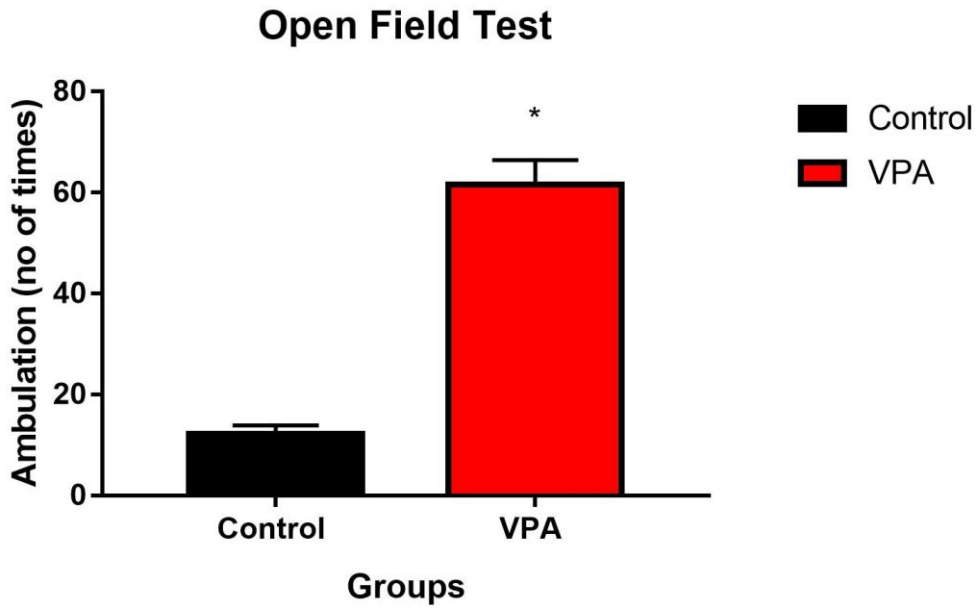

Figure 2: The number of lines crossed by the animals in the control and VPA group during the Open field test (n=12). *(Control vs VPA; p<0,0001)

The Novel Object Recognition Test

The novel object recognition test was performed in the control and VPA groups to determine the discrimination index.

Discrimination index from the Novel Object Recognition Test:

The VPA group had a significantly lower discrimination index when compared to the control group *(Control vs VPA; p = 0,0431, Fig 3) which indicates a VPA effect.

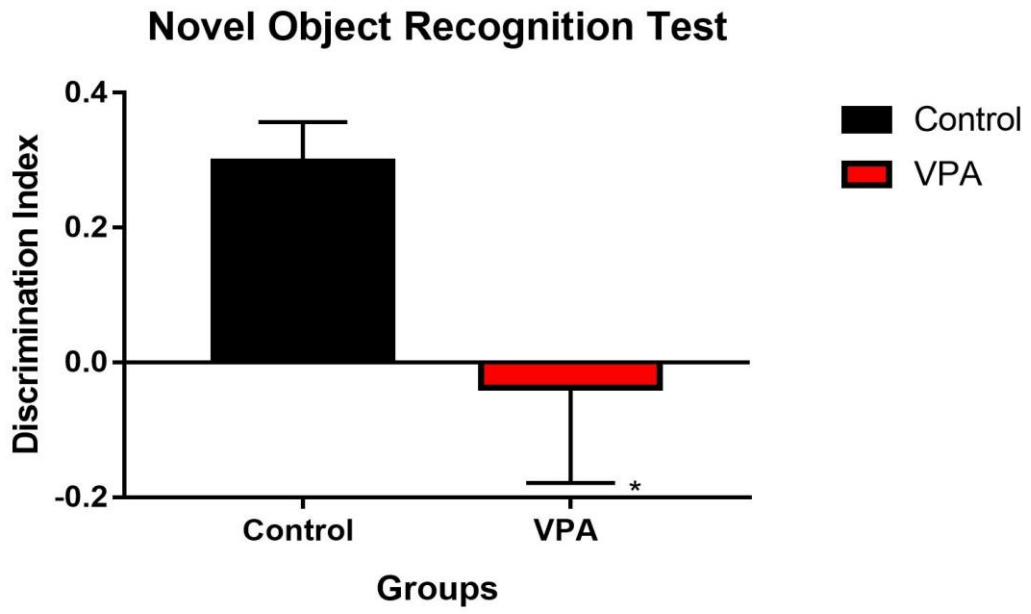

Figure 3: The Discrimination indexes obtained for the control and VPA group during the novel object recognition test (n=12). *(Control vs VPA; p = 0,0431)

Glutamate concentration

A Glutamate assay was conducted to determine the glutamate concentration in the control and VPA groups.

The Glutamate concentration in the prefrontal cortex and hippocampus:

The VPA group exhibited significantly lower glutamate concentrations in their prefrontal cortices*(control vs VPA; p= 0,0095, Fig 4A) and hippocampi *(control vs VPA; p= 0,0036, Fig 4B) when compared to the control group which indicates a VPA effect.

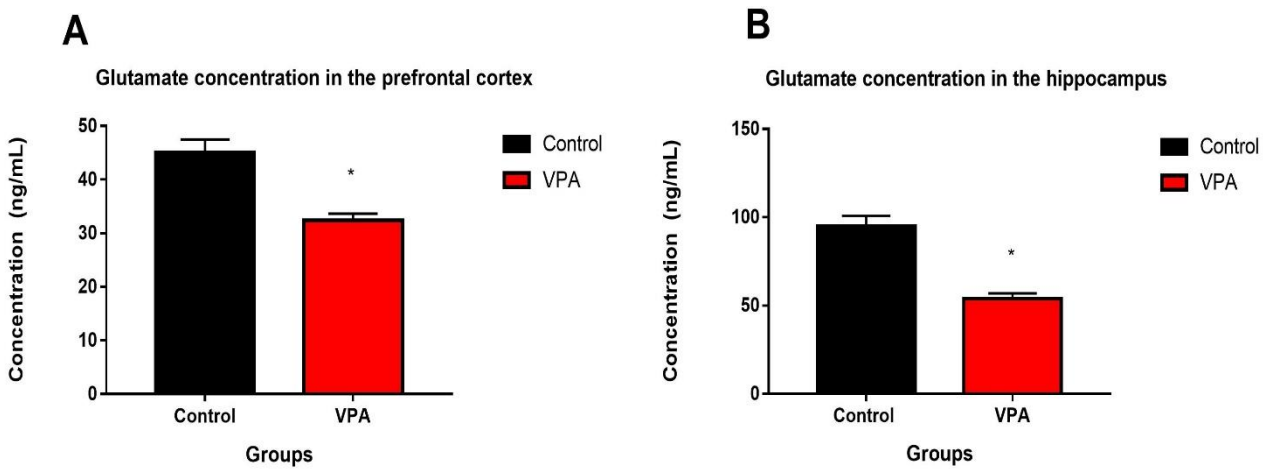

Figure 4: The concentration (ng/mL) of glutamate in the prefrontal cortex (A) and hippocampus (B) observed in the control and VPA group (n=3). 4(A) (control vs VPA; p=0,0095) 4(B) *(control vs VPA; p= 0,0036)

GABA Receptors

RT PCR was conducted to determine the of Gamma-aminobutyric acid B Receptor 1 (GABBR1) and Gamma-aminobutyric acid type A receptor subunit beta-3 (GABRB3) concentrations in the control and VPA groups.

The GABA B Receptor 1 concentration in the hippocampus and cerebellum:

There were no differences in the hippocampi and cerebellums of the VPA and control groups ($p>0,05$).

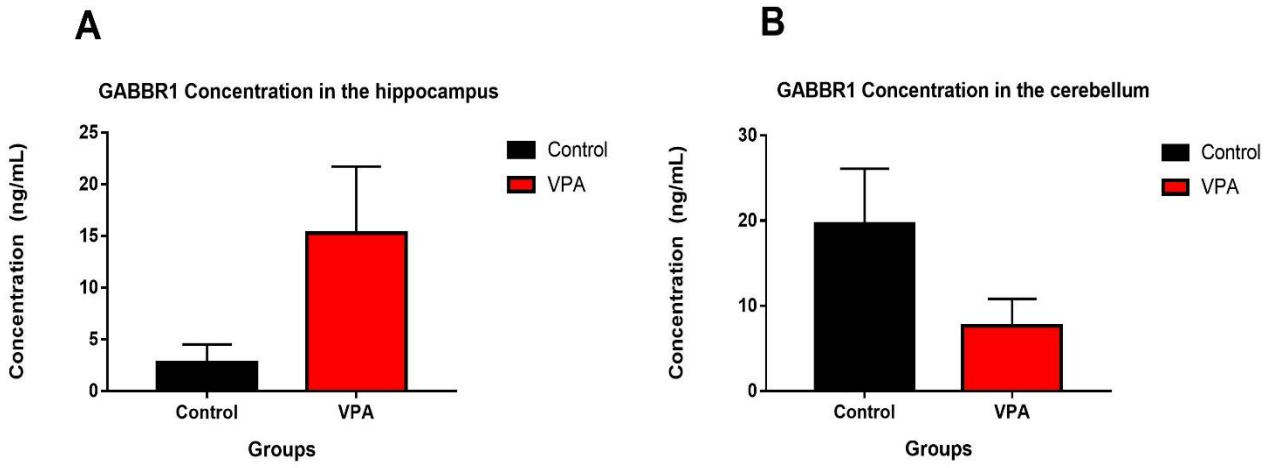

Figure 5: The concentration (ng/mL) of GABBR1 in the hippocampus (A) and cerebellum (B) of the control and VPA group (n=6).

The GABA type A receptor subunit beta-3 concentration in the hippocampus and cerebellum:

The VPA group exhibited significantly higher GABRB3 concentrations in their hippocampi when compared to the control group *(control vs VPA; p =0,0029, Fig 6A) which indicates a VPA effect.

There were no differences in the GABRB3 concentration in the cerebellums of the control and VPA groups (p>0,05) indicating that there was no VPA effect.

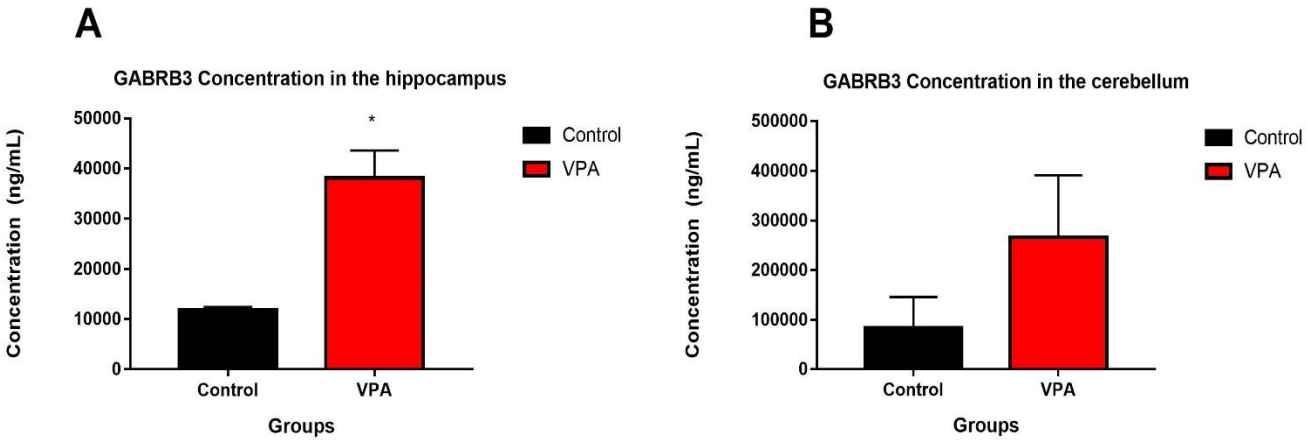

Figure 6: The concentration (ng/mL) of GABRB3 in the hippocampus(A) and cerebellum (B) of the control and VPA group (n=4/6). 6(A) *(control vs VPA; p =0,0029)

DISCUSSION

The current study investigated the mechanism of action used by VPA to cause an autistic-like phenotype in a rat model by assessing the behaviour, glutamatergic and GABAergic systems.

The open field test was conducted to assess rearing and ambulation. Our results show an increase in rearing following exposure to VPA. Several studies have documented rearing to be a repetitive behaviour (Ryan *et al.*, 2010, Kim *et al.*, 2016, Hisaoka *et al.*, 2018). This result is similar to Hisaoka *et al.* (2018), who also found an increase in rearing. Repetitive behaviour is one of the core symptoms of ASD and different models have been used to assess this phenomenon (Ryan *et al.*, 2010, Kim *et al.*, 2016, Hisaoka *et al.*, 2018). ASD models have observed ambulation as a sign of locomotive function, exploration and even as being a form of emotional behaviour but there is inadequate proof (Archer, 1973, Tatem *et al.*, 2014). Our results show an increase in ambulation following VPA exposure. This is a result of hyperactivity in a new environment, which has been previously documented by Narita *et al.* (2010) and also observed in human autism.

We further conducted the novel object recognition test to determine the discrimination index as a measure of memory. Our study documented positive discrimination indexes for the control group in contrast to the VPA group. This indicated that the control pups spent more time with the novel object whilst the VPA pups spent more time with the familiar object. According to Lueptow (2017) if novel object recognition memory is intact then the animals will remember the familiar object and will therefore spend more time with the novel object. Hence this suggests that the memory of the control group was intact, whilst the VPA animal's memory was impaired (Antunes and Biala, 2012, Lueptow, 2017). Hou *et al.* (2018) and Banerjee *et al.* (2014) documented that rats exposed to VPA also exhibited memory impairments. This result is imperative since past studies have documented that prenatal exposure to VPA results in the offspring's exhibiting autistic behaviour and autistic individuals have been associated with episodic memory deficits which includes explicit retrieval of past experiences (Cooper *et al.*, 2017, Fujimura *et al.*, 2017).

To further confirm the behaviour observed in this study we assessed the glutamate neurotransmitter system as repetitive behaviour is due to an imbalance of the excitatory/inhibitory signalling in the developmental period (Mehta *et al.*, 2011). This study observed abnormally decreased glutamate levels in the hippocampi of the VPA exposed animals which is in agreement with numerous past studies (Carlsson, 1998, Purcell *et al.*, 2001, Jamain *et al.*, 2002, Cetin *et al.*, 2015). Disrupted signalling of glutamate is imperative in repetitive behaviour as hypo-glutamatergic animal models have been documented to exhibit autistic characteristics including restricted behaviour with the inability to change behaviour and deficient habituation (Jamain *et al.*, 2002, Koppers, 2010). Evidence suggests that

glutamate experiences disrupted signalling in autism, especially the signalling of its metabotropic glutamate receptors, which plays a part in dendritic translation (Mehta *et al.*, 2011, Peralta *et al.*, 2016). In addition, the amygdaloid-hippocampal pathway is overly activated in ASD models (Kim *et al.*, 2016). VPA has been documented to cause mGluR1a (metabotropic glutamate receptor type 1) over-functioning in the hippocampus which alters definite regulatory pathways that affect the processing of the hippocampus which may be the cause of autistic behaviour (Peralta *et al.*, 2016). This study observed memory deficits in the VPA rats which are also seen in autistic individuals whom are associated with episodic memory deficits (Cooper et al., 2017, Fujimura et al., 2017). The glutamate concentration was used to confirm this change as glutamate is essential for cognitive functions like learning and memory (Carlsson, 1998, Jamain *et al.*, 2002, Koppers, 2010, Cetin *et al.*, 2015, Horder *et al.*, 2018). Our study observed that the VPA exposed rats had decreased glutamate in the PFC. This finding was also documented by several other studies (Carlsson, 1998, Jamain *et al.*, 2002, Koppers, 2010, Cetin *et al.*, 2015, Horder *et al.*, 2018). This alteration in the glutamate neurotransmitter system suggests that there are alterations in the glutamate receptors (Jamain *et al.*, 2002, Tarabeux *et al.*, 2011, Uzunova *et al.*, 2014). Uzunova *et al.* (2014) documented alterations of the NMDAR in the PFC which is essential for cognitive processes and these alterations could possibly result in memory deficits. Whilst Jamain *et al.* (2002) documented that a dysfunction of the glutamate receptor 6 (GluR6), which is a member of the ionotropic kainate receptors, plays a role in learning impairments of autistic subjects. Furthermore, mutations of this receptor could pose consequences on the integration of excitatory synaptic signals which control these behavioural aspects and alterations in the editing process of GluR6 mRNA, can also play a role in the autistic phenotype (Jamain *et al.*, 2002). Our results suggest that autism is a Hypo-glutamatergic condition which is in agreement with numerous past studies (Carlsson, 1998, Purcell *et al.*, 2001, Jamain *et al.*, 2002, Cetin *et al.*, 2015).

This study observed hyperactivity in VPA-rats which is also seen in human autism and is linked to cerebellar abnormalities (Pierce and Courchesne, 2001, Narita *et al.*, 2010). We further confirmed this behavioural change by assessing the GABAergic neurotransmitter system as numerous studies have shown that disruption in this pathway is associated with the autism pathogenesis (Chen *et al.*, 2014). The current study found that the rats exposed to VPA showed no difference, however a decreasing trend in the GABBR1 concentration was seen in the cerebellum. Autism presents abnormal cerebellar development and these abnormalities are considered to be responsible for the dysfunction of the motor system commonly observed (Nayate *et al.*, 2005, Fatemi *et al.*, 2009b, Rogers *et al.*, 2013). The motor deficits seen in autism is linked to cerebellar irregularities like Purkinje cell loss (Fatemi *et al.*, 2012, Rogers *et al.*, 2013). In addition, Fatemi et al. (2009a) found significantly decreased levels of GABBR1 in the cerebellums of individuals with autism. The current study however, did not document a significant difference which could possibly be due to using rat brain tissue as compared to human brain tissue used by Fatemi *et al.* (2009a) or because our autistic rats did not comorbidly experience seizures, which cause

a long-term decrease in GABBR1 (Han *et al.*, 2006). Another candidate gene for autism is GABRB3, which is essential during development for mediating excitatory signalling, differentiation and neuronal growth (Warrier et al., 2013, Chen et al., 2014). Our study found that the VPA exposed rats showed no difference, however an increasing trend for the GABRB3 concentration was observed in the cerebellum. We found that the VPA exposed rats had an increase in GABRB3 and showed no difference, but an increasing trend for the GABBR1 concentration in the hippocampus. Dickerson *et al.* (2011) found a significant increase of GABAB receptor 1 whilst, in 2014 (Chen *et al.*) found that increased expression of GABRB3 contributes to the pathogenesis of autism. Chen *et al.* (2014) suggested that missense mutations and rare variants in the 5′ regulatory region of GABRB3 is associated with autism which implies altered gene expression of GABRB3 is involved in autism's neurobiology. Such mutations can influence the structure of the secondary protein and change a phosphorylation-based binding motif or phosphorylation-based substrate motif which could have a functional effect on GABRB3 (Chen *et al.*, 2014). Furthermore, the dysfunction in the neurons of the GABAergic system has been suggested to cause a sensitization and/or upregulation of the postsynaptic GABAA receptors, which is possibly the reason for increased expression of GABRB3 (Rudolph and Möhler, 2014). Gaetz *et al.* (2014) found that individuals with autism exhibited decreased levels of GABA in the motor cortex and this could possibly be the reason for the upregulation of GABRB3. In addition, Rudolph and Möhler (2014) documented that an upregulation of α5 GABAA receptors impaired cognitive performance, which is significant in the current study where we see an upregulation of GABA receptors and impaired cognitive function. Impaired memory may be a result of disruptions in the firing activity of the cortical networks which is usually arranged by the parvalbumin interneurons, the latter of which is insufficient in GABA release due to reduction in GAD67 expression (Charych *et al.*, 2009). Mice lacking GABRB3 and GABBR1 have been documented to exhibit several behavioural abnormalities including deficits in memory and learning therefore, alterations in the expression of these receptors may lead to these memory and learning deficits (Fatemi *et al.*, 2009a, Chen *et al.*, 2014). In addition, mutations of these receptors may be associated with autism and even their expression may be affected by epigenetic alterations (Fatemi et al., 2009a, Chen et al., 2014). Our study however, did not document a significant difference for the GABRB3 concentration in the cerebellum which could be due to the fact that Chen *et al.* (2014) used human patients from Taiwan and genomic DNA was prepared from the peripheral blood.

CONCLUSION

This study observed that exposure to VPA results in alterations in the functioning of the GABAergic and glutamatergic systems which result in the autistic-like phenotype. This was evident as exposure to VPA resulted in decreased glutamate in the hippocampus and PFC. VPA exposure also resulted in increased GABRB3 in the cerebellum and hippocampus as well as in GABBR1 of the hippocampus whilst it caused a decrease in GABBR1 of the cerebellum. These alterations resulted in repetitive

behaviour, hyperactivity and impaired memory. Our results show that valproic acid exposure alters the glutamatergic and GABAergic systems which results in the autistic-like phenotype.

DECLARATION OF INTEREST

We hereby disclose that there are no known financial and personal conflict of interests in the work of this project.

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CHAPTER THREE

Manuscript 2

The manuscript consists of a brief introduction, a detailed materials and methods section followed by the results and discussion sections. Previously, we have established that valproic acid results in the autistic-like phenotype. This manuscript focuses on the effects of maternal separation stress with prenatal exposure to VPA.

Evaluating the effects of maternal separation stress on prenatally exposed valproic acid rats

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Abstract

Introduction: There are numerous risk factors for autism, including the exposure to stress. The HPA axis, which is the primary stress system, is activated in the event of stress. However prolonged stress and early traumatic life events can result in the dysregulation of the HPA axis. Maternal separation is an example of prolonged stress in the first two weeks of life. Stress has also been found to exacerbate autism symptoms. The valproic acid animal model is used globally to induce autism. This study was conducted to determine the effects of maternal separation stress with VPA.

Methods: This study used forty-eight rat pups which were divided in to control (saline-0.9%, 3.3 ml/kg), control stressed, VPA (VPA-500 mg/kg) and VPA stressed groups. The stressed groups were subjected to maternal separation stress from postnatal day 2-14 for 3-hours (9h00 to 12h00) each day. The open field test was conducted to assess the pup's behaviour. Corticosterone and Serotonin Transporter ELISA's were conducted to measure their concentrations.

Results: Stress exposure with VPA caused a dysregulation in the HPA axis resulting in a sensitized stress response. Exposure to stress and VPA also resulted in decreased Serotonin transporter concentrations in the amygdala and prefrontal cortex. These alterations were accompanied by anxiety-like behaviours, namely a decrease in the time spent in the centre and total excretion.

Conclusion: This study showed that valproic acid together with maternal separation stress causes dysregulation in the HPA axis and altered serotonergic signalling.

Key Words: Autism, Valproic acid, Maternal Separation Stress, Corticosterone, Serotonin Transporter

Introduction

Stress can occur in different forms (Dietert *et al.*, 2011). The hypothalamic-pituitary-adrenocortical (HPA) axis regulates stress by stimulating the release of glucocorticoids known as cortisol or corticosterone (Corbett and Simon, 2014). These glucocorticoids are essential for re-establishing homeostatic conditions (Aguilera, 2011, Stephens and Wand, 2012). During the first 14 days of life, the stress system is developed and there are low glucocorticoid levels hence being termed the stress-hyporesponsive period (Lai and Huang, 2011, Schmidt, 2019). If stress is experienced in the early years of life or over a prolonged period of time it can result in the dysregulation of the HPA axis causing sensitized stress response, adrenal exhaustion and can further lead to alterations in brain functions and neurobehavior (Thomas and Lena, 2010, Herman *et al.*, 2011, Lai and Huang, 2011). Chronic stressors can change the responsiveness of the HPA axis, causing it to shift from being over-responsive to non-responsive (Thomas and Lena, 2010). Prolonged exposure to stress hormones can also predispose psychological, metabolic or immune alterations (Aguilera, 2011). The environment to which an individual is exposed to during their first years of life has a powerful influence on the development of their behavioural responses in adulthood (Weiss *et al.*, 2011). Maternal separation is an example of prolonged early life stress, which disturbs the mother infant bond during the stress-hyporesponsive period to model postnatal stress (Weiss *et al.*, 2011, Nishi *et al.*, 2013). Stress and trauma are not only a risk factors for autism, but have been documented to exacerbate autistic symptoms (Fuld, 2018).

Autism is a pervasive developmental disorder which is characterized by impaired social reciprocity, deficits in communication and by abnormal repetitive, restricted behaviours and interests (Lord *et al.*, 2000, Frith and Happé, 2005, Faras *et al.*, 2010). A dysfunction in the major neurotransmitter systems like the GABAergic, glutamatergic and serotonergic are linked to autism pathogenesis (Trottier *et al.*, 1999). Serotonin is particularly important in autism as it plays a role in the development of social skills and alterations in serotonin during early life can cause irregularities in its metabolism (Brummelte *et al.*, 2017, Cetin *et al.*, 2015). There is no general agreement with regard to the risk factors or what causes autism (Hrabovska and Salyha, 2016). However, exposure to certain environmental conditions like stress, chemicals or drugs are risk factors for autism (Dietert *et al.*, 2011). Valproic acid (VPA) is an anticonvulsant drug, however usage of this drug by pregnant women is associated with the increased risk of autism (Gottfried *et al.*, 2013, Mabunga *et al.*, 2015, Ornoy *et al.*, 2019). VPA is used therefore used to create autism animal models, as it can result in epigenetic modifications (Gadad *et al.*, 2013, Mabunga *et al.*, 2015). In rats, embryonic exposure to VPA, approximately at the time of closure of the neural tube, results in autistic-like behaviour and anatomical abnormalities in the progeny (Favre *et al.*, 2013). In addition, VPA alters the GABAergic and glutamatergic neurotransmitter systems which are linked to the core symptoms experienced in autism (Carlsson, 1998, Jamain *et al.*, 2002, Koppers, 2010, Cetin *et al.*, 2015, Horder *et al.*, 2018).

This study aims to determine the effects of maternal separation stress together with prenatal exposure to VPA.

Materials and Methods

Mating

Rats were obtained from the Biomedical Resource Unit of the University of Kwa-Zulu Natal and were kept in a humidity-and-temperature-controlled facility with a 12-hour light/dark cycle with water and food *ad* libitum. All the procedures that were performed were in accordance with the Animal Research Ethics Committee (AREC) regulations for animal experimentation, in South Africa (AREC/044/018M). Eight adult female rats were placed together for 5 days to synchronise their oestrous cycle, which is described as the "special period of sexual desire of the female" by Westwood (2008). For mating, a ratio of 2 females: 1 male was used. Positive mating was indicated through the presence of sperm in the vaginal smears. The initial positive mating represented the gestational day zero of the study (GND 0).

Valproic acid model

On gestational day 12.5 (GND 12.5), the pregnant dams in the experimental group were injected intraperitoneally with a once off 500 mg/kg valproic acid (Sigma-Aldrich, SA) injection in 0.9% saline (100 mg/ml, pH 7.3), whilst the pregnant dams in the control group were injected with a once off saline injection (0.9%, 3.3 ml/kg). Rats were housed/kept 2 per cage until birth.

Study Subjects

A total of 48 male and female Sprague-Dawley rat pups were obtained from the pregnant dams and used as test subjects in the following study. The control and VPA non-stressed pups remained undisturbed, with the exception of routine cleaning of the cages once a week, with their mothers in clean cages that contained bedding, water and food. Whilst the control and VPA stressed pups were separated from their mothers to conduct the maternal separation stress protocol. All pups were weaned on PND 21.

Maternal separation stress protocol

The rat pups were separated in to groups, shown in table 1, according to the injections their mothers were given, namely VPA and control (saline). These groups were then further split in-to groups that were stressed and groups that were not exposed to stress.

CONTROL GROUP		VPA GROUP	
Control Non-stressed	Control Stressed	VPA Non-stressed	VPA Stressed
Mother had saline injection	Mother had saline injection	Mother had VPA injection	Mother had VPA injection
No Stress	Stressed	No stress	Stressed

Table 1: How the different groups were split based on injections and stress exposure

2 days after birth, the rat pups that were to be stressed in the experimental and control groups were subjected to stress through maternal separation from postnatal day (PND) 2-14. These pups were stressed by using the 3-hour model (9h00 to 12h00). This essentially means that the dams were placed in to clean cages, whilst the pups remained in the home cage alone for 3-hours for each day. Dams and litters were situated in such a way that there was no olfactory and visual contact. After the 3-hour interval the dams were returned to their home cage with the pups.

Assessment of pups

The Open Field Test was performed on PND 26 to the pup's anxiety and stress.

Open field test

The open field test was used to assess the pup's stress and anxiety (Kristina *et al.*, 2017). Each rat was placed inside a 1m x 1m plexi-glass box, with 50cm high sides. The box was demarcated with tape into 25 squares of equal size. The rat was placed in the centre square and allowed to move freely for five minutes, during which the time in the centre of the square, total number of lines crossed, rears on hind limbs, number of times of urination and defaecation was recorded. The box was cleaned with 70% ethanol after each rat.

Sacrifice and tissue collection

On PND 27 all the pups were euthanised by decapitation. Blood was collected in EDTA coated collecting tubes using a funnel. Once the blood was in the collecting tubes, it was then shaken and stored in a Bio freezer until it was required for neurochemical analysis. Brain tissue was removed and placed in frozen saline in order to stop all biological processes including loss of proteins. This brain tissue was then dissected, to acquire and remove the amygdala and prefrontal cortex for further analysis.

Neurochemical analysis

The following study used a corticosterone enzyme-linked immunosorbent assay (ELISA) kit and Serotonin Transporter ELISA kit to determine the concentration of corticosterone and serotonin transporter respectively.

Corticosterone ELISA

The Corticosterone (CORT) levels were measured in plasma samples using an ELISA kit (Elabscience Biotechnology, USA) which used the competitive ELISA principle. The samples and standards (50µl each) were pipetted into the respective wells on the precoated ELISA plate. Detection Ab (50µl) was immediately added to each well, followed by incubation for 45-minutes at 37°C. After incubation, the wells were then washed 3 times using a Wash Buffer (350µl per wash) and thereafter Enzyme Conjugate was added to each well (100µl). This was followed by an incubation period of 30-minutes at 37°C. The wash step was repeated and the Substrate Solution was added (90µl). After a 15-minute incubation period the Stop Solution (50µl) was added to each well and the Optical Density was measured immediately utilizing a micro-plate reader set at 450nm.

Serotonin Transporter ELISA

The Serotonin Transporter levels were measured in the amygdala and prefrontal cortex using a Rat Serotonin Transporter (SERT) ELISA kit (Elabscience Biotechnology, USA) which used the Sandwich-ELISA principle. The tissue was homogenized in PBS (tissue weight (mg): PBS (µl), 1:9) through sonication. The samples and standards (100µl each) were pipetted into the respective wells on the precoated ELISA plate and was then incubated for 90-minutes (37°C). Liquid was removed without washing and Detection Ab (100µl) was then added to each well, followed by incubation for 60-minutes (37°C). Wells were then washed 3 times using Wash Buffer (350µl per wash) and thereafter Enzyme Conjugate was added (100µl). This was then incubated for 30-minutes (37°C). Wells were washed again and Substrate Solution was added (90 µl). Following a 15-minutes incubation period at 37°C, Stop Solution (50µl) was added to each well and the Optical Density was measured immediately using a micro-plate reader set at 450nm.

Statistical analysis

Graph Pad Prism Version 7 was used for statistical analysis. For all the tests performed, groups were compared using a Two-way ANOVA. The Turkey's post-hoc test was then performed to determine significance between groups. P values of <0.05 was considered significant. All data sets are shown as the mean ±SEM.

Results

The Open Field Test

The open field test was performed in the control non-stressed (NS), control stressed (S), VPA non-stressed (VPA NS) and VPA stressed (VPA S) groups to assess the time spent in the centre and total excretion.

The time spent in centre during the Open field test:

The VPA S group spent significantly less time in the centre when compared to the VPA NS group *(VPA NS vs VPA S; p=0,0002; $F_{(1, 44)} = 32,21$, Fig 1) which indicates a stress factor. Furthermore, The VPA S group also spent less time in the centre when compared to the control NS group, however this result is not statistically significant (p>0,05). There were no differences between the NS groups (p>0,05).

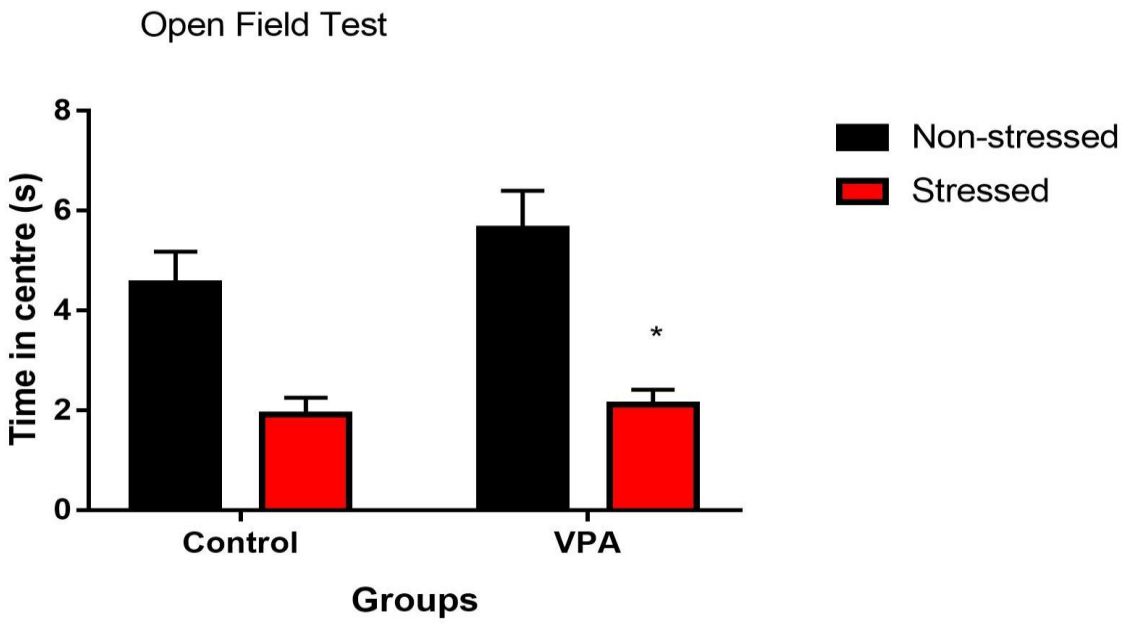

Figure 1: The time spent in the centre by the respective groups (control NS, control S, VPA NS and VPA S) during the Open field Test. All data are expressed as mean ±SEM, (n=12). *(VPA NS vs VPA S; p=0,0002)

The Total excretory waste during the Open field test:

The VPA S group had a significant reduction in waste when compared to the VPA NS group *(VPA NS vs VPA S; p=0,0044; $F_{(1, 44)} = 13,85$, Fig 2) which indicates a stress effect. The VPA S group also excreted less waste when compared to the control NS group, whilst the VPA NS group excreted more waste when compared to the control NS group however these results are not statistically significant (p>0,05).

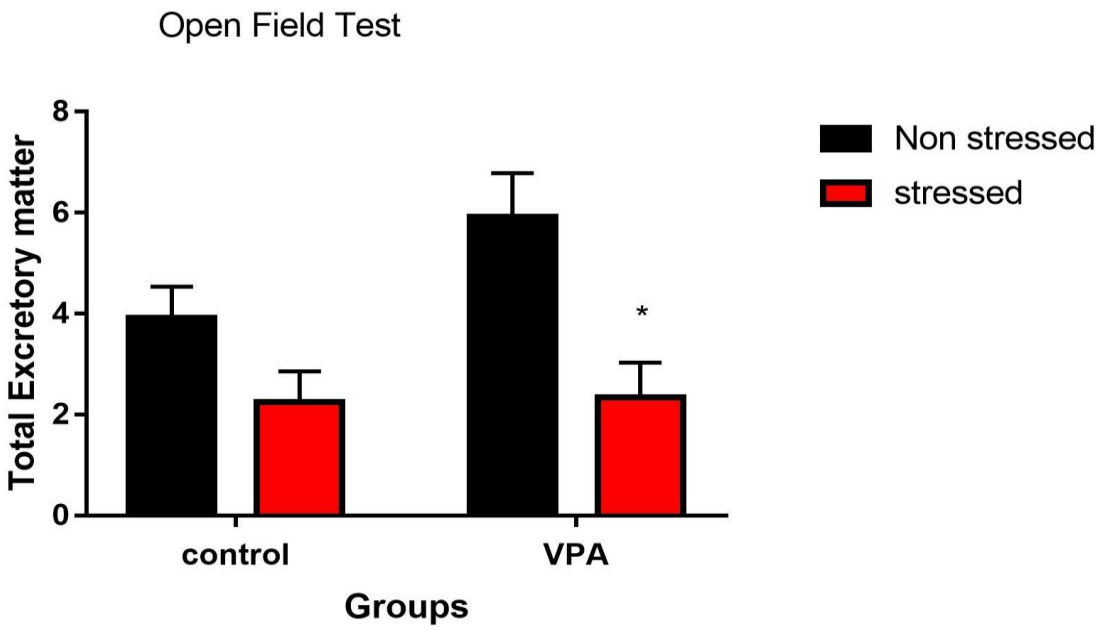

Figure 2: The total excretory matter of animals in the respective groups (control NS, control S, VPA NS and VPA S) during the Open field test. All data are expressed as mean ±SEM, (n=12). *(VPA NS vs VPA S; p=0,0044)

Corticosterone concentration

The Corticosterone concentration observed in the blood:

A corticosterone ELISA was conducted to determine the corticosterone concentration in the control non-stressed (NS), control stressed (S), VPA non-stressed (VPA NS) and VPA stressed (VPA S) groups.

The VPA NS group also exhibited a significantly higher corticosterone concentration when compared to the control NS group **(Control NS vs VPA NS; p=0,0025; $F_{(1, 8)}$ = 17,8, Fig 3) which indicates a VPA effect.

The control S group exhibited a significantly higher corticosterone concentration compared to the control NS group *(Control NS vs Control S; p=0,0337; $F_{(1, 8)}$ = 1,822, Fig 3) whilst the VPA S group exhibited a significantly lower corticosterone concentration when compared to the VPA NS group ****(VPA NS vs VPA S; p<0,0001; $F_{(1, 8)}$ = 1,822, Fig 3), which indicates a stress effect.

Furthermore, the VPA S group exhibited a significantly lower corticosterone concentration compared to the control S group ****(Control S vs VPA S; p=0,0003; $F_{(1, 8)}$ = 83,64, Fig 3) which indicates an overall effect from both stress and VPA.

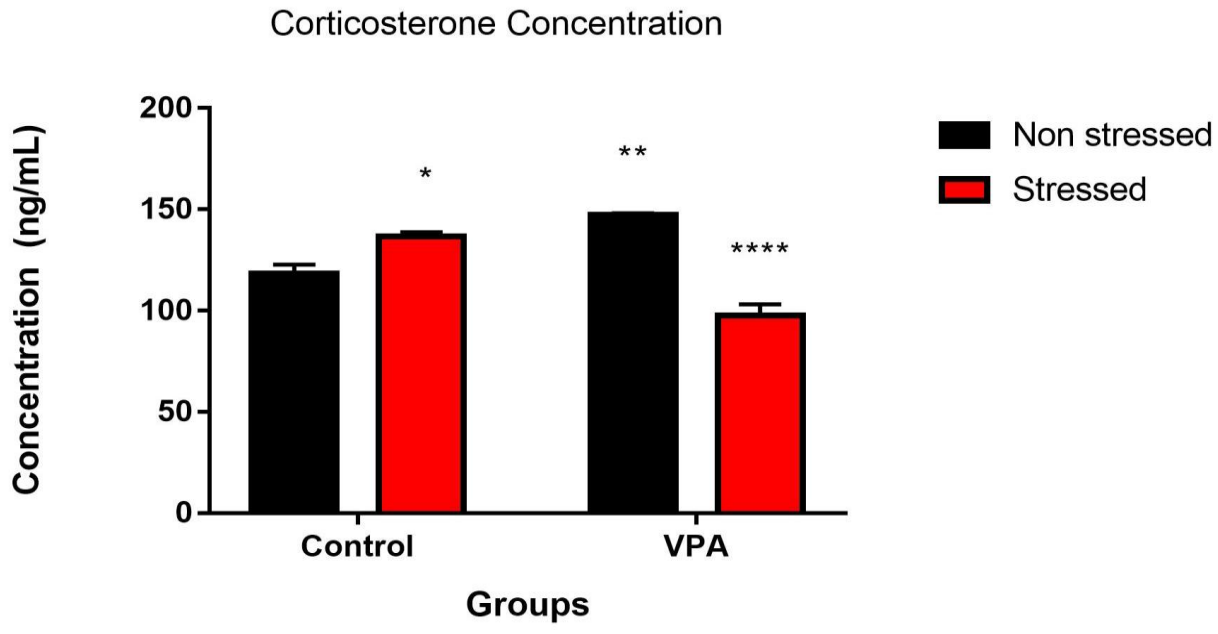

Figure 3: The concentration (ng/ML) of corticosterone observed in the blood from animals of the respective groups (control NS, control S, VPA NS and VPA S). All data are expressed as mean ±SEM, (n=3). *(Control NS vs Control S; p=0,0337), ****(VPA NS vs VPA S; p<0,0001), **(Control NS vs VPA NS; p=0,0025), ****(Control S vs VPA S; p=0,0003)

Serotonin transporter concentration

The Serotonin transporter concentration in the amygdala and prefrontal cortex:

A serotonin transporter ELISA was conducted to determine the SERT concentration in the control non-stressed (NS), control stressed (S), VPA non-stressed (VPA NS) and VPA stressed (VPA S) groups.

The control S group exhibited significantly higher concentrations of SERT in their amygdalae compared to the control NS group *(control NS vs control S; p = 0,0195; $F_{(1, 8)}$ = 1,115, Fig 4A) which indicates a stress effect.

Whilst the VPA S group exhibited significantly lower concentrations of SERT in their amygdalae when compared to the control S group **(control S vs VPA S; p = 0,0013; $F_{(1, 8)}$ = 19,68, Fig 4A) which indicates an overall effect from both stress and VPA.

The VPA S group exhibited the lowest levels of SERT in their PFC's when compared to all the other groups, however these differences observed are not statistically significant (p>0,05) which indicates that there was neither a stress or prenatal exposure to VPA effect.

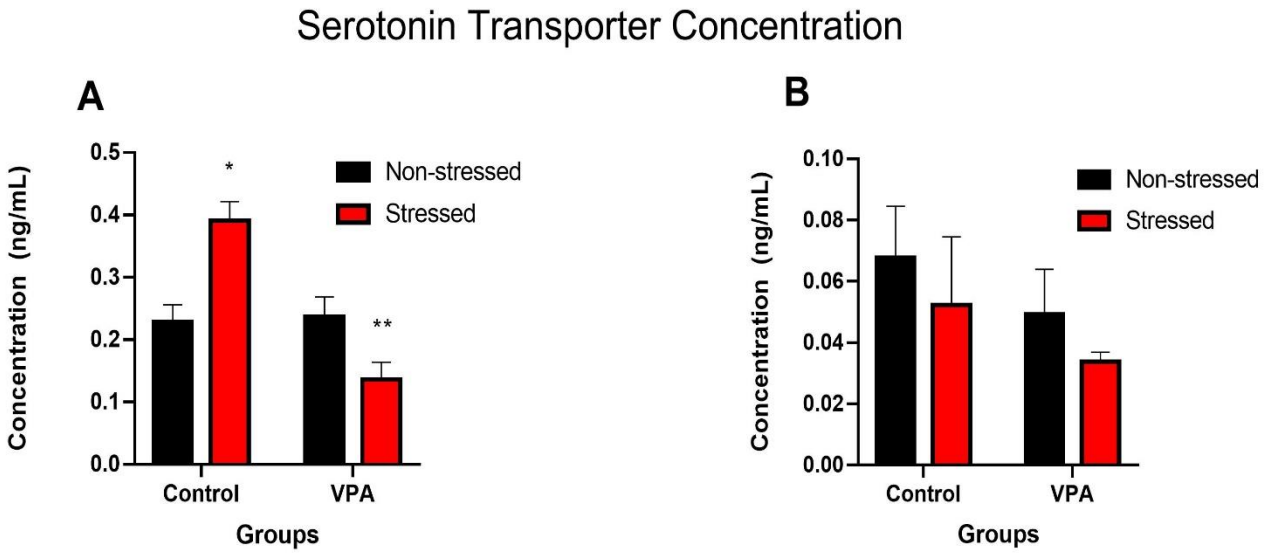

Figure 4: The concentration (ng/mL) of SERT observed in the amygdala (A) and prefrontal cortex (B) of the respective groups (control NS, control S, VPA NS and VPA S). All data are expressed as mean ±SEM, (n=3). 4(A) *(control NS vs control S; p = 0,0195), **(control S vs VPA S; p = 0,0013)

Discussion

The current study investigated how maternal separation stress affected rats that were prenatally exposed to VPA by assessing behaviour, corticosterone and the serotonergic system.

The open field test is one of the most commonly used tests in behavioural research which examines anxiety-related responses and emotionality in rats (Sestakova *et al.*, 2013). The current study observed that the VPA stressed group spent less time in the centre when compared to the VPA non-stressed group. This is an important finding since Sestakova *et al.* (2013) interpreted less time spent in the centre and more time spent in the periphery/corners to be a sign of anxiety. In addition, stress and the emerging rise in glucocorticoid levels are said to be involved in depressive disorder's pathophysiology (Chiba *et al.*, 2012). Chiba *et al.* (2012), documented that chronic stress in rats resulted in depressive and anxiety-like behaviour. These findings mentioned above are in agreement to the current study which observed anxiety-like behaviour due to stress. Defaecation and urination is a negatively associated measure of a rodent's emotions and is also a measure of anxiety (Seibenhener and Wooten, 2015). According to Tobach (1966) urination and defaecation can be merged together to generate a composite elimination - score. The current study found that the VPA stressed group excreted less when compared to the VPA non-stressed group, which is in agreement to Roth and Katz (1979), who documented that stressed animals defaecated less during the open field test. The increase in excretion in the non-stressed group could be a result of the animal being fearful/anxious about its exposure to the new and possibly unsafe environment, which is further confirmed by the neurochemical analysis (Sestakova *et al.*, 2013).

Stress leads to the activation of the hypothalamic–pituitary–adrenal (HPA) axis which is responsible for regulating the glucocorticoid levels and the main circulating glucocorticoid in rodents is corticosterone (Raubenheimer *et al.*, 2006, Mishima *et al.*, 2015). Corticosterone is the crucial stress hormone which is manufactured in the adrenal glands' cortex and has a regulating responsibility for the HPA axis activity in rodents (Mishima *et al.*, 2015). In the event of stress, corticosterone is responsible for promoting self-maintenance as well as escape behaviours, increasing certain immune parts and makes stored energy readily available (Lattin and Romero, 2014). Therefore, to further confirm the stress observed in the pup's behaviour we assessed the corticosterone concentration where we observed a higher corticosterone concentration in the control stressed group when compared to the control non-stressed group. This result is expected since the exposure to stress activated of HPA axis which resulted in the production of corticosterone (Raubenheimer et al., 2006, Mishima et al., 2015). Whilst, the VPA non-stressed group exhibited a higher corticosterone concentration than the VPA stressed group. If stress is experienced in the early years of life or over a prolonged period of time it can result in the dysregulation of the HPA axis causing sensitized stress response as seen in the current study (Thomas and Lena, 2010, Herman *et al.*, 2011, Lai and Huang, 2011). Many studies with chronic stress have

documented decreased corticosterone negative feedback and even expression of receptors in the related brain areas (Sapolsky *et al.*, 1984, Mizoguchi *et al.*, 2003, Dickens *et al.*, 2009). In addition, Dickens *et al.* (2009) found that exposure to more than one stressor can possibly alter the stress response (HPA axis) which can then result in the negative feedback system experiencing decreased sensitivity. Essentially the decreased corticosterone concentration exhibited by the stressed VPA group of the current study could be as a result of decreased sensitivity of the negative feedback system (Sapolsky et al., 1984, Mizoguchi et al., 2003, Dickens et al., 2009). The current study also observed that the control non-stressed group exhibited a significantly lower corticosterone concentration when compared to the VPA non-stressed group. This is a result of exposure to VPA which has been documented to cause hyperactivity in a new environment (Narita *et al.*, 2010). This increase in locomotive activity has been documented to result in correlated differences in the HPA axis which leads to increased corticosterone concentrations (Malisch *et al.*, 2006). In addition, the VPA non-stressed group of this study exhibited the highest corticosterone concentration which further conveyed these animal's fear due to exposure to the new and a possibly unsafe environment (Sestakova *et al.*, 2013). This increase in corticosterone concentration could also be as a result of mild novelty, such as the cleaning of cages, which has been shown to increase corticosterone concentrations (Hennessy and Levine, 1977, Malisch *et al.*, 2006). Our study also noted a significant increase in the corticosterone concentration in the control stressed group, when compared to the VPA stressed group. This finding tells us that not only does chronic stress result in decreased corticosterone negative feedback, but exposure to VPA with maternal separation stress yields the same result (Sapolsky *et al.*, 1984, Mizoguchi *et al.*, 2003, Dickens *et al.*, 2009).

Serotonin is one of the most well-known neurotransmitter's and is responsible for regulating neural activity and an assortment of neuropsychological processes (Berger *et al.*, 2009). The neuropsychological and behavioural processes regulated by serotonin include fear, mood, memory, anger, perception, attention, aggression and any dysregulation of the serotonergic system has been linked to the pathogenesis of several neurological and psychiatric disorders (Berger *et al.*, 2009). The serotonin transporter (SERT or 5-HTT) is responsible for the accumulation of serotonin in to the cells after 5-HT1is released, therefore regulating the serotonergic neurotransmission plus signalling and was therefore assessed to confirm the anxious behaviour seen in this study (Ramsey and De Felice, 2002). The amygdala, which is part of the limbic system, has been revealed to play a role in emotional response, with proof of its amygdaloid subnuclei playing a role in the regulation of fear, attention, memory and sexually-related behaviours in rats (Rasia-Filho *et al.*, 2000). In this study we found that the control non-stressed rats exhibited a lower SERT concentration when compared to the control stressed group. Oler *et al.* (2009), found that the availability of SERT in the amygdala area has a positive correlation with behaviour of anxious temperament. Our findings are in agreement with that of Oler *et al.* (2009), as we see that the anxious behaviour of the control group resulted in more SERT being available in their amygdalae. We also observed that the control stressed group exhibited higher amygdala SERT

concentrations when compared to the VPA stressed group. Prenatal exposure to VPA has been shown to result in decreased SERT levels (Dufour-Rainfray *et al.*, 2010). In addition, mice and rats lacking SERT have been found to present autistic-like features (Homberg *et al.*, 2007, Moy *et al.*, 2009).

We further confirmed the SERT concentrations in the prefrontal cortex (PFC) which is linked to cognitive functions, memory, general intelligence and behavioural inhibition (Siddiqui *et al.*, 2008, Wilson *et al.*, 2010). The current study observed that the non-stressed groups had a tendency to exhibit higher SERT concentrations in their PFC's when compared to their respective stressed groups. This is in accordance with Murphy and Lesch (2008) who documented that stress will result in decreased serotonergic activity and therefore less SERT. The VPA groups of this study also exhibited lower SERT concentrations when compared to the control groups, as VPA exposure has been documented to decrease SERT levels, as mentioned previously (Dufour-Rainfray *et al.*, 2010).

Conclusion

This study observed that the exposure to stress and VPA resulted in anxiety-like behaviour. We found that VPA exposure resulted in an increased corticosterone concentration whilst stress with VPA resulted in the dysregulation of the HPA axis as we observed decreased corticosterone concentration. We also found that when VPA and stress exposure was coupled, it resulted in decreased SERT concentration. Our results show that maternal separation stress with VPA causes a sensitized stress response with alterations in the serotonergic system.

Declaration of Interest

We hereby disclose that there are no known financial and personal conflict of interests in the work of this project.

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CHAPTER FOUR

Chapter four consists of a synthesis which identifies the link between the two studies in this dissertation, summarizing the overall findings and states the conclusions from these studies.

Synthesis

Autism is a neurodevelopmental disorder which is becoming more prevalent every day and has therefore caused a fear of an epidemic (Frith and Happé, 2005). Autism South Africa has found that 160 autistic children are born weekly and 1 child with autism every hour. Autistic individuals display communication deficits, repetitive behaviours, impairments in social reciprocation and executive function (Lord *et al.*, 2000, Frith and Happé, 2005). However, the display in these impairments vary greatly amongst different individuals therefore the autism spectrum was developed (Frith and Happé, 2005, Faras *et al.*, 2010). VPA is a medication used for epilepsy, however using it during pregnancy has caused concern as it is related with the increased risk of autism (Mabunga *et al.*, 2015). There are also environmental conditions which make individuals more prone to becoming autistic (Baron-Cohen and Bolton, 1993). Disturbances during early development can play a role in causing neurodevelopmental disorders (Mpofana *et al.*, 2016). Maternal separation is such a disturbance, as it a form of early life stress which can cause changes in the stress response of individuals (Weiss *et al.*, 2011). This study intended to find out how VPA changed the functioning of the important neurotransmitter systems, which caused autistic-like behaviour. This study also intended to find out what happens to individuals when exposed to both VPA and maternal separation stress. Autistic-like symptoms were induced though a once of VPA injection in the pregnant mothers. Maternal separation stress was carried out by separating the pups from their mothers for 3 hours on postnatal days 2-13.

Following the injection of VPA we recorded number of rearing's, ambulation and discrimination index by carrying out the open field and novel object recognition tests. The concentrations of glutamate and 2 GABA receptors (Gamma-aminobutyric acid B Receptor 1: GABBR1 / Gamma-aminobutyric acid type A receptor subunit beta-3: GABRB3) were measured. We found that VPA causes a decrease in the glutamate concentration in the hippocampus which leads to the repetitive behaviour, commonly observed in autistic individuals. From this finding we deduced that autism is a hypo-glutamatergic condition. We also found that VPA caused a decrease in GABBR1 and an increase in the GABRB3 concentrations in the cerebellum which resulted in hyperactivity. Lastly, we found that VPA caused a decrease in the glutamate concentration in PFC whilst it decreased the GABRB3 and GABBR1 concentrations in the hippocampus which lead to impaired learning or memory.

We also looked at the effects of maternal separation stress together with VPA exposure. Following maternal separation stress and VPA exposure we recorded the time spent in the centre and total excretion by carrying out the open field test. The corticosterone and serotonin transporter concentrations were measured. We found that stress together with VPA caused a decrease in the time spent in the centre plus total excretion, when compared to VPA without stress, which are signs of anxiety and stress. VPA exposure lead to an increased corticosterone concentration as it caused hyperactivity thereby increasing

movement which in turn caused correlating changes in the stress axis. However, stress with VPA exposure lead to a decrease in corticosterone concentration as these factors together caused a dysfunction in the stress response, therefore it did not respond normally when stress occurred. Lastly, we found that stress together with VPA caused a decrease in the serotonin transporter concentration in the amygdala and prefrontal cortex.

This study shows that VPA causes changes in the GABAergic and glutamatergic systems which are responsible for causing the autistic-like behaviour seen in autism, however VPA together with maternal separation stress causes a dysfunction the stress response plus changes in the serotonergic system.

Conclusions

This study showed just how relevant and powerful Valproic acid really is in modern times, as this drug is commonly used for treatment globally. However, the consequences of using this drug during pregnancy are severe, as shown in this study. Whereby it is evident that prenatal exposure to valproic acid results in the autistic-like phenotype. The Valproic acid model is therefore valuable in autism research as, it is able to mimic some of the core symptoms of autism allowing us to investigate the physiology underlying autistic-like behaviour. This model therefore holds great importance for therapeutic measures, as it allows us to uncover the complex pathways affected in autism which can then be used in future treatments to address the core symptoms of autism. This study also shows that stress is an important factor in autism, as when it is combined with the autistic phenotype it presents more severe consequences to the pathways and functioning in the brain. Therefore, it is crucial to consider stress in autism therapy because when autistic individuals are exposed to early or prolonged stress, this can lead to abnormalities in their stress response, which could result in dire consequences and even worsen their autistic symptoms.

Limitations and Future Recommendations

Over the years there has been extensive research conducted on autism symptoms, risk factors etc. However, there is still a large gap in the knowledge of how this neurodevelopmental disorder causes dysfunction in the neurotransmitter systems of the brain. Hence, autism research should prioritise filling this gap as this knowledge is imperative for the future treatment of autism. Our study will bring light to how detrimental stress is, especially to individuals with autism. Therefore, this study can be used to enlighten pregnant mothers, especially those with epilepsy, on the effects that stress may have on their children and can therefore be used a preventative measure. As better understanding of autism can improve early detection methods and also inform treatment modalities. In addition, this study can then inspire future research on autism with stress, especially whether stress may exacerbate autistic-like symptoms. Such research is crucial in the present, as stress is affecting both young and old which is resulting in more diseases and disorders.

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Appendices

Appendix 1: Ethics Approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

29 June 2018

Ms Chanel Heeralall (214528607)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Ms Heeralall,

Protocol reference number: AREC/044/018M
Project title: Evaluating the role of maternal separation stress on the development of autistic behaviour utilizing Sprague-Dawley rats

Full Approval – Research Application

With regards to your revised application received on 19 June 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 authorised 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 29 June 2019.

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

.....

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Dr Thabisile Mpofana and Dr Litile Qulu
Cc Academic Leader Research: Dr Michelle Gordon
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Anita Engelbrecht

cc SRC – Dr Linda Bester

Animal Research Ethics Committee (AREC)
Ms Marietta Snyman (Administrator)
Westville Campus, Govan Mbeki Building
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Website:

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Appendix 2: Corticosterone ELISA Protocol

Elabscience®

4th Edition, revised in May, 2019

(FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSTICS !)

Rat CORT(Corticosterone) ELISA Kit

Synonyms: CORT

Catalog No : E-EL-R0269
96T

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help (info in the header of each page).

Phone: 240-252-7368(USA) 240-252-7376(USA)
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please refer to specific expiry date from label on the side of box.

Please kindly provide us with the lot number (on the outside of the box) of the kit for more efficient service.

Appendix 3: Serotonin Transporter ELISA Protocol

Elabscience®

4th Edition, revised in May, 2019

(FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSTICS !)

Rat SERT(Serotonin Transporter) ELISA Kit

Synonyms: SLC6A4, HTT, SERT, 5HTT, 5-HTT

Catalog No : E-EL-R1140
96T

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help (info in the header of each page).

Phone: 240-252-7368(USA) 240-252-7376(USA)
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please refer to specific expiry date from label on the side of box.

Please kindly provide us with the lot number (on the outside of the box) of the kit for more efficient service.

Appendix 4: Glutamate Assay Protocol

ab83389

Glutamate Assay kit (Colorimetric)

Instructions for Use

For the rapid, sensitive and accurate measurement of Glutamate in various samples.

This product is for research use only and is not intended for diagnostic use.

Appendix 5: RNA Extraction Protocol

ZYMO RESEARCH

The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

Quick-RNA™ Miniprep Kit

Catalog Nos. **R1054 & R1055**

Highlights

- High-quality total RNA (including small RNAs) from a wide range of samples.
- You can opt to isolate small and large RNAs in separate fractions.
- *DNA-free* RNA is ready for use in any downstream application. *DNase I included.*

Contents

Appendix 6: CDNA Synthesis Protocol

BIO-RAD

iScript™ cDNA Synthesis Kit

Catalog #	Description
1708890	**iScript cDNA Synthesis Kit**, 25 x 20 µl reactions
1708891	**iScript cDNA Synthesis Kit**, 100 x 20 µl reactions

For research purposes only.

Introduction

iScript cDNA Synthesis Kit provides a sensitive and easy-to-use solution for two-step reverse transcription quantitative PCR (RT-qPCR). This kit includes three tubes, which contain all the reagents required for successful reverse transcription.

The iScript Reverse Transcriptase is RNase H+, which provides greater sensitivity than RNase H– enzymes in qPCR. iScript is a modified Moloney murine leukemia virus (MMLV) reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA. The enzyme is provided preblended with RNase inhibitor. The unique blend of oligo(dT) and random hexamer primers in the iScript Reaction Mix works exceptionally well with a wide variety of targets. This blend is optimized for the production of targets <1 kb in length. iScript cDNA Synthesis Kit produces excellent results in both real-time and standard RT-qPCR.

Storage and Stability

Store at –20°C. Guaranteed for 12 months at –20°C in a constant temperature freezer. Nuclease-free water can be stored at room temperature.

Note: Kits whose six-digit lot number begins with a 2 are not compatible with kits whose six-digit lot number begins with a 1. Please make note of this distinction if you have multiple lots of this kit in storage.

Kit Contents

Reagent	Volume for 25 Reactions	Volume for 100 Reactions
5x iScript Reaction Mix	100 µl	400 µl
iScript Reverse Transcriptase	25 µl	100 µl
Nuclease-free water	1.5 ml	1.5 ml

Reaction Setup

Note: The 5x iScript Reaction Mix may generate some precipitation upon thawing; this does not affect the quality

Component	Volume per Reaction, µl
5x iScript Reaction Mix	4
iScript Reverse Transcriptase	1
Nuclease-free water	Variable
RNA template (100 fg–1 µg total RNA)*	Variable
Total volume	**20**

* When using larger amounts of input RNA (>1 µg), the reaction should be scaled up (for example, 40 µl reaction for 2 µg, or 100 µl reaction for 5 µg) to ensure optimum synthesis efficiency.

Reaction Protocol

Incubate the complete reaction mix in a thermal cycler using the following protocol:

Priming	5 min at 25°C
Reverse transcription	20 min at 46°C
RT inactivation	1 min at 95°C
Optional step	Hold at 4°C

Recommendation for Optimal Results Using the iScript cDNA Synthesis Kit

The maximum amount of the cDNA reaction that is recommended for downstream PCR is one-tenth of the reaction volume, typically 2 µl.

Related Products

Catalog #	Description
Reverse Transcription Reagents for Real-Time qPCR	
1708840	iScript Reverse Transcription Supermix for RT-qPCR
1725037	iScript Advanced cDNA Synthesis Kit for RT-qPCR
1708896	iScript Select cDNA Synthesis Kit
1725034	iScript gDNA Clear cDNA Synthesis Kit
Reagents for Real-Time qPCR	
1725270	SsoAdvanced™ Universal SYBR® Green Supermix
1725280	SsoAdvanced Universal Probes Supermix
1725120	iTaq™ Universal SYBR® Green Supermix
1725130	iTaq Universal Probes Supermix
1725160	SsoAdvanced PreAmp Supermix

Visit bio-rad.com/web/iscriptcDNA for more information.

Appendix 7: PCR Protocol

For general laboratory use. Not for use in diagnostic procedures. FOR *IN VITRO* USE ONLY.

Roche

Roche Applied Science

LightCycler® 480 SYBR Green I Master

Version June 2005

Easy-to-use hot-start reaction mix for PCR using the LightCycler® 480 System

Cat. No. 04 707 516 001

Kit for 5 × 100 reactions (20 µl each)

Store the kit at −15 to −25°C

⚠ Keep LightCycler® 480 SYBR Green I Master (vial 1, green cap) away from light!

Appendix 8: Conference Output

Oral Presentation:

UKZN Neuroscience Symposium 2019

Nelson R Mandela School of Medicine, K-RITH Building

July 2019