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Using a Valproic acid-induced rat model to investigate the effects of litter size on behaviouraland morphological outcomes

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USING A VALPROIC ACID-INDUCED RAT MODEL TO INVESTIGATE THE EFFECTS OF LITTER SIZE ON BEHAVIOURAL AND MORPHOLOGICAL OUTCOMES.

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A dissertation submitted to the Faculty of Health Sciences, the University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine by Research.

Johannesburg, 2024







DECLARATION

I, [**Christine Dignon**] declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of [**MSc Medicine**] at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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(Signature of candidate)

_____day of_____20_____in_____

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ABSTRACT

Autism Spectrum disorder (ASD) constitutes a variety of neurodevelopmental disorders such as autism, Asperger's syndrome and Rett's syndrome, all characterised by three main features: deficits in social interaction, impaired communication and language, and the presence of repetitive and restrictive behaviours. Genetic factors, in association with environmental insults during key moments in development can result in the development of autism in early childhood. Valproic acid (VPA), an anticonvulsant drug used in the treatment of epilepsy, has been linked to an increased risk of autism when used during pregnancy. For this reason, VPA has been increasingly used to create models of autism in rodents, to understand further the behavioural characteristics, cellular and molecular underpinnings, and potential therapies for autism. An understudied area of ASD research is the variation in behavioural phenotypes exhibited by individuals with autism. Not only are males more likely to be diagnosed with ASD, but there may be many differences in the *severity* of ASD symptoms. This study utilised the VPA-induced autism model for analysis of the behavioural and morphological autistic-like characteristics in the offspring of valproic acid exposed dams. In addition to the repetitive and restricted behaviours, as well as cognitive, locomotor and exploratory behaviours assessed; gross anatomical characteristics were also analysed such as tail length, "the crooked tail phenotype" and relative brain mass. In addition, ELISA was used to detect and quantify whether there was residual VPA within blood sera, and what concentrations would be found in individuals of different litter sizes. Comparisons were made between neurotypical and VPA-exposed pups, as well as within the VPA litters to determine the effects of sex and litter size on autistic-like traits. Morphological findings seemed to confirm that individuals from smaller litter sizes were likely exposed to more VPA *in utero*, as these individuals showed the greatest tail abnormalities (significantly decreased length and the presence of tail *kinks)* as well as significantly larger brain mass relative to body mass. The main behavioural difference between VPA-exposed individuals and controls was seen in locomotor activity. VPA-exposed females showed greater locomotor activity than controls, however, it was those from the largest litter size that appeared to drive the trend, showing greater average speed as well as greater distance travelled in the hole board assay. In contrast, no clear pattern was seen in the performance of assays assessing cognitive,

repetitive, or exploratory behaviour of VPA-exposed individuals from different litter sizes. Similarly, no differences were seen in the reactivity to heat in the hotplate thermal assay. Importantly, ELISA did not provide reliable data on serum levels of VPA in pups. The findings of this study indicate that the autism-like behavioural phenotypes are not limited to males only and provide support for the inclusion of females in ASD studies. Furthermore, the study demonstrates the need for the inclusion of litter size as a variable in the assessment of all analyses pertaining to the features of ASD-like in rodents.

CHAPTER ONE - INTRODUCTION

1.1. GENERAL INTRODUCTION

Autism spectrum disorder (ASD) encompasses a variety of disorders that include autism, Asperger's syndrome, Rett's syndrome, and Fragile X syndrome. The disorders are characterized by persistent deficits in social communication and interaction (including reciprocation and non-verbal communication), and the presence of repetitive and restrictive behavioural patterns. Repetitive and restrictive behaviours encompass the insistence on sameness or routine, repetitive movements, fixated interests and hyper/hypo-reactivity to sensory input (American Psychiatric Association, 2022). The disorders may also be associated with a decline in cognitive ability (seen in approximately two thirds of cases), and in many other cases, may be associated with other maladaptive behaviours such as anxiety, irritability/mood instabilities including temper outbursts and aggression, and self-injury (Brentani et al., 2013). Between the ages of 18 to 24 months, there is often a regressive decrease of 30% in developmental skills (Blaylock, 2008), as a result of abnormal brain development causing marked delays in development (Blaylock, 2008; Nemechek & Nemechek, 2017). Autism can develop before the age of 36 months and has a prevalence of occurring more often in males, with a ratio of 4:1, than females (Blaylock, 2008; Geier & Geier, 2006). It is known to be the most common pervasive developmental disorder worldwide, and there is much ongoing research about its causes and treatments (Won et al., 2013). Currently, there are several hypotheses as to the aetiology of autism including genetic components (Taleb et al., 2021) and exposure to teratogens such as thalidomide, valproic acid (Schneider & Przewłocki, 2005) and ethanol (Schneider & Przewłocki, 2005; Williams & Hersch, 1997) during development. In the 1980s, ASD was shown to be highly heritable with strong genetic components along with its association with other heritable neurodevelopmental and neuropsychiatric disorders adopted through genetics such as schizophrenia (Shifman et al., 2008; Won et al., 2013), X chromosome-linked intellectual disability (Won et al., 2013) and ADHD and depression (Caspi et al., 2003; Gillberg & Wahlstrom, 1975; Manor et al., 2001; Won et al., 2013). Given the frequency with which conditions like ASD tend to occur in families of affected children, there is clear evidence that genetic factors are at play (Buie, 2013).

Environmental factors implicated in the development of autism include foetal exposure to drugs such as valproic acid (VPA) (Hirsch et al., 2020; Schneider & Przewłocki, 2005). Valproic acid (VPA) is an anticonvulsant drug, is widely used as an active ingredient in the pharmaceutical treatment for epilepsy, mood instabilities and migraines (Taleb et al., 2021; Williams et al., 2001). However, when used for treatment in pregnant women, the drug increases the risk of autism development significantly, especially when taken during the first trimester (Christensen et al., 2013; Hirsch et al., 2020; Williams et al., 2001). Valproic acid is a salt known also as sodium valproate salt or valproate sodium salt and is contained in medications such as Depakene® (oral, Abbott laboratories), Stavzor® (oral, Biopharma Inc.) or Depacon® (injection, Fresenius Kabi USA). Due to its role in the treatment of neural disorders, its teratogenic effects are more severe, often resulting in neural tube defects (Gotlib et al., 2017; Qiu et al., 2021; Taleb et al., 2021). The use of VPA to treat maternal epilepsy was initially recognised as causing Foetal Valproate Syndrome (FVS), which was clinically documented by congenital malformations, developmental delay and an increased risk of autism (Meador, 2008; Meador et al., 2009; Ornoy, 2009; Roullet et al., 2013; Shallcross et al., 2011), in addition to abnormalities within the phenotypic characteristics of the face (Williams et al., 2001). A report from the UK Epilepsy and Pregnancy Register showed that higher dosages of >1000 mg per day increased the risk of malformations significantly (Mawhinney et al., 2012; Roullet et al., 2013). Other drugs researched, such as Phenobarbital, Carbamazepine and Lamotrigine, showed a lower percentage of major congenital malformations, and are thus not as reliable in inducing ASD-like symptoms (Ornoy, 2009; Tomson & Battino, 2012). Valproic acid (VPA) is the most commonly used teratogen, it acts as a maternal immune activator, and is more reliable in inducing autistic-like behavioural, immunological and neurological abnormalities. The teratogenic effect of a drug is measured by the rate and degree of teratogenic potential, and sufficient data indicates that VPA is highly teratogenic (Diav-Citrin et al., 2008; Kaneko et al., 1999; Morrow et al., 2006; Ornoy, 2009).

In rodents, prenatal exposure to VPA can induce autism-like characteristics, including brain abnormalities with an increase in anxiety and decreased social behaviour skills (Bambini-Junior et al., 2011; Hirsch et al., 2020). Therefore, this prenatal exposure to VPA in rodents is one of the best characterized models for studies of autism, (Hirsch et al., 2020; Mabunga et al., 2015; Roullet et al., 2013) with earlier research using this rodent model to examine the

clinical link of VPA and autism through the observation of developmental disabilities (Bambini-Junior et al., 2011; Christianson et al., 1994). Thus, the VPA model is ideal in investigations that aim to compare morphological or physiological features associated with ASD. However, recent attempts at reproducing findings of behavioural and physiological characteristics related to VPA-induced autism model, pointed to the need for further enquiry into how studies that make use of the model are designed, and how the data are analysed. For example, as males are generally more prone to develop ASD than females, many studies only report on findings making use of male pups; but there is mounting evidence that this sex bias may be for many other reasons such as that within the diagnostic criteria and existing assessment tools (Begeer et al., 2013; Evans et al., 2019; Giarelli et al., 2010; Rutherford et al., 2016). Similarly, it is not clear to what extent each individual in a litter is exposed to VPA, and whether this can account for the large variation in the behavioural phenotypes and performance in behavioural assays (Lazic & Essioux, 2013). In the present study, we examined behavioural and morphological findings in both male and female pups exposed to VPA during uterine development, as well as specifically compared individuals in large and small litters in an attempt to characterise potential VPA effects more accurately on development in rats. The resulting differences in morphological and behavioural characteristics provide further insight into how VPA affects individuals in small and large litter sizes.

1.2. LITERATURE REVIEW

1.2.1. Autism Spectrum Disorder: The Diagnostic and Statistical Manual of Mental Disorders (DSM)

Although there are morphological indicators and changes that occur in the autistic brain, the diagnosis of ASD is based primarily on behavioural evaluation and analysis, with potential biomarkers still under investigation (Chang et al., 2017; Varcin & Nelson, 2016).

The Diagnostic and Statistical Manual of Mental Disorders (DSM) version five (V) compiled in 2013, categorises ASD as a neurodevelopmental disorder with its onset during the early developmental period with deficits in social, personal, and occupational functioning (American Psychiatric Association, 2013, 2022). The DSM-V elaborates two main categories

of diagnostics, i) deficits in social communication and interaction, with abnormal verbal and non-verbal communication and ii) restrictive and repetitive patterns of behaviour, which include hypo/hypersensitivity to sensory input and repetitive motor movements (American Psychiatric Association, 2022).

To investigate the contribution of environmental or genetic factors along with potential therapeutic interventions, it is important to attempt to replicate the equivalent behavioural traits in an animal model (Chang et al., 2017). The VPA animal model of autism allows for further study of the mechanisms and risks underlying autism development and supports the close association of autistic behavioural traits (Favre et al., 2013). The inclusion of certain behavioural tests and their associated relevance to the categories of the diagnostic symptoms of autism is important and adds value to the use of animal models in ASD research (Crawley, 2012).

1.2.1.1. Sociability

Children with ASD struggle to develop and maintain relationships due to difficulty in reciprocating socially and emotionally, which includes abnormal social approach and difficulty in reading body language and making eye contact (American Psychiatric Association, 2022; Coeckelbergh et al., 2016). There is also a reduction in sharing of interests which can result in the lack of back-and-forth conversation and deficits in nonverbal communicative behaviours with reduced understanding and use of gestures and facial expressions (American Psychiatric Association, 2022). Sociability is one of the most complex constructs concerning autism, with a wide range of severity and type (Moy et al., 2008). Rodent models can be used as a useful research tool to investigate aspects of sociability, including characterising some social behaviours that can be qualitatively similar to social deficits seen in autism (Insel, 2001; Moy et al., 2008).

1.2.1.2. Restrictive and Repetitive Behaviours

ASD diagnoses require the combination of social communicative deficits accompanied by excessive repetitive behaviours and insistence on sameness (American Psychiatric Association, 2013, 2022). These behaviours include stereotyped motor movements (pacing, finger flicking, hand flapping), at times with the use of objects or speech (American Psychiatric Association, 2013, 2022; Brentani et al., 2013). An inflexible adherence to routine or rituals

which include both non-verbal and verbal behaviour such as greeting rituals or same food (American Psychiatric Association, 2013, 2022). Diagnosis requires the careful evaluation of all aspects of social and repetitive behaviours, along with the diagnosis of other features and specifiers associated with ASD such as intellectual impairment, language impairment, or known genetic or medical specifiers such as epilepsy, Down Syndrome, attention deficit disorder or Tourette's syndrome (American Psychiatric Association, 2013, 2022).

1.2.2. Autism Spectrum disorder: Sex discrepancies and prevalence

Males are generally more prone to develop autism spectrum disorders with most neurodevelopmental disorders, including attention deficit disorder (ADHD) and intellectual disabilities, having a male bias (Ferri et al., 2018; Werling & Geschwind, 2013). However, the statistics of male to female bias changes depending on the lack of intellectual disability, with a 16:1 ratio or the presence of moderate to severe intellectual disability, with a ratio 1-2: 1 (Ferri et al., 2018; Werling & Geschwind, 2013). Girls are more often undiagnosed, compared with boys, or diagnosed at a later age due to more intact cognitive and language skills and ASD symptoms that are less severe (Begeer et al., 2013; Evans et al., 2019; Giarelli et al., 2010; Rutherford et al., 2016). Evidence suggests that girls tend to mask ASD symptoms due to better performance in non-verbal communication with increased demands in language and social aspects (Evans et al., 2019; Rynkiewicz et al., 2016). Another possible explanation for this sex-gender difference is the "extreme male brain theory" which highlighted the weakness in empathic traits and a strength in systematising compared to females (Auyeung et al., 2006; Baron-Cohen et al., 2003, 2011; Evans et al., 2019; Wheelwright et al., 2006). Systemising is the ability to "analyse and or construct rule-based systems" and refers to the drive to construct systems and predict the behaviour of a system, measured by the systemising quotient (SQ) where males typically score higher (Baron-Cohen et al., 2003, 2011).

1.2.3. Autism Spectrum Disorder: Aetiology

Autism Spectrum Disorder (ASD) has become the most commonly diagnosed neurodevelopmental disorder, with its prevalence increasing rapidly (Kim et al., 2018). This increase in diagnoses is seen as a result of complex combinations of genetic and environmental factors, rather than due to increased awareness or improved diagnostics

(Ashwood et al., 2006; Fombonne, 2003). The exact aetiology of ASD is largely unknown; however, the likely cause is the result of combinations of immunological, environmental, neurological, and genetic factors that affect the developing brain (Ashwood et al., 2006; Hodges et al., 2020). Ongoing research into ASD seems to point to the likelihood that the development and progression of ASD is likely due to the cumulative effects of heterogenous factors, with no one unifying cause. Genetic predisposition through parents with an environmental trigger during prenatal or postnatal periods is understood as the main risk factors making it more likely that children will develop the condition.

1.2.3.1. Autism Spectrum Disorder: Environmental Factors

The exposure to environmental neurotoxicants such as prenatal exposure to teratogens, infections and vitamin D deficiency plays a role in the development and exacerbation of ASDs. One example of environmental factors includes the role of vitamin D in exacerbating the symptoms of ASD. For many years, the role of vitamin D was known to metabolise phosphorus and regulate calcium for the acquisition of bone mass and bone mineralisation (Principi & Esposito, 2020; Saggese et al., 2018). In more recent years, studies show vitamin D to be more essential in extra skeletal activity as well as in physical and mental health with its deficiency being associated with several diseases such as asthma, metabolic syndromes, and neurodevelopmental disorders such as ASD (Jia et al., 2018; Mazahery et al., 2016; Principi & Esposito, 2020; Saggese et al., 2018). Further functions in the body involve modulating immune function, brain cell proliferation and neuroprotective properties (Holick, 2007; Pioggia et al., 2014). It has been reported that vitamin D deficiency during pregnancy increases the chances of offspring developing ASD and leads to decreased levels of serum vitamin D in offspring compared with that of typically developing children (Principi & Esposito, 2020; Stubbs et al., 2016; Wang et al., 2016). Furthermore, some evidence also suggests that vitamin D deficiency during pregnancy affects both the maternal immune system as well as foetal brain development (Grant & Soles, 2009; Pioggia et al., 2014). Another environmental factor related to the development of ASD is maternal exposure to teratogens.

Valproic acid (VPA) is a teratogenic chemical (Diederich et al., 2010; Romoli et al., 2018a; Taleb et al., 2021) that is used in the treatment of neural disorders such as epilepsy and seizures. It is well known that prenatal exposure to VPA induces neurotoxicity within the development of the central nervous system (CNS) (Ornoy et al., 2015; Taleb et al., 2021) when administered

at a critical period in the first trimester, before the closure of the neural tube. Children exposed to such medications are more vulnerable and show poor cognitive development and impaired cognitive function. Valproic acid also increases the risk of development of autism resulting in lifelong behavioural impairments, deficits in motor performance and anxiety-like behaviour (Bambini-Junior et al., 2011; Nicolini & Fahnestock, 2018).

A genetic component has been attributed to the aetiology of only 5 to 15% of autism cases. Therefore, an accumulation of exposure of environmental factors such as maternal vitamin D deficiency and maternal VPA exposure and genetic factors can increase the risks of autism development.

1.2.3.2. Autism Spectrum Disorder: Genetic Factors

The most common aetiology of the development of ASD is through a number of high-risk genes (Choi et al., 2016). The high-risk genes, whether associated with ASD in males or females, can be aggravated by environmental insults and prenatal injuries which imposes a significant contribution to ASD development during prenatal periods (Bailey et al., 1995; Choi et al., 2016). Although the genetic aetiology is mainly linked to males (Giarelli et al., 2010), there is also growing concern that cognitively able females are diagnosed less frequently, with under-diagnosis or later diagnosis becoming more common (Giarelli et al., 2010; Petrou et al., 2018). Autism is considered the most severe disorder within the autism spectrum however, other specifiers of ASD are associated with other known genetic conditions such as Down's syndrome, Rett's syndrome and fragile X syndrome (American Psychiatric Association, 2022; R. A. Kumar & Christian, 2009). The most common heritable single-gene disorder is Fragile X syndrome (FXS) and classified as an intellectual disability, termed as a "full mutation", and occurs in 1 to 6 % of all cases of autism (Kaufmann et al., 2017; Muhle et al., 2004; Schaefer & Mendelsohn, 2013). Studies on males with FXS showed that 30 to 53% also met the criteria for autism with more prominent social withdrawal and higher levels of anxiety (Clifford et al., 2007; Hagerman & Harris, 2008; Hall et al., 2008; Kaufmann et al., 2017).

Epigenetic processes refer to genetic changes that occur outside of conventional understanding of genetic mutations and typically involve alterations and regulation of gene expression (Jaenisch & Bird, 2003; Wiśniowiecka-Kowalnik & Nowakowska, 2019). Valproic acid (VPA) exhibits more than one mechanism of action, a major one of which is the inhibition

of class-I histone deacetylases (HDACs), which subsequently increases histone acetylation, specifically H3 and H4 histones (Kataoka et al., 2013; Moldrich et al., 2013; Nicolini & Fahnestock, 2018), an epigenetic marker that activates transcription (Delcuve et al., 2012; Konopko et al., 2017; Monti et al., 2009). Histone deacetylases, in conjunction with decreasing histone acetylation, affect the interaction of transcription factors and RNA polymerase with DNA via the induction of chromatin changes resulting in the modulation of gene transcription (Iwata et al., 2010). Evidence reported by Kataoka and colleagues (2013) showed a transient increase in the levels of acetylated histones H3 and H4, resulting in a decrease in cell proliferation in the embryonic brain after the administration of VPA at gestational day 12.5. The study also showed evidence that a VPA analogue that cannot inhibit HDACs did not cause behavioural alterations (Kataoka et al., 2013). Thus, exposure to teratogens at key points in embryonic development, has the potential to alter gene expression, resulting in widespread changes to how the brain develops, grows and functions overall.

1.2.4. The VPA-induced rodent model of Autism.

Exposure to valproate at a specific stage of pregnancy, and its reproductive toxicity (Kim et al., 2011), results in various defects in the foetus, which include behavioural alterations and skeletal malformation (Favre et al., 2013). The first trimester is an integral stage in development resulting in the closure of the neural tube followed by structured and elaborate development to ensure functional and structural organisation of the central nervous system (Kim et al., 2011). Animal models have been developed to elucidate the underlying neurobiology of autism with the identification of major symptoms to allow for the study of behavioural, cellular and molecular alterations (Favre et al., 2013). The use of the teratogen thalidomide was originally used to explore the effects on limb development, however, the exposure of thalidomide to rabbits and rats, and its resulting deficits, were shown to be vastly different to that of humans (Rodier et al., 1996; Schumacher et al., 1972). In contrast, Rodier and colleagues found that exposure to valproic acid (VPA) did produce many of the same external features associated with autism in humans (Rodier et al., 1996) along with its brain-damaging effects (Vorhees, 1987). In the rat, the neural tube closes on gestational day 11 along with the formation of the earliest nuclei in the early embryonic stage, therefore VPA is administered between day 11 and 12.5 of gestation to produce similar behavioural and cellular alterations as seen in autism (Favre et al., 2013; Rodier et al., 1997). The treatment of

VPA during this time period, the early embryonic stage, results in the significant loss of neurons in various regions of the brain responsible for motor and sensory functions as well as altering the conditions necessary for neuron production (Rodier et al., 1997). VPA significantly reduces the number of neurons during the time of closure due to the altered conditions such as alteration in embryonic genes and protein patterns that assist in neurulation and neural tube closure (Ornoy, 2009). The neural tube is completely closed by day 11 of gestation of rodents (Morriss-Kay & Tuckett, 1985) when nerve cell migration has not yet been completed, resulting in hyper-reactivity and hyper-plasticity due to the hyper-connectivity between neurons (Favre et al., 2013). Sakai (1989) describes eight phases of neurulation in the mouse embryo occurring between gestational day seven and nine, therefore full closure of the neural tube occurs before day 11.

1.2.5. VPA Metabolism

Valproic acid is a known anti-convulsant and short-chain fatty acid derivative of valeric acid, and is a prominent organic solvent used in pharmaceutical manufacturing (Romoli et al., 2018b; Shnayder et al., 2023). Not only is the teratogenic drug used for the treatment of seizures and epilepsy, but it is also used in the treatment of migraines, bipolar disorder, mood and other psychiatric disorders (Chateauvieux et al., 2010; Ghodke-Puranik et al., 2013). Recently, VPA has been established as an adjuvant agent in cancer and neurodegenerative diseases because of its action as a histone deacetylase inhibitor (Ghodke-Puranik et al., 2013; Terbach & Williams, 2009). The use of VPA is chronic in order to treat the above-mentioned disorders, however, this requires a balance that is optimal between its safety and effectiveness as a treatment. This effectiveness is dependent on the metabolism of VPA and its transport in the human body (Shnayder et al., 2023; Zhu et al., 2017). Valproic acid and its metabolites are of great interest since they can have both therapeutic effects and unexpected toxic effects (Perucca, 2002; Shnayder et al., 2023). The toxic effects of VPA, specifically the toxic metabolites, are well documented (Kudin et al., 2017; Shnayder et al., 2023; Tang & Abbott, 1996) in increasing the severity of neurological diseases, mental disorders, abnormal and delayed development, and organ malformations (Kim et al., 2022; Silva et al., 2008). Valproic acid is one of the four most commonly prescribed medications for epilepsy (Kacirova et al., 2015, 2019), with different dosages showing various effects on the development of neurodevelopmental disorders and neural tube deformities. However, VPA has been a gold

standard for treatment in generalised epilepsy disorder, with other anti-epileptic drugs not performing as effectively as VPA (Cerulli Irelli et al., 2020; Tomson et al., 2015). Valproic acid is normally considered as the first-line treatment due to its efficacy (Cerulli Irelli et al., 2020; Tomson et al., 2015); however, it is recommended that valproic acid not be used in treatment of epilepsy for girls and women of childbearing age (Tomson et al., 2015). Regular therapeutic drug monitoring is recommended during pregnancy due to the increased risk of intrauterine growth restrictions even at doses of 70 mg per litre (Kacirova et al., 2015, 2019). There are reports of correlation between the concentration of VPA in maternal blood and cord blood, with cord blood containing up to three times the serum concentrations of maternal blood at birth, which is the reason why therapeutic drug monitoring is important to limit the effects of VPA on the foetus (Kacirova et al., 2015, 2019). Kacirova et al. (2015) also show an inverse relationship between the serum concentrations of VPA and birth length and weight. Similarly, there are also reports of VPA occurring in maternal breast milk and how this affects VPA concentration levels in infant blood serum at different ages after birth (Kacirova et al., 2015, 2019). It is noted that 67 % of the milk samples in the study done by Karcirova (2019) had VPA levels below the limit of quantification and the quantification of the remaining samples showed the lowest concentrations between maternal blood serum and infant blood serum at < 1.0 to 16.7 mg/L. The adverse effects of VPA exposure in breastfed infants is dependent on multiple factors such as the volume of milk ingested, metabolism, excretion of the drug and the maternal VPA dosage (Kacirova et al., 2019). A higher infant or maternal serum level of VPA can result in higher exposure of breastfed infants which can indicate a possible higher risk of adverse effects (Kacirova et al., 2019). The exposure of infants to VPA through breastmilk varies depending on the daily dose and resultant VPA serum concentration, the volume of milk ingested and the metabolism of VPA in the infant (Kacirova et al., 2019). There has been a significant decrease in prescription of VPA in many countries to girls and women in general and specifically during pregnancy, however, in low to middle income countries, this change is generally slower.

1.2.6. VPA-induced autism model and rodent litter size

Numerous animal models have been developed for the evaluation of disease pathology on neurodegenerative, psychiatric, and neurodevelopmental disorders, however, these models

are less useful when determining potential therapies and predicting clinical efficacy (Lazic & Essioux, 2013). Animal models do provide a means to examine the potential impact of pharmacologically based treatments, with the assumption that animal models provide construct, face, and predictive validity that allows for the extrapolation to humans (Chaliha et al., 2020). However, there is growing concern in the scientific community that there are an increasing number of studies that are hard to replicate when looking at neurodevelopmental disorders (Begley & Ioannidis, 2015; Jiménez & Zylka, 2021; Kafkafi et al., 2018). In 2015, there was a large, scientific community effort to attempt to replicate the findings of 100 psychological papers which found that 64% of the replications did not report significant differences, i.e. failed to find the previously reported outcomes (Aarts et al., 2015). However, Gilbert et al. (2016) disputes this claim of replicability of previous reports, stating that one cannot determine whether the replicability of psychological science is high or low and that the uncertainty is much larger. The authors further suggest that another study that improves on the research design may be able to replicate such studies (Aarts et al., 2015; Gilbert et al., 2016). This improvement on research design does include the basics such as increasing the sample size, the inclusion of both sexes and improving statistical instruction (Kafkafi et al., 2018).

With the above improvements to the research design and statistical instruction, assists in understanding the overall outcomes of animal models. In revisiting the ASD rodent model and other related disorders, the conclusion made was that rat and other rodent preclinical models are suitable for general neurodevelopmental insult rather than a specific disorder, and perfectly encapsulating ASD is unobtainable (Silverman et al., 2022). Nevertheless, the epidemiological findings continue to show a strong association of maternal gestational VPA and the later development of autism in the offspring (Chaliha et al., 2020; Christensen et al., 2013; Hirsch et al., 2020; Nicolini & Fahnestock, 2018), suggesting that the VPA animal model of autism appears to possess construct validity to model human autism as it seems to express similar, superficial behavioural phenotypes as those possessed by humans with ASD (Chaliha et al., 2020). Lazic and Essioux (2013) discuss two issues when looking at the design and preclinical study analysis and how this affects the validity and resulting reproducibility. The first issue deals with the design and experimental treatment being applied to whole litters rather than individual animals from different litters, based off their behavioural phenotypic

results (Lazic & Essioux, 2013). The second addresses the natural litter-to-litter variation resulting in the value of the measured experimental outcome which is potentially affected by the litter from which that subject came (Lazic & Essioux, 2013). However, the question remains as to the existence and degree of the variation, whether behavioural or anatomical, amongst individuals within the same litter, before the addition of any clinical treatment.

The VPA-autism rat model most commonly includes the assessment of male pups, due to the prevalence of autism occurring more often in males than females. The rodent model allows for an increased sample size, with one litter containing between 9 and 13 pups and the presence of both male and female pups. However, there is no way to determine beforehand the number of males born within one litter, resulting in the culling of litters to a certain number with equal numbers of males and females, to avoid variability and a statistically viable sample size (Olexová et al., 2016; Shahrbabaki et al., 2022), or the rat pups being chosen from multiple litters to allow for natural variability (Banerjee et al., 2014). However, the varying litter does allow for analysis of the effect of VPA on the dam, the average litter size produced (Shahrbabaki et al., 2022; Snow et al., 2008), and the survival rate determined by the number of pups that reached the weaning age (Banerjee et al., 2014). Rodents from the same litter share prenatal and early postnatal environments and are genetically similar; however, the statistical analysis and methods are assuming that the data are from unrelated individuals. Therefore, studies that involve individuals from various litters need to be designed and analysed in a way that does not produce bias or confound results (Festing, 2006; Festing & Altman, 2002; Haseman & Hogan, 1975; Holson & Pearce, 1992; Lazic & Essioux, 2013).

There is a need for better consideration of litter-to-litter and within-litter sex variation in the VPA-induced rodent model of autism. It is important to take into account the litter and litter size of an individual when analysing the results of behavioural and morphological assessments in animal studies, as was done in the current study. The lack of research on the effects of overall sample size, litter size, VPA exposure, and the inclusion of both male and female subjects, highlights the need for further investigation into the impact of these factors on assessing the model.

1.3. AIMS

This study aimed to determine whether there exists a difference in behavioural and morphological characteristics related to autism spectrum disorder (ASD) by comparing the valproic acid (VPA)-induced rat model (*Rattus norvegicus*) and their neurotypical counterparts. This includes specific focus on whether the litter size influences the severity of behavioural and morphological outcomes in rodents.

1.3.1 Objectives

To meet these aims, the objectives of the study were:

1. To induce ASD using VPA and conduct behavioural assessments, using the DSM-5/DSM-V criteria as a guide to examine ASD related behaviours in the valproic acid induced autism model.
    1.1. To analyse behavioural assessments, to assess the relative behavioural outcomes between the different litter sizes of the VPA-induced rat model.
2. Using ELISA, to investigate any remaining levels of VPA within serum, and compare these between litter sizes in the VPA cohort.
3. To assess selected morphometric features (i.e. tail length and brain mass) between the VPA-induced rat model and control.
    3.1. Further, to investigate the effect of litter size on morphometric features between varying litter sizes.

CHAPTER TWO – METHODS AND MATERIALS

2.1. SAMPLING

All animals were cared for within the Central Animal Services, Wits University, under animal ethics approval number 2022/03/07/B (see **APPENDIX A**).

Fertile, adult Sprague-Dawley (*Rattus norvegicus*) nulliparous female or dam rats (10- to 12-weeks old) were acquired from the Wits Animal Research Facility (WRAF), University of the Witwatersrand, South Africa, and were left to mate overnight with a male in a metabolic cage. The occurrence of conception was confirmed by locating the vaginal plugs from the females and considered as the indication of mating, therefore considered as gestational day 0 (GD0) (Figure 1). Once pregnancy was confirmed, each female rat was individually caged with shredded paper and wood chippings, as well as environmental enrichment which includes a tunnel and other colourful objects and given access to food and water *ad libitum*. Cages were kept at room temperature or 23-25 °C, regulated by a thermometer and temperature gauge, along with a 12-hour dark and light cycle (6:00 hours to 18:00 hours) for the duration of the study. Cages were cleaned twice a week to maintain a healthy environment. Animals were weighed on every second or third day as part of monitoring their health status. Animal mass was also used for estimation of the concentration of valproic acid that was administered on gestational day 12.5 (GD12.5). The body mass of each rat was divided by the concentration (600 mg/kg) to obtain the weight of VPA required for each rat. A total of 21 rats were assigned of which, eight dams had successful pregnancies and were used in the study. Each dam was randomly assigned into two groups, valproic-acid exposed (VPA, n= 4) and control (CON, n= 4). The VPA group received a single, subcutaneous injection of 600mg/kg valproic-acid sodium salt (per body weight) (Sigma-Aldrich, P4543 – 100 g)(Figure 1) dissolved in 0.9% sterile isotonic saline (2 ml) and the CON group received a single, subcutaneous injection of 0.9 % sterile isotonic saline (Figure 1) on the same day. Birth of pups occurred naturally between GD 19 and 21 and were left with the dams to suckle from postnatal day 1 (PND1) to PND21 under normal conditions, before weaning on PND23. The VPA dams produced litter sizes of

10, 6, 5 and 12 individuals, and the saline dams produced litter sizes of 10, 12, 13 and 7. All 75 rat pups were used in testing unless otherwise specified.

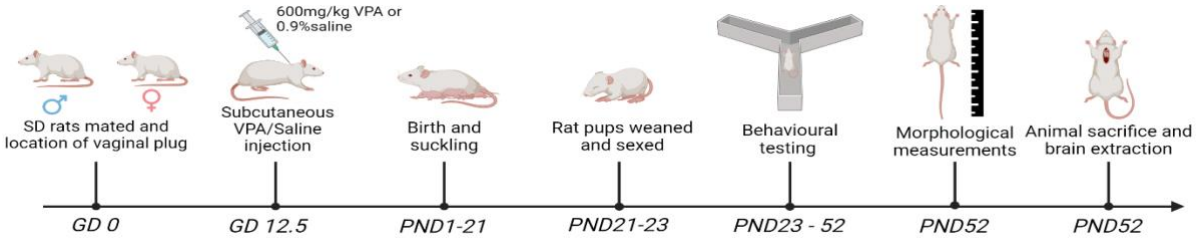


Figure 1: Diagram depicting the timeline of events from the mating of a male and female Sprague-Dawley (SD) rat to the presence of the vaginal plug labelled as gestational day 0 (GD). On gestation day 12.5 (GD12.5) each rat was randomly selected and administered with a subcutaneous injection of 2 ml of either VPA (600mg/kg) or a 0.9% saline solution. The birth of the pups was labelled as post-natal day (PND) 1 following which natural suckling was established between PND1 and PND21 from the mother. The SD rats were then weaned from their mothers and sexed before being placed in individual cages between PND21 and PND23. Behavioural testing took place between PND23 and PND52 following which morphological measurements were taken of each rat and then followed by SD sacrifice and extraction of the brain.

2.2. BEHAVIOURAL ASSESSMENTS

All behavioural testing was completed on rat pups weaned at postnatal day 21 (PND21) where each pup was sexed and placed in individual cages. Behavioural testing commenced from PND23 until PND56. Prior to each test, each pup was transported in its cage to the testing room and acclimatised for 30 minutes to 1 hour prior to training or testing, depending on the behavioural test. A total of 75 rat pups were used in testing, with a total of 32 VPA-exposed and 43 controls. There was a total of 15 males and 17 females for VPA-exposed group and 20 males and 23 females for the control group. The litter sizes varied between each dam. The VPA dams produced litter sizes of 5, 6, 10 and 12 individuals, and the saline dams produced litter sizes of 7, 10, 12 and 13. All 75 rat pups were used in testing unless otherwise specified.

2. Repetitive Behaviour: Marble Burying Test

The marble burying test was used to infer repetitive and compulsive-like behaviour, these methods were adapted from Angoa-Perez and colleagues (2013). This test was completed on PND41 on all 75 rat pups. The test utilized a standard housing cage filled with fresh, unscented

bedding to a depth of 6 cm and pressed down firmly. The animal was placed in the cage with the fresh bedding and allowed to acclimatize for 10 minutes. After the acclimatization time, the animal was removed and twelve marbles with a diameter of 35 mm, thoroughly cleaned with ethanol, were placed in 4 x 3 arrangement on top of the bedding (Figure 2). The animal was placed back in the cage and allowed to explore for 20 minutes. A picture was taken after the 20 minutes had elapsed and the animal was removed, the number of marbles that were 2/3 buried in the bedding was counted. The number of marbles buried were used to determine the extent of repetitive behaviour exhibited by each animal.



Figure 2: Image depicting the marble arrangement and bedding type for the marble burying assay.

2.2.2. Exploratory behaviour: Hole board Assay

A willingness or lack thereof to explore an unknown environment has been documented in VPA-induced ASD in rodents. This behaviour can be assessed using the hole board exploratory apparatus, herein adapted from Kumar and Sharma (2016), and completed on 35-day old rats, (PND35). The hole board arena consisted of a black board of 1-cm thickness placed on the floor of a Perspex box (53 x 44 x 19cm) painted all black. The black board contained 16 holes of 4 cm diameter spaced equally (Figure 3A). Four of the holes were each chosen to contain a food incentive (peanut butter and seed balls) which were placed diagonally through the centre of the board, these were named the incentive holes, and used to encourage exploration. The remaining holes were left empty, to determine the level of exploratory behaviour. A decrease in exploratory behaviour is hypothesised to result in a decreased

number of investigations of the all the holes present. Subject rats were acclimatized to the testing room for 1-hour prior to testing. Each subject rat was placed in the centre of the apparatus and allowed to explore for 5 minutes whilst being recorded via a webcam (Figure 3B). The video was then analysed for number of head dips, total distance travelled and average speed of travel in Any-maze software (Stoetling Co.).

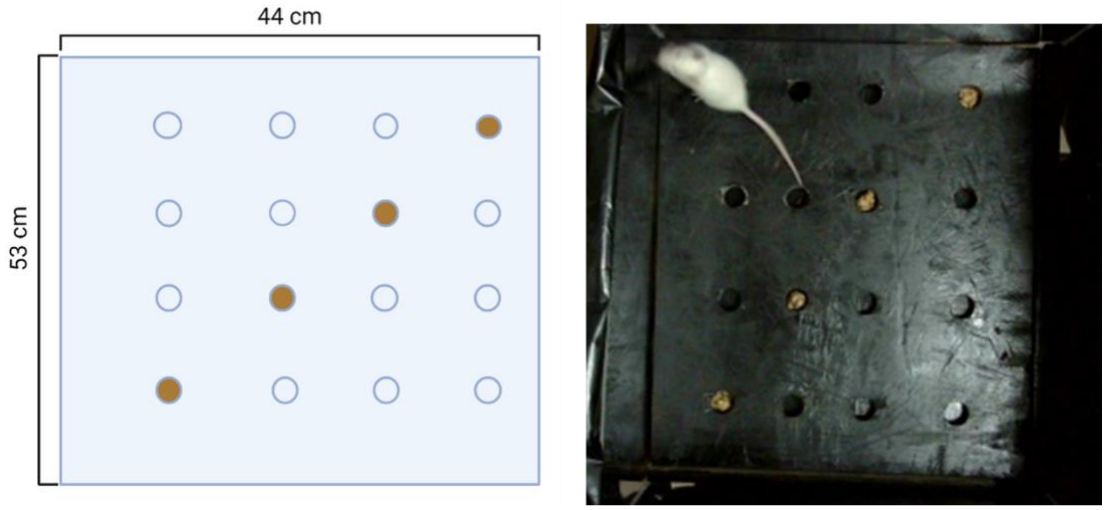


Figure 3: **A**, the hole board assay setup created using BioRender.com. **B**, the final hole board apparatus on test day, with a test rat pup undergoing the hole board test.

2.2.3. Sensory Stimuli: Hotplate Thermal Flick Test

The hotplate test is used to determine nociceptive response to a noxious thermal stimulus, analogous to the human response of removal of a hand from a heat source, which is a unconscious, natural response to thermal pain. This assay was completed on 52-day old rats, PND52. A metal, square hotplate was set to a temperature of 54.5 – 55.5 °C and monitored using an infrared thermometer (Dynamic Instrumentation, DT8380AH) (Figure 4). A subject rat was removed from its home cage and placed directly on the plate surrounded by mesh wire (Figure 4). The nociception was measured as the time elapsed or latency to respond (in seconds) from when the animal was placed on the hot plate and when it licks either of its hind paw (Kerr et al., 2013). The cut off time was set to 30 seconds to avoid any tissue damage to test animals. Each animal was recorded via a webcam and then analysed on Any-maze Software (Stoetling Co.).

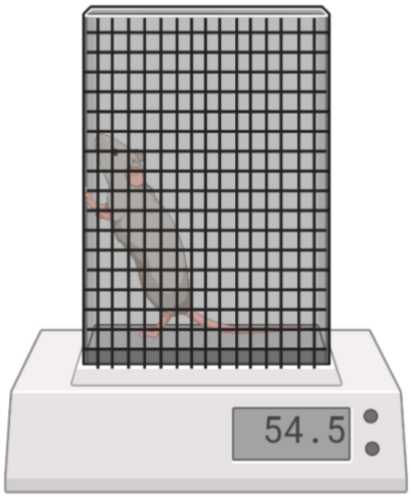


Figure 4: The hotplate thermal assay set up, with temperature expressed in degrees Celsius. Created using BioRender.com

2.2.4. Repetitive behaviour: Y-Maze Test

This test is used to determine spontaneous alternations where a decrease in the percentage of spontaneous alterations were taken as an indicator to an increase in sameness. This assay was completed on 32-day old rats. The Y-maze consisted of three equal arms at 120 ° to each other and dimensions of 30 x 9 x 15 cm (Figure 5A). All rat subjects were acclimatized to the testing room for 30 minutes prior to testing and were not exposed to the testing apparatus. Each rat was exposed individually to the maze, placed in the centre of the maze and allowed to explore freely for 8 minutes (Kumar & Sharma, 2016a), while being recorded by a webcam (Figure 5B). The series of arm entries were analysed using Any-maze software to determine the number of spontaneous alternations, an alternation occurring when the animal entered the arms in succession. The total distance travelled, and percentage of spontaneous alternations were determined via Any-maze software (Stoetling Co.). The percentage of spontaneous alternations were calculated using the following equation (Kumar & Sharma, 2016a; Merali et al., 2014):

$$\%Spontaneous\ alterations = \frac{Total\ alterations}{Total\ arms\ entered - 2}\ x\ 100$$

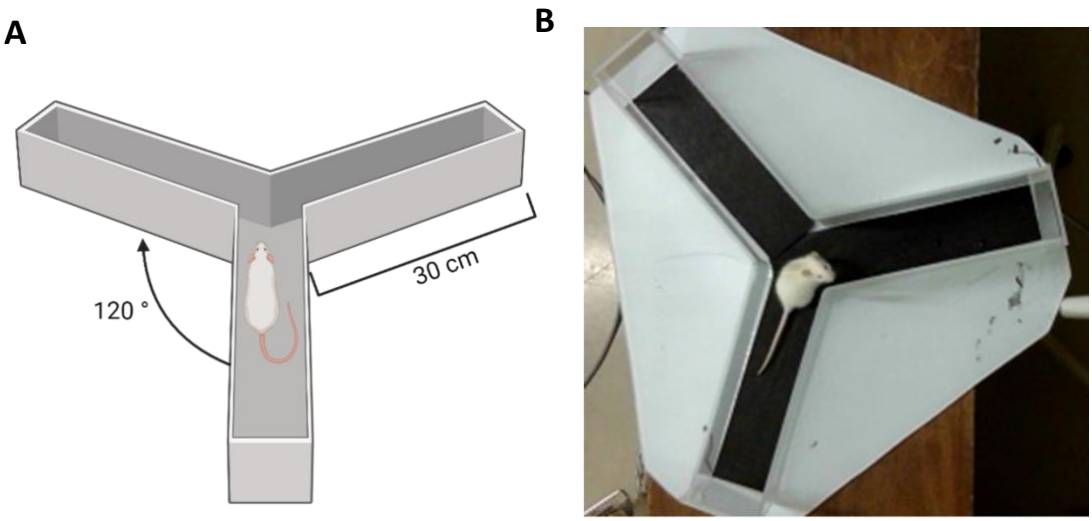


Figure 5: **A**, the y maze assay set up, created using BioRender.com. **B**, the final y maze apparatus set up on test day with a subject rat undergoing the test.

2.3. MORPHOLOGY

2.3.1. Tail length

After anaesthesia but before sacrifice, each rat subject, male and female, was laid out on a black piece of cardboard with their tail straightened out, and a ruler placed vertically and horizontally to the body surface in order to measure the length of the tail (Figure 6).

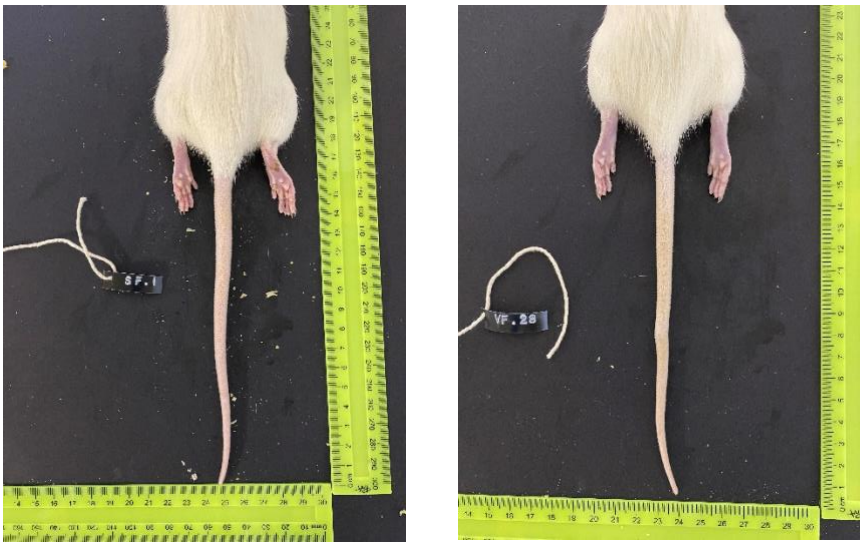


Figure 6: Images depicting the layout of each rat with the tail straightened out, with rulers placed vertically and horizontally in order to measure the length of the tails.

2.3.2. Tail Scoring

All tails were scored according to the number of kinks, number of bulges (i.e. no kink) (Figure 8) and the extremity of the tail kink. The extremity of the kink was scored as a straight tail (Figure 7A), a minor (Figure 7B), medium (Figure 7C) or extreme kink (Figure 7D).

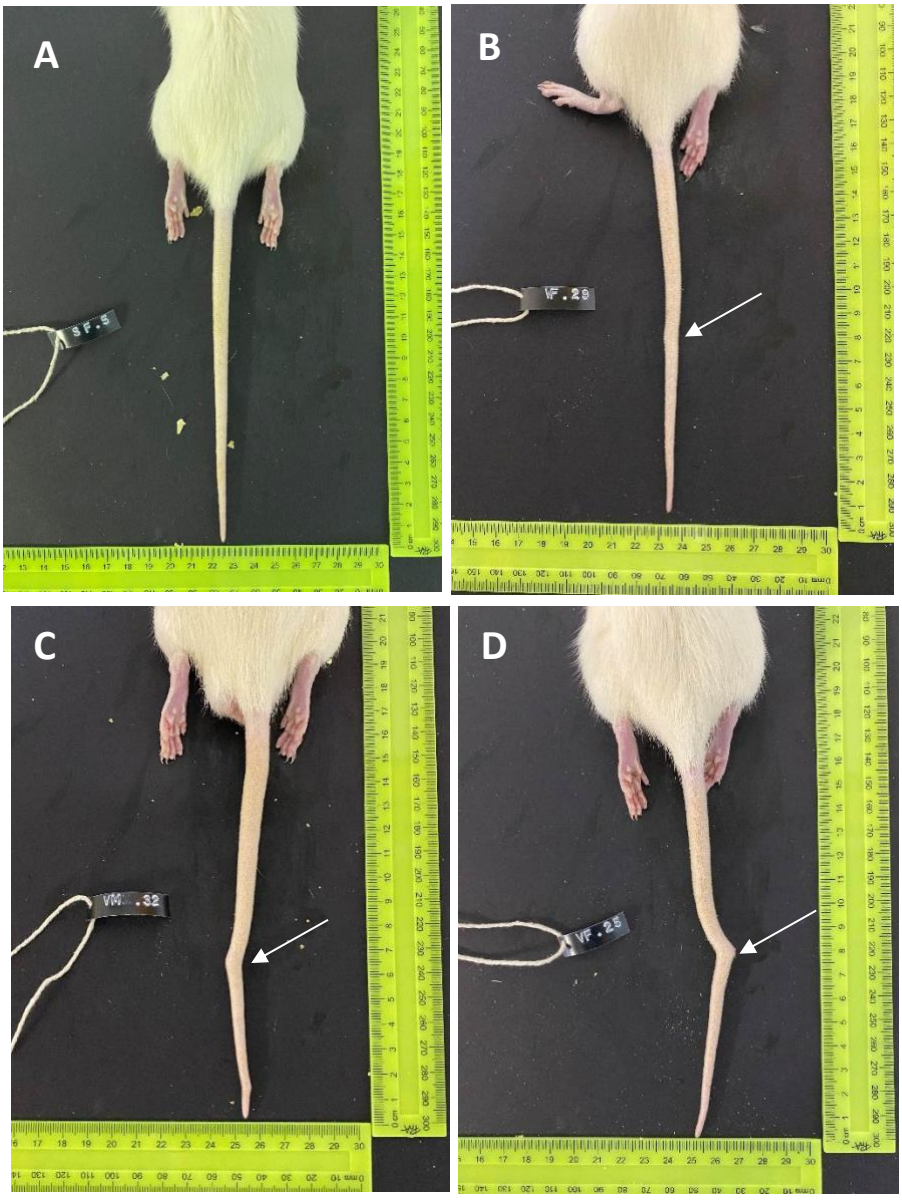


Figure 7: Images showing the extremity of the kinks of the tails of the VPA-exposed autism model only, each indicated by the white arrows. **A**, straight tail, **B**, minor kink; **C**, medium kink and **D**, extreme kink.

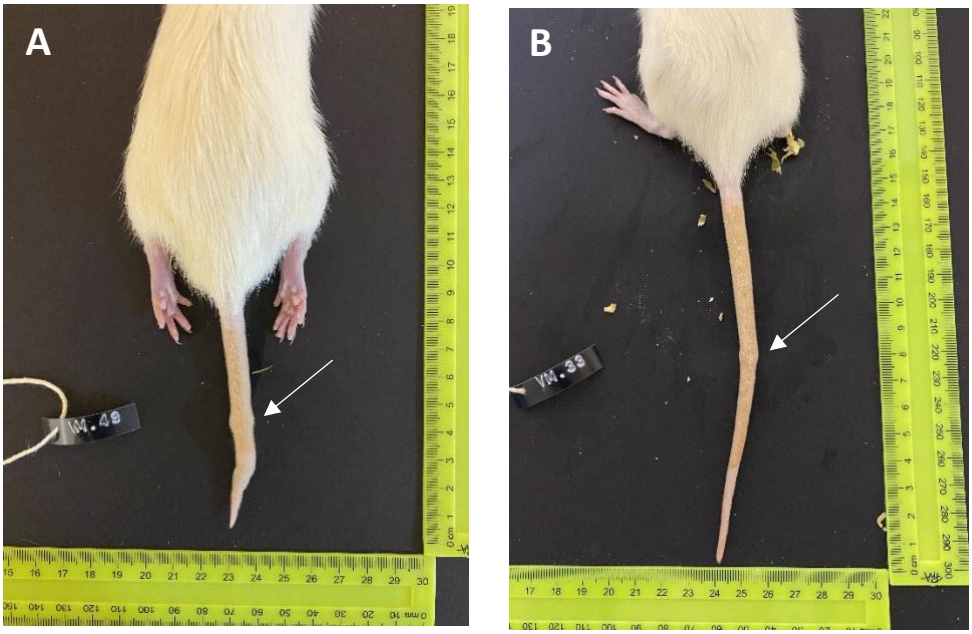


Figure 8: Images portraying the bulge that is present on the animal's tail, defined as an increase in width of either side of the tail but with no kink in the tail. Both **A** and **B** show the bulge presented by the white arrows.

Finally, each animal's tail was given a score for the number of kinks, number of bulges and the extremity of the kink in the tail. A minor kink is scored if there are at least 1 minor kink. A medium kink is scored if there are at least 1 or 2 medium kinks and 1 minor kink. Finally, an extreme kink is scored if there is 1 or 2 extreme kinks and 1 medium kink. Following which a combined score was given from the addition of the above scores together. The overall scores were determined following the rules given in Table 1.

Table 1: Table showing the tail score and description of each tail score to determine level of overall severity.

Overall Score	Description	Reference Image
0	at least 1 bulge but no kinks	Figure 8B
1	1 or more kinks or bulge, but minor kink score	Figure 7B
2	1 - 2 kinks with medium kink score	Figure 7C
3	1 - 2 kinks with extreme kink score	Figure 7D

2.4. ANIMAL EUTHANISATION

Subject rats received a subcutaneous injection of sodium pentobarbital (calculated according to mass of 800 mg/kg) until the body was limp and there was no response to pressure or pain. Once breathing had ceased, and the heart still beating, a thoracoabdominal incision was made to each animal and arterial blood was drawn from each left atrium. Following which two methods were completed to preserve the brains of each subject rat:

2.4.1. Transcardial Perfusion

A transcardial perfusion was completed on 32 subject rats of both VPA-exposed (*n* = 13) and control (*n* = 19), using ice cold 0,9 % isotonic saline, pushed through the blood vessels via injection through the right ventricle until a clear liquid was observed through the incision. This was followed by perfusion of ice cold 4 % paraformaldehyde (PFA) solution in 0.1M PB (pH 7.4) until the lungs appeared white. The brain was then extracted using small ronjeurs, weighed and then placed in a vial containing PFA solution before being prepared for cryo-storage. For cryo-storage, brains were placed in a 30 % sucrose solution in 0.1M phosphate buffer (PB) until the brain was saturated and had sunk to the bottom of the vial. The brains were then placed in antifreeze solution (consisting of ethylene glycol and glycerol) for storage in -20 °C.

2.4.2. Liquid Nitrogen Snap freezing

The remaining brains of VPA-exposed (*n* = 19) and control (*n* = 24) pups were not perfused and extracted immediately after blood collection and weighed. The brains were then carefully wrapped in foil and placed in a sealed plastic bag, with some holes created for air to escape, and placed in liquid nitrogen and then stored at -80 °C. The brains weighed before snap freezing were pooled together with the brains weighed after perfusion with PFA, assuming there is minimal effect of the two preparation methods.

Brain mass relative to body mass was calculated via the following formula:

$$Percentage\ brain\ mass = \frac{Brain\ mass}{Body\ weight} \times 100$$

2.5. VPA ELISA

The VPA ELISA kit was purchased from MyBioSource, the procedure followed the manufacturers protocol instructions from the Goat Valproic acid ELISA kit (MBS7271085) (**APPENDIX B**). All reagents were brought to room temperature before use, including the 96-well plate. The microplate was preheated for 15 minutes prior to use. This test was done solely on the VPA-exposed rats to determine the difference in VPA concentrations to compare litter sizes.

To determine the different VPA concentrations between litter sizes, blood serum from all 32 VPA-exposed rats, 15 males and 17 females, and two control samples, both male, were centrifuged at 3000 rpm for 15 minutes, of which 80 µl of serum, from each sample, was used for the ELISA. The serum samples were then diluted at a 1:2 with 80 µl of serum and 160 µl of PBS. A standard solution of 100 µl was added in duplicates to rows 1 and 2 of the 96-well plate, and 100 µl of PBS was added to the blank wells (standard 0 ng/l). Immediately after, 50 µl of conjugate was added to each well, excluding the blank wells and mixed well for 60 seconds. The plate was covered and incubated for 1 hour at 37 °C. The solution was decanted from each well and placed in the plate washer for automated washing of the 96-well plate. The addition of 400 µl of wash buffer was added to each well and soaked for 10 seconds, with a shaking time of 5 seconds between each wash. The plate was patted dry on absorbent paper. Substrates A and B were then added to each well, including the blank wells, and incubated for 20 minutes at 37 °C under foil for protection from light. A measurement of 50 µl of stop solution was added to each well including the blank wells and mixed well. The optical density of each sample was then determined using a micro plate reader at 450 nm. A standard curve was completed in GraphPad Prism using the concentrations and optical densities of the standards following which the concentrations of the unknown samples were calculated using the best fit line of the standard curve.

2.6. STATISTICAL ANALYSIS: MORPHOLOGY

Statistical analysis was completed in STATA/SE 17 software. The Shapiro-Wilk test for normality was done to determine normality of data, where normal distribution is $p > 0.05$. Following which a one-way ANOVA was completed to determine whether there were any significant differences between VPA-exposed and the control groups. This was followed by a post-hoc Bonferroni test to determine significant differences between each group of VPA-exposed male and females and control males and females.

The above statistical analysis was completed to compare litter-to-litter variations and within-litter variations based on sex.

All graphs were completed in GraphPad Prism using the mean and standard deviation values.

2.7. STATISTICAL ANALYSIS: BEHAVIOURAL ASSAYS

All statistics were completed in STATA/SE 17 statistics software. A comparison was done between sex, drug and the sex/drug interaction for behavioural tests completed on both VPA-exposed and control rats, followed by within group comparisons.

A Shapiro-Wilk test was completed to determine normality of data, where normal distribution is $p > 0.05$. If normally distributed, a two-way ANOVA was completed for sex interaction, drug interaction and the sex/drug interaction, followed by Tukey's Pairwise-test (post-hoc) to determine significant differences between groups of litter size and sex. Graphs were completed in GraphPad Prism, using the mean and standard deviation from the STATA program software. If data were not normally distributed, the Friedman's test was completed followed by the Dunn's Pairwise Comparison to determine significant differences between groups of litter size and sex. Graphs were completed in GraphPad Prism, using the Median and the Inter-quartile range (IQR) from the STATA program software.

However, when comparing litter sizes, a one-way ANOVA was completed followed by a Bonferroni post-hoc test for between group comparisons. The litter-to-litter and within litter

variation has the following abbreviations: litter size 10, male and female are 10M and 10F respectively, litter size 6 are 6M and 6F and so on.

CHAPTER THREE - RESULTS

3.1. VPA-EXPOSED VS CONTROL

3.1.1. Morphology

Tail length: the length of the tail was measured from the base of the tail that connects to the body to the very tip of the tail using a ruler. The results showed that there was a significant difference between all groups of VPA-exposed and control counterparts, male and female [χ^2 (3) = 13.76, p = 0.0000]. The post hoc comparisons between groups, there was a significant difference between VPA-exposed and control males (p = 0.001) (Figure 9A) where the VPA-exposed had shorter tails compared to their control counterparts. There was also a significant difference between the VPA-exposed and control females (p < 0.001) (Figure 9A), with the VPA-exposed having a shorter tail than the controls. However, there were no significant differences between the VPA-exposed male and females' groups (p = 1) (Figure 9A) and between the control male and females (p = 1) (Figure 9A).

Brain mass: all brain masses, with both perfused and non-perfused pooled together, were measured on a scale immediately following dissection. The results showed that there was a significant difference between all four groups of VPA-exposed male and female, and control group male and female [$F_{(3, 57)}$ = 7.58, p = 0.0003]. The post hoc comparison test showed that there was a significant difference between the males and females of the VPA-exposed group (p = 0.038) (Figure 9B) and between the males and females of the control group (p =0.016) (Figure 9B), with the males of both groups having a lower brain mass relative to body weight. There were no significant differences between the males of the VPA-exposed group (p = 0.646) (Figure 9B) and between the females of both groups (p = 0.663) (Figure 9B).

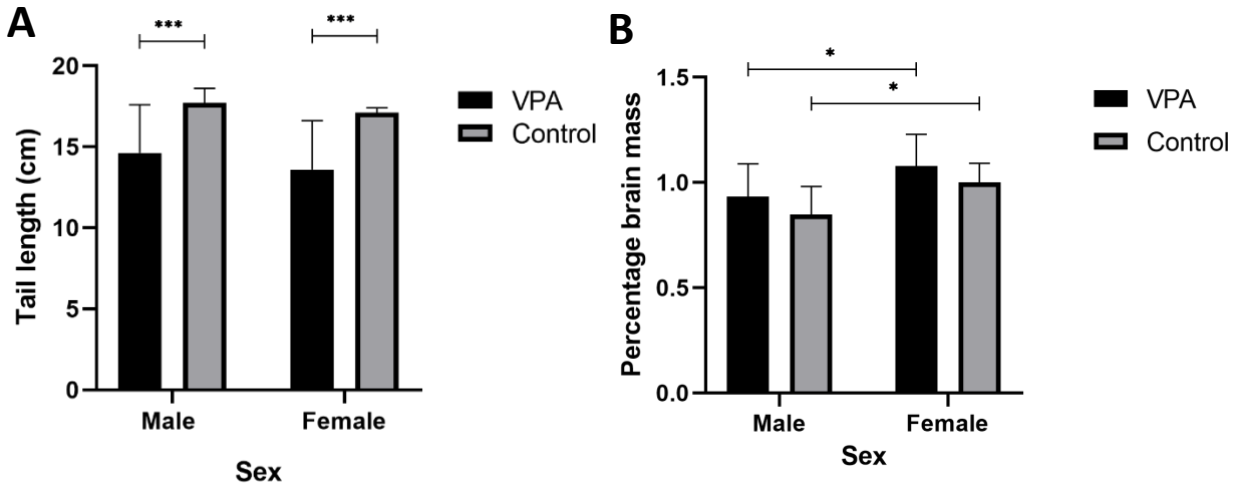


Figure 9: **Morphology**. Morphological features compared between VPA-exposed and control subjects. **A,** tail length measured in cm of the VPA-exposed males and females compared with the control males and females. **B**, percentage brain mass relative to body weight of the VPA-exposed males and females, and the control males and females, with males having smaller brains relative to body weight. Error bars indicate standard deviation. Data is presented as non-parametric data with mean and IQR and where * indicates $p < 0.05$ and *** indicates $p < 0.001$.

3.1.2. Behavioural Assays

3.1.2.1. The Marble Burying Assay

Following a 20-minute period while left uninterrupted in the marble burying apparatus, the marbles were photographed to note degree of concealment from the surface of the sawdust. The urge to bury marbles shows a tendency towards repetitive behaviour, which is equivalent to the repetitive behaviour associated with one of the symptoms of autism in humans. There was no drug effect on the number of marbles buried between the controls and the VPA-exposed autism model [χ^2 (1) = 0.42, p = 0.52]. In addition, sex played no role in the number of marbles buried [χ^2 (1) = 0.49, p = 0.49]. Within the VPA-exposed animals, the males and females exhibited no significant difference in degrees of marble burying (p = 0.13) (Figure 10); similarly, in the control group, the males and females buried marbles to the same (p = 0.48) (Figure 10). The level of repetitive behaviour was found to be similar across all four groups in the study.

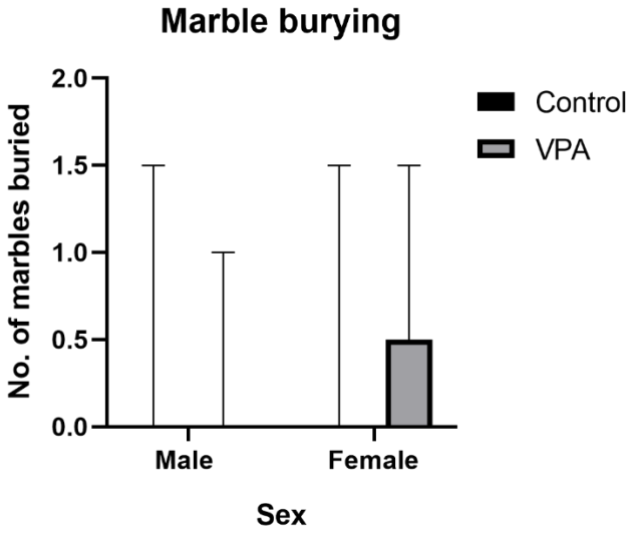


Figure 10: **Marble burying.** Graph depicting the marble burying assay results comparing VPA-exposed males and females and the control group males and females, showing no increased marble burying by any group. The absence of bars indicates zero data. Error bars indicate standard deviation. Data is presented as non-parametric data with mean and IQR.

3.1.2.2. Hole Board Assay

Total number of investigations (head dips): The hole board test simulates a novel environment and tests the willingness to explore, this represents the human autism characteristic of lack of openness to explore the unknown. With regards to number of investigations in the hole board apparatus, there were no significant differences when compared along drug exposure [$F_{(1, 71)}$ = 3.64, p = 0.06], sex [$F_{(1, 71)}$ = 2.47, p = 0.12] or interaction between sex and drug exposure [$F_{(1, 71)}$ = 2.31, p = 0.13] (Figure 11A).

Distance travelled: The total distance travelled within the apparatus indicated a significant difference when drug exposure [$F_{(1, 71)}$ = 7.92, p = 0.006] and sex [$F_{(1, 71)}$ = 16.98, p < 0.001] were considered, but not the interaction between the two variables [$F_{(1, 71)}$ = 3.23, p = 0.08]. The VPA-exposed group was found to have travelled greater distances than their control counterparts, while the females had travelled significantly longer distances than their male counterparts. *Post hoc* comparisons showed that within the VPA-exposed group, the females travelled significantly more than the males (p = 0.01) (Figure 11C), while the control males and females showed no differences in travel distance (p = 0.34) (Figure 11C). The males across

drug exposure were not statistically different from each other (p = 0.91) (Figure 11C), while the VPA-exposed females travelled longer than the control females (p = 0.005) (Figure 11C).

Speed of travel: For average speed of travel within the hole-board apparatus, differences were observed along drug exposure [$F_{(1, 71)} = 7.71$, p = 0.007], sex [$F_{(1, 71)} = 16.98$, p < 0,001], and none for the interaction between the two variables [$F_{(1, 71)} = 3.68$, p = 0.06]. *Post hoc* comparisons showed that within the sexes, the VPA-exposed males showed no difference in speed of travel from the control males (p = 0.94) (Figure 11B); while the VPA-exposed females travelled significantly faster than the control females (p = 0.004) (Figure 11B). Within the VPA-exposed group, the females showed higher speeds than the males (p < 0,001) (Figure 11B). Within the control group, we found no difference in speeds of travel between the two sexes (p = 0.35) (Figure 11B). Taken together, the parameters assessed in the hole board test suggest the VPA-exposed female group displaying hyperlocomotion.

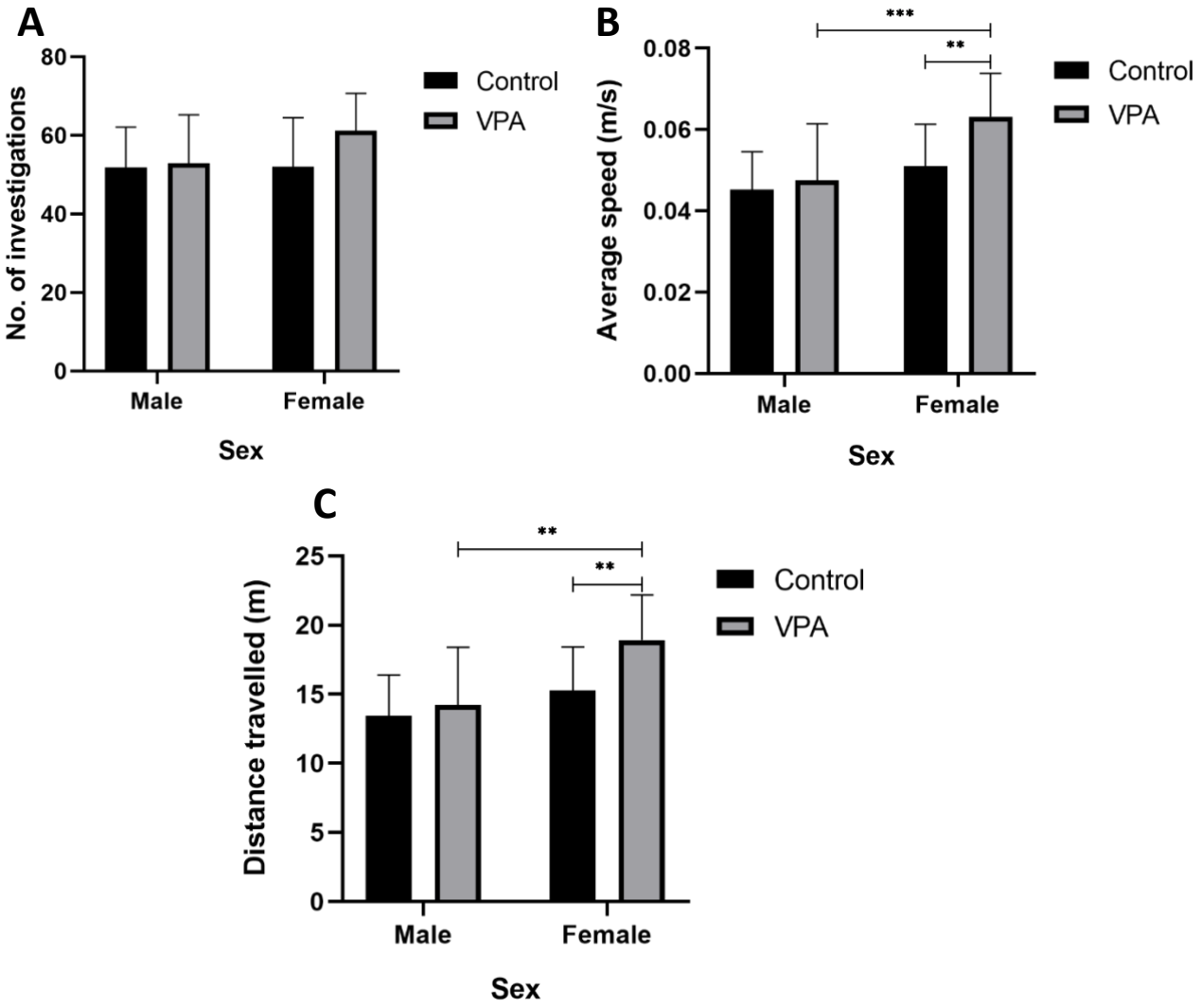


Figure 11: **Hole board**. The outcomes of the hole board assay compared between the VPA-exposed autism model and the control group. **A**, quantification of the total number of investigations (head dips) into the investigation holes indicating no significant differences between groups. **B**, average speed travelled while exploring the hole board apparatus, indicating that VPA-exposed females travelled significantly faster than their male counterparts as well as the control females. **C**, the distance travelled during the hole board test, indicating that VPA-exposed females travelled significantly further distances than the control females and VPA-exposed males. Error bars indicate standard deviation. Data is presented as mean ± standard deviation where ** indicates $p < 0.01$ and *** indicates $p < 0.001$.

3.1.2.3. Hotplate Thermal Assay

The hotplate test was used to assess the nociceptive threshold to a mildly painful thermal stimulus. Across the drug exposure, no differences in the threshold were observed [χ^2 (1) = 1.43, p = 0.23], while in between the sexes, no differences were found either [χ^2 (1) = 2.28, p =0.13]. However, within the control group, the males showed a significantly higher

nociceptive threshold than the females (p = 0.03) (Figure 12); while the VPA-exposed males and females showed no difference in their nociceptive thresholds (p = 0.44) (Figure 12).

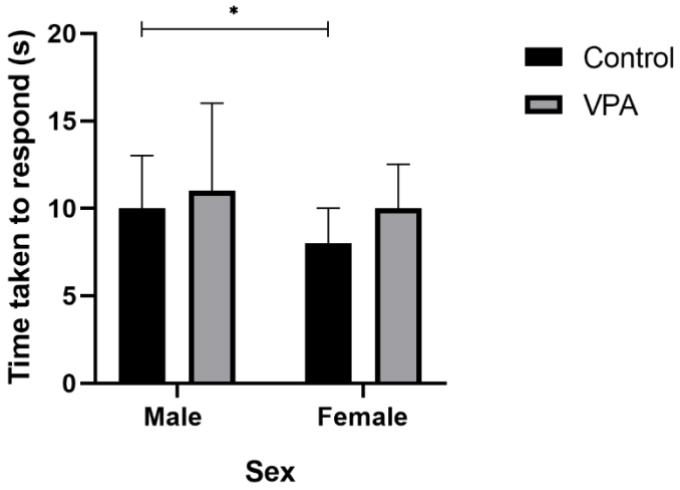


Figure 12: **Hot plate**. Graph indicating the time taken to react to the hot plate in seconds, comparing the VPA-exposed autism model and the control counterparts, where there were no significant differences between the VPA-exposed and control groups, while the control males showed a longer reaction time. Error bars indicate standard deviation. Data is presented as mean ± standard deviation, where * indicates $p < 0.05$.

3.1.2.4. Y-Maze Assay

Percentage of spontaneous alternations: No differences were observed when assessing the level of spontaneous alternations by drug exposure [$F_{(1, 71)} = 0.18$, $p = 0.68$], sex [$F_{(1, 71)} = 0.04$, $p = 0.84$], or by sex and drug exposure interaction [$F_{(1, 71)} = 0.12$, $p = 0.73$] (Figure 13). The Y-maze test revealed the same levels of spatial short-term memory and no heightened repetitive behaviours in any of the groups.

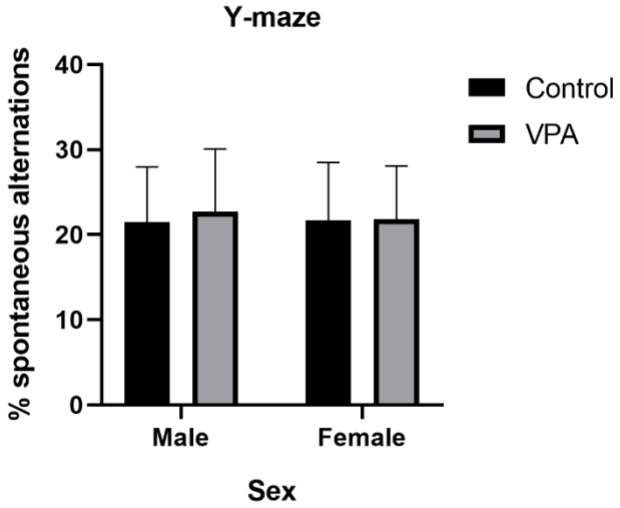


Figure 13: **Y maze**. Graph showing the outcome for the y maze assay representing the percentage of spontaneous alternations for the VPA-exposed autism model and control groups, for both males and females. The graph indicates that there were no significant differences between the four groups. Error bars indicate standard deviation. All data presented as mean ± standard deviation.

3.2. VPA LITTER VARIATION

3.2.1. Morphology

With respect to morphology, the present study selected a few key features to examine which are indicative of neural tube development (tail length, tail morphology and brain mass), and quantified these accordingly for a comparative analysis between the experimental and control cohorts, as well as with respect to varying litter sizes.

3.2.1.1. Tail Length

The measurement of tail length for each offers further information on the impact of VPA-exposure on the development and closure of the neural tube. Therefore, this important measurement was undertaken and compared across the various litter sizes and within each litter to assess whether the same effects could be seen in the same litter, and whether a litter size would necessarily have a predictive value on the impact of VPA on specific litter sizes, i.e. large vs small litter sizes. The litter size, i.e. number of individuals in each litter, is indicated by the number and the sex is indicated by the letter M, for male and F, for female.

First we compared tail lengths between the various litters, where we noted that litter-to-litter comparisons showed a significant difference between the four groups, which contained litters of five (“litter 5”), six (“litter 6”) , ten (“litter 10”) and eleven (“litter 11“) [χ^2 (3) = 26.083, p = 0.0001]. The *post hoc* comparisons of all groups showed that there was a significant difference between litter 5 and 10 (p < 0.0001) (Figure 14A) and litter 5 and 11 (p = 0.028) (Figure 14A), where litter size 5 had the shortest tails. There was also a significant difference noted between litter 6 and 10 (p < 0.0001) (Figure 14A) and litters 6 and 11 (p < 0.0001) (Figure 14A), where litter size 6 also had the shortest tails. In addition, litters size 10 and 11 also showed a significant difference in tail length (p = 0.001) (Figure 14A), with litter 10 having the longest tails, however, there was no significant difference seen between litters 5 and 6 (p = 0.053) (Figure 14A). The smaller litters displayed an effect of shortening of the tail as an impact of VPA. However, there was an indication that the larger litter sizes do vary in their effects from the VPA. Although litter 10 showed significantly longer tails than litter 11, both the litters are large and show consistently longer tails than the smaller litter sizes.

Still considering the litter-to-litter variation, sex-specific analyses showed a significant difference between the same sexes of each litter size [χ^2 (3) = 27.822, p = 0.0002]. *Post hoc* test comparisons showed that there was a significant difference between the males of litter 5 and 10 (5M and 10M, p = 0.003) (Figure 14B) and the females of litter 5 and 10 (5F and 10F, p < 0.001) (Figure 14B), where litter 5 had the shortest tails in both sexes. For litters 6 and 10, the males showed a significant difference (p < 0.001) (Figure 14B) and so did the females (p < 0.001) (Figure 14B) with litter 6 having the shortest tails in both sexes. In addition, there was a significant difference between the males of litter 6 and 11 (p = 0.007) (Fig. 14B) and the females displayed the same pattern (p < 0.001) (Figure 14B), the smaller litter i.e. litter 6 again having the shorter tails than litter 11, for both male and female subjects. This indicated that the smaller litter sizes had significantly shorter tails in both sexes relative to the larger litters. When the larger litters 10 and 11 were compared to one another, there was a significant difference observed between both males (10M and 11M, p = 0.03) (Figure 14B) and the females (10F and 11F, p = 0.022) (Figure 14B), an indication that the larger litter sizes are effected by the VPA differently. However, there was no significant difference in tail length between the males of litter 5 and 11 (5M and 11M, p = 1) (Figure 14B) and the females (5F and 11F, p = 0.424) (Figure 14B), as well as no significant difference between the males of

litters 5 and 6 (5M and 6M, p = 1) (Figure 14B) and the females (5F and 6F, p = 0.077) (Figure 14B) in terms of length of tails.

When comparisons were done within each litter i.e. the male and female subjects of each litter size, there was a significant difference between the sexes within each litter [χ^2 (3) = 27.822, p = 0.0002]. The *post hoc* comparisons showed no significant differences between the sexes within each litter size, namely within litter 10 (10F and 10M, p = 1) (Figure 14B), litter 6 (6F and 6M, p = 0.22) (Figure 14B), litter 5 (5F and 5M, p = 1) (Figure 14B) and litter 11 (11F and 11M, p = 1) (Fig. 14B). This indicates that there is no significant variation of tail length within each litter with male and females showing similar morphological effects of VPA exposure.

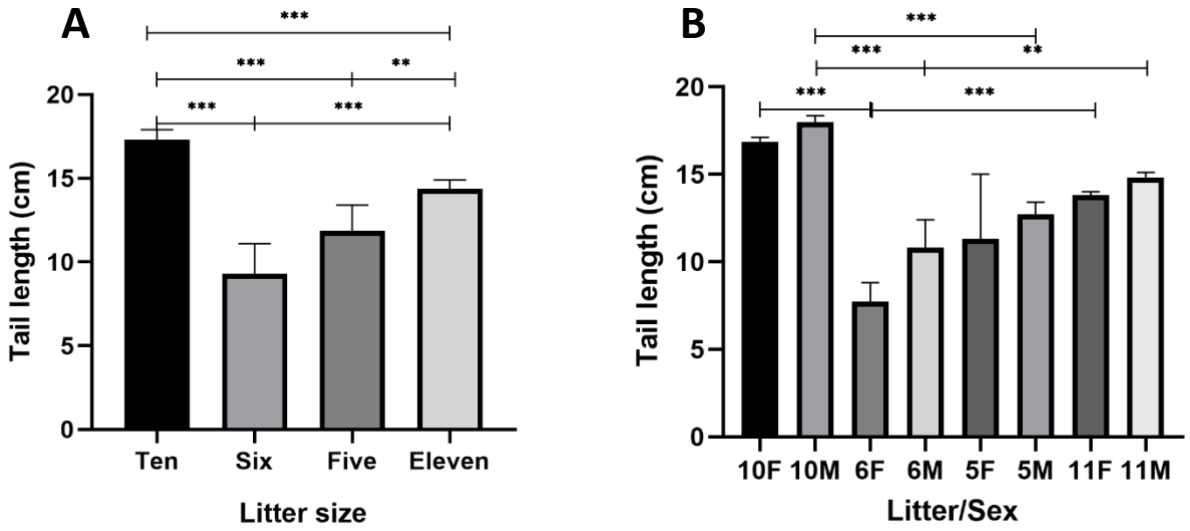


Figure 14: **Tail length (cm)**. Comparing the tail lengths amongst litter sizes within the VPA-exposed autism model. The x-axis represents the size of the litter, and the M and F indicates the sex, male and female respectively**. A**, litter-to-litter variation of tail length, with litter ten having the longest tails. **B**, within-litter variation between the sexes, and litter-to-litter comparisons by sex. Litter ten males (10M) had the longest tails and litter six female (6F) had the shortest tails. Error bars indicate standard deviation. All data presented as mean ± standard deviation where ** indicates p < 0.01 and *** indicates p < 0.001.

3.2.1.2. Tail Scores

The morphological appearance of the tails was then analysed for litter-to-litter variation and within-litter variation. The observation noted the tails' straightness or lack there of, including features such as bulging or thickening, angling along its length to form "kinks", and the number of kinks formed along the length of the tail. Based on the morphological features, a scoring system was developed to quantify the variations between the subjects' tail morphologies, which ranged from "typical", where the tail had no kinks, to "mild" where the tail had one kinkand onwards to "severe" where the tail had two kinks or more, see Table 1. There was observed a significant difference in tail morphology between the litter sizes [$F_{(3, 28)} = 4.83$, $p = 0.0079$]. *Post hoc* comparsions indicated a signifciant difference between litters 6 and 10 ($p = 0.014$) (Figure 15) and between litters 6 and 11 ($p = 0.012$) (Figure 15), where litter size 6 had the most severe tail scores. There was also a significant difference between litters 6 and 5 ($p = 0.049$) (Figure 15), where litter 5 had less severe tail scores, which were similar to litters 10 and 11's scores.

For within-litter variation, there was a signifcant difference between the groups when each litter was separated by sex [$F_{(7, 24)} = 3.44$, $p = 0.0108$]. *Post hoc* Bonferroni comparisons specified that there was a significant difference between the males and females of litter 6 and 5 (6F and 5F, $p = 0.022$) (Figure 15), with males having the highest severity tail score. However, there were no significant differences between male and female tail scores in all the other litters (10, 11, 6 and 5, $p = 1$).

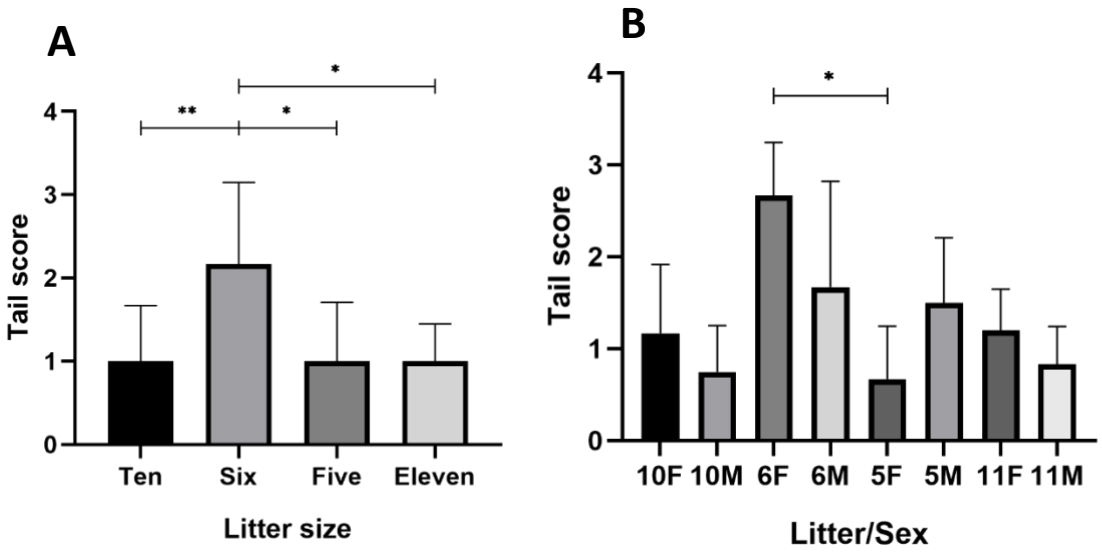


Figure 15: **Tail Score**. The comparison of tail scores for litter-to-litter variation and within-litter variation between males and females, i.e. the severity of the kink and number of kinks, for the VPA-exposed group. The x-axis represents the size of the litter, and the M and F indicates the sex, male and female respectively. **A**, comparison amongst the different VPA-exposed litter sizes and litter-to-litter comparison by sex where litter 6 had the highest tail score. **B** within-litter variation between the sexes, and litter-to-litter comparisons by sex, where the females of litter 6 (6F) had the highest tail score. Error bars indicate standard deviation. Data is presented as mean ± standard deviation where * indicates $p < 0.05$ and ** indicates $p < 0.01$.

3.2.1.3. The effect of VPA exposure on brain mass

Brain mass is another morphological feature was assessed herein to investigate the effects of VPA exposure on the developing brain, and whether such effects vary according to the different litter sizes. This measure was expressed herein as a percentage relative to body mass. The litter-to-litter variation and comparison showed a significant difference between the litter sizes [$F_{(3, 26)} = 5.71$, $p = 0.0045$]. The *post hoc* analysis showed a significant difference between litter size 11 and 10 ($p = 0.005$) (Figure 16A), with litter size 10 having smaller brains than litter 11, relative to body mass. However, there was no significant difference between litter 5 and 11 ($p = 0.504$) (Figure 16A), litter size 6 and 11 ($p = 0.064$) (Figure 16A) and litter size 5 and 6 ($p = 1$) (Figure 16A).

The litter-to-litter comparisons between the same sexes revealed significant differences between the same sexes of each litter size [$F_{(7, 26)} = 8.53$, $p = 0.0001$]. Post hoc comparisons showed a significant difference between 11F and 10F ($p = 0.041$) (Figure 16B), with 10F having

smaller brains relative to body mass compared to 11F. There was also a significant difference between the males of litter size 10 and 11 (p = 0.005) (Figure 16B) with the males of litter 10 having smaller brains relative to body mass. Finally, there was a significant difference between the females of litter size 6 and 11 (p = 0.023) with litter 11F having larger brains relative to body mass (Figure 16B).

Looking at a sex comparison within each litter, results showed no significant differences between the sexes of any litters (p = 1) (Figure 16B). This lack of variation within each litter in terms of brain size, where male and female have similar brain weights, indicates that both sexes had similar effects from VPA exposure.

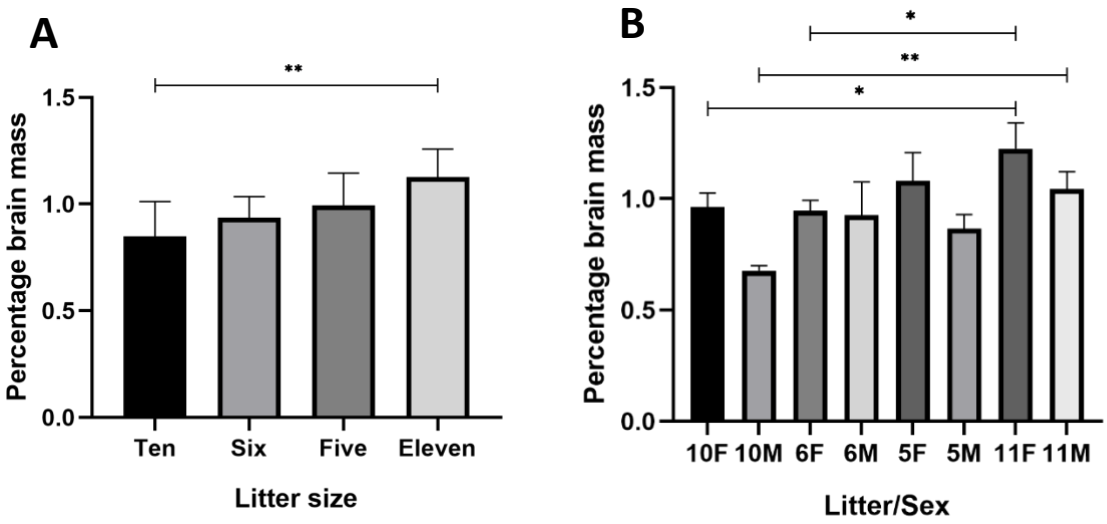


Figure 16: **Percentage brain mass**. The percentage brain mass relative to body mass amongst the different litter sizes and sex within the VPA-exposed autism model. The x-axis represents the size of the litter, and the M and F indicates the sex, male and female respectively. **A**, litter-to-litter variation between litter sizes only with litter size eleven having the largest brains relative to body mass. **B**, within-litter variation between the sexes, and litter-to-litter comparisons by sex, with litter ten, male and female (10M and 10F) having the largest overall brain mass. Error bars indicate standard deviation. All data presented as mean ± standard deviation where * indicates $p < 0.05$, ** indicates $p < 0.01$.

3.2.2. Behavioural Assays

With respect to behavioural analysis, the present study selected a few key features to examine which are indicative of the autism-like phenotype (repetitiveness, exploration,

sensory processing, and special memory), and then compared them between the experimental and control cohorts, as well as with respect to varying litter sizes.

3.2.2.1. Marble Burying Assay

With respect to behavioural analysis, the present study selected a few key features to examine which are indicative of the autism-like phenotype (repetitiveness, exploration, sensory processing and special memory), and then compared them between the experimental and control cohorts, as well as with respect to varying litter sizes. When comparing only the VPA litters in the marble burying assay, where the number of marbles buried indicates repetitive behaviour, the results showed that there was no significant difference in marbles buried between the litter sizes [$F_{(3,28)} = 0.18$, $p = 0.9071$] (Figure 17A). Furthermore, there was no significant difference between the four different litter sizes, between both male and female, exposed to VPA [$F_{(7,24)} = 0.79$, $p = 0.6017$] (Figure 17B). This indicates that there was no effect of VPA on repetitive behaviour observed between the different litter sizes.

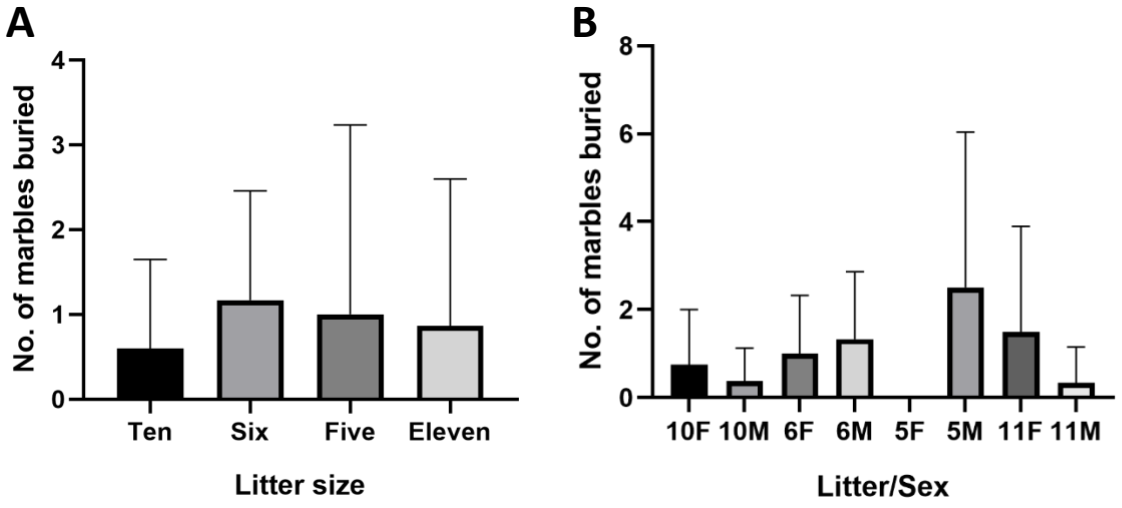


Figure 17: **Marble burying**. Graphs quantifying the number of marbles buried amongst litter sizes for the VPA-exposed autism model. The x-axis represents the size of the litter, and the M and F indicates the sex, male and female respectively. **A**, number of marbles buried comparing the different litter sizes. **B**, number of marbles buried comparing within-litter variation between the sexes, and litter-to-litter comparisons by sex. Error bars indicate standard deviation. Data is presented as mean ± standard deviation showing no significant differences.

3.2.2.2 Hole Board Assay

the hole board assay was employed herein to assess interest or lack thereof in exploring a novel environment in the subject rats, which when excessive, is seen as hyperactivity in humans, and when depressed is seen as insistence on sameness and lack of interest in humans. For this assay, a few parameters were noted and quantified, namely total number of investigations, distance travelled and speed of travel.

Total number of investigations: this parameter measured the total number of investigations of the holes presented to the subject rats within the hole board apparatus. When assessing the VPA-exposed litter sizes only, or litter-to-litter variation, there was no significant difference observed in terms of investigation numbers between litter sizes [$F_{(3, 24)} = 1.41$, $p = 0.2606$] (Figure 18A). When comparing within-litter variation when separated by sex, there was also no significant difference between the groups [$F_{(7, 24)} = 1.32$, $p = 0.2817$] (Figure 18A).

Distance travelled: this parameter indicates a hyperactivity, when increased, or hypoactivity as a response to the novel environment. The litter-to-litter variation for the total distance travelled amongst each VPA-exposed litter showed a significant difference between the litter sizes [$F_{(3, 28)} = 3.11$, $p = 0.0423$]. The *post hoc* comparisons showed a significant difference between litter size 6 and 11 ($p = 0.041$) (Figure 19A), but no significant difference between litter 5 and 11 ($p = 1$) (Figure 19A) and between litter 5 and 6 ($p = 0.343$) (Figure 19A). There were no significant differences between litter size 5 and 10 ($p = 1$) and litter 6 and 10 ($p = 0.107$). Furthermore, there were significant differences between litter size, when comparing males and females [$F_{(7, 24)} = 6.92$, $p = 0.0001$]. The *post hoc* comparisons showed a significant difference between males of litters 6 and 10 (6M and 10M, $p = 0.017$) (Figure 19B), with 6M showing a significantly shorter distance travelled within the hole board. There were no significant differences in distance travelled between litters 5M and 10M ($p = 1$) (Figure 19B), litters 6F and 11F ($p = 0.336$) (Figure 19B). The results for within-litter variation showed no significant difference between litter 10 (10F and 10M, $p = 0.685$) (Figure 19B), litter 6 (6M and 6F, $p = 0.051$) (Figure 19B), and litter 5 (5M and 5F, $p = 1$) (Figure 19B). However, there was a significant difference between litter 11 (11F and 11M, $p = 0.012$) (Figure 19B), where 11F showed a significantly longer distance travelled compared with 11M. An increased distance travelled indicates hyperactivity when combined with no change in the number of investigations.

Speed of travel: this parameter also indicates hyper/ hypoactivity with an increased speed indicating hyperactivity and a decreased speed indicating the latter. The speed of travel was measured, and for litter-to-litter variation showed a significant difference between litters [$F_{(3, 28)} = 3.12$, $p = 0.0420$]. The post-hoc group comparison showed a significant difference between litter 6 and 11 ($p = 0.043$) (Figure 20A), where litter 6 showed a significantly slower speed of travel indicating a lack of interest and hypoactivity when exploring the novel environment. However, there was no significant differences between litter 5 and 10 ($p = 1$) (Figure 20A), litter 5 and 11 ($p = 1$) (Figure 20A) and litter 6 and 10 ($p = 0.098$) (Figure 20A). The litter-to-litter variation separated by sex showed a significant difference between litter 6M and 10M ($p = 0.017$) (Figure 20B), where 6M had a significantly slower speed of travel. However, there was no significant difference between litter 6M and 11M ($p = 0.08$) (Figure 20B), litter 6F and 11F ($p = 0.352$) (Figure 20B), litter 5M and 11M ($p = 0.359$) (Figure 20B), and 5F and 11F ($p = 1$) (Figure 20B). There was also no significant difference regarding litter 6F and 10F ($p = 1$) (Figure 20B), litter 5F and 10F ($p = 1$) (Figure 20B) and litter 5M and 10M ($p = 1$) (Figure 20B). Litter 6, with a decreased speed of travel, indicates that VPA had more of an effect on the smaller litter size compared with litter 11, a larger litter size. No significant differences indicate no change in the speed of travel and no increased interest in exploring the novel environment.

The results for within-litter variation showed a significant difference between groups [$F_{(7, 24)} = 6.86$, $p = 0.0002$]. The *post hoc* comparisons showed as significant difference between litter 11M and 11F ($p = 0.013$) (Figure 20B), where litter 11M had a significantly slower speed of travel. However, there was no significant difference between litter 6M and 6F ($p = 0.055$) (Figure 20B), 5M and 5F ($p = 1$) (Figure 20B) and 10M and 10F ($p = 1$) (Figure 20B). The significantly slower speed of travel in the males of litter 11 indicates a reduced interest in exploratory behaviour and hypoactivity compared with the females of the same litter. This indicates that VPA-exposure had different effects on the males and females of litter 11.

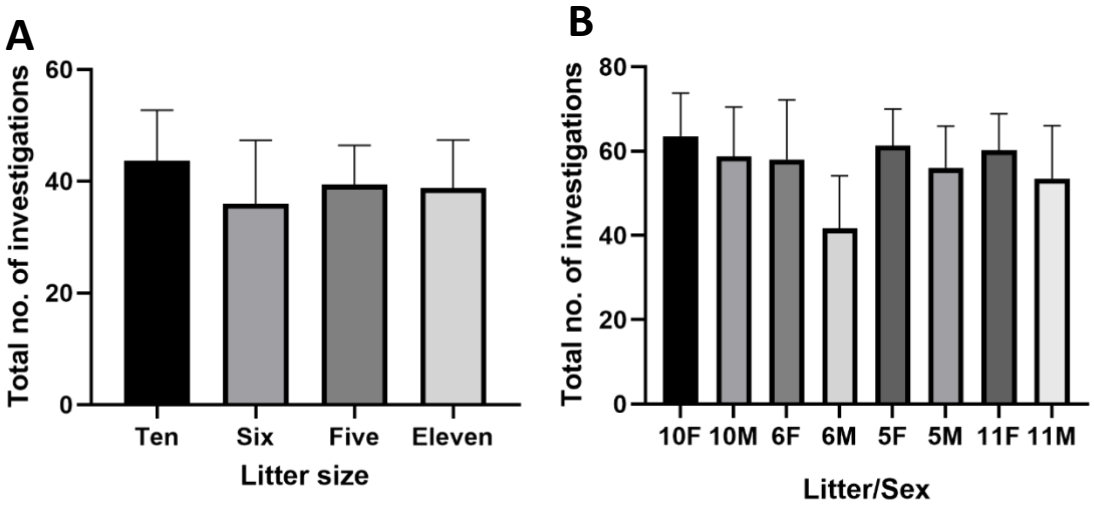


Figure 18: **Hole board: total number of investigations**. The total number of investigations amongst the litter sizes of the VPA-exposed autism model. The x-axis represents the size of the litter, and the M and F indicates the sex, male and female respectively. **A**, total number of investigations comparing the different litter sizes with no significant differences. **B**, total number of investigations comparing within-litter variation between the sexes, and litter-to-litter comparisons by sex, with no significant differences. Error bars indicate standard deviation. Data is presented as mean ± standard deviation.

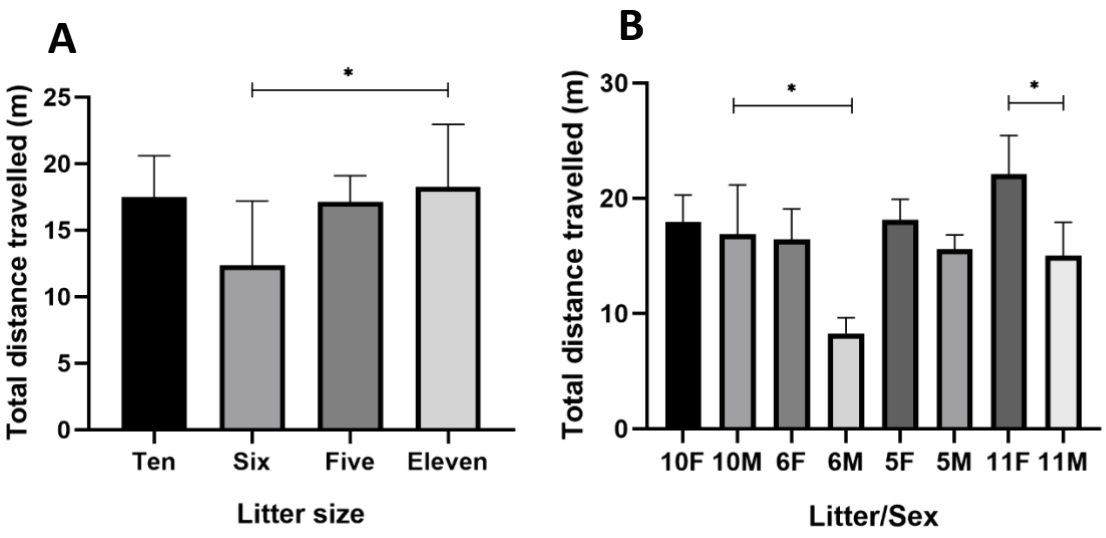


Figure 19: **Hole board: total distance travelled (m)**. The total distance travelled comparing litter sizes and sex of the VPA-exposed autism model. The x-axis represents the size of the litter, and the M and F indicates the sex, male and female respectively. **A**, the total distance travelled comparing the different litter sizes with litter 6 travelling the shortest distance compared to litter 11. **B**, total distance travelled within-litter variation between the sexes, and litter-to-litter comparisons by sex, with litter 6, males (6M) travelling the shortest distance. Error bars indicate standard deviation. Data is presented as mean ± standard deviation where * indicates $p < 0.05$.

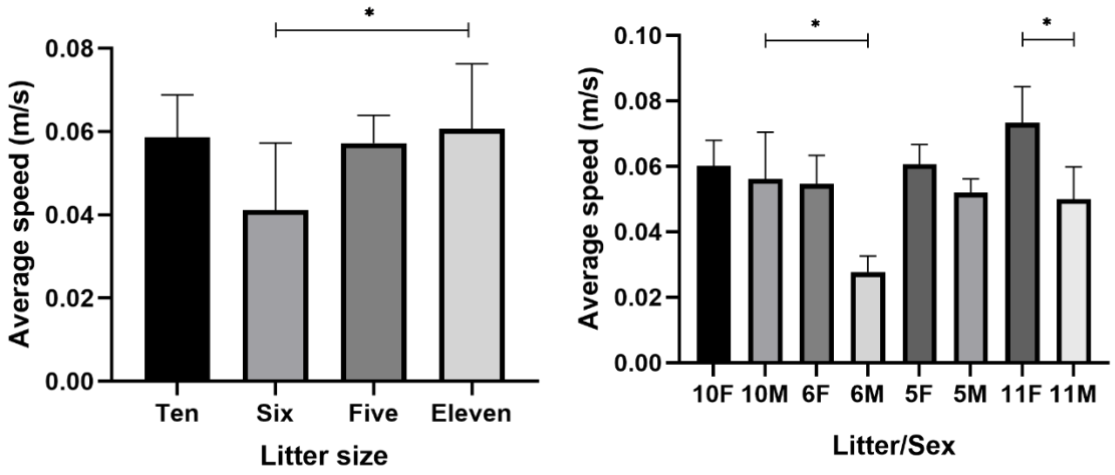


Figure 20: **Hole board: average speed (m/s)**. The average speed travelled amongst litter sizes of the VPA-exposed autism model. The x-axis represents the size of the litter, and the M and F indicates the sex, male and female respectively. **A**, the average speed comparing the different litter sizes with litter 6 travelling the slowest compared to litter 11. **B**, comparing the average speed within-litters between the sexes, and litter-to-litter comparisons by sex, with litter 6, male (6M) travelling the slowest. Error bars indicate standard deviation. Data is presented as mean ± standard deviation where * indicates $p < 0.05$.

3.2.2.3. Hotplate Thermal Assay

When comparing the litter-to-litter variation in the time taken to react from the hotplate thermal test, there was no significant difference between the different litter sizes [$F_{(3, 27)} = 1.54$, $p = 0.2264$] (Figure 21A). When looking at within-litter variation where sex is compared, there was also significant differences between litters [$F_{(7, 23)} = 1.96$, $p = 0.1051$] (Figure 21B). This shows no change in response time of the VPA-exposed subjects indicating no hyper/hypoactivity in response to VPA, and no change in the processing of sensory stimuli.

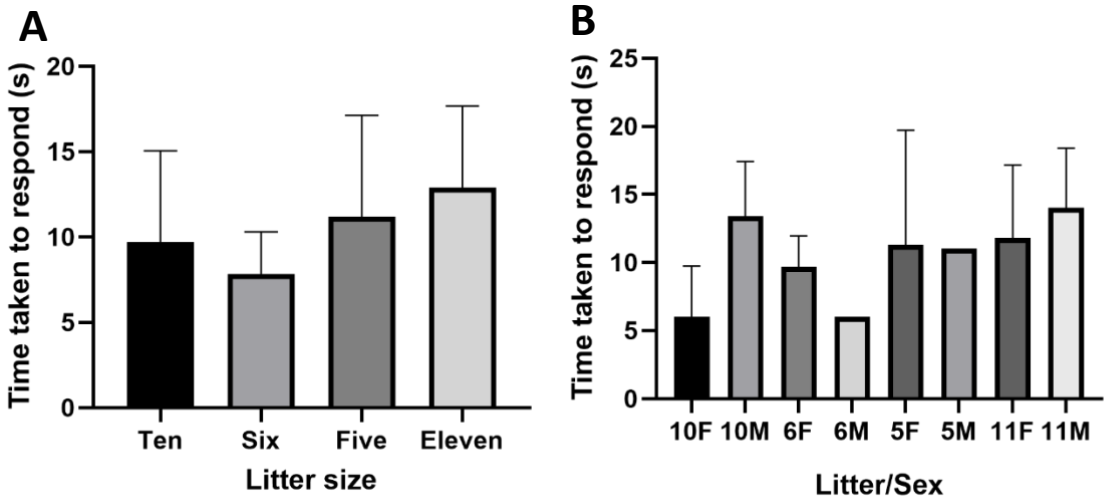


Figure 21: **Hotplate thermal assay**. The total time taken to react to the hotplate in seconds for the VPA-induced autism model. The x-axis represents the size of the litter, and the M and F indicates the sex, male and female respectively. **A**, time taken to react comparing litter-to-litter variation with no significant differences. **B**, time taken to react comparing within-litter variation between the sexes, and litter-to-litter comparisons by sex, with no significant differences between groups. Error bars indicate standard deviation. Data is presented as mean ± standard deviation.

3.2.2.4. Y-Maze Assay

The Y-Maze test was employed to assess the spatial memory and repetitive behaviour of the VPA-exposed rats and whether litter size would have any effect on memory and the insistence of sameness. For this, the parameters of distance travelled, and percentage spontaneous alternations were quantified.

Total distance travelled: the distance travelled is used in the same way as for the hole board assay, indicating hyper/hypoactivity. When comparing the litter-to-litter variation, the total distance travelled showed a significant difference between the litter sizes [$F_{(3, 28)} = 5.37$, $p = 0.0048$]. Further *post hoc* comparisons showed a significant difference between litter sizes 5 and 10 ($p = 0.015$) (Figure 22A), where litter size 5 had a significantly shorter total distance travelled, and litter size 5 and 11 ($p = 0.025$) (Figure 22A). However, there was no significant difference between litter size 6 and 10 ($p = 0.118$) (Figure 22A) and litter size 6 and 11 ($p = 0.195$) (Figure 22A). Further litter-to-litter variation when separating sex in post hoc analysis showed no significant differences between 5M and 11M ($p = 0.242$) (Figure 22B), 5M and 10M ($p = 0.458$) (Figure 22B) and no significant difference between 6M and 11M ($p = 1$) (Fig. 22B) and 6M and 10M ($p = 1$) (Figure 22B). The decrease in the distance travelled in litter 5 indicates a hypoactive response compared with the larger litter 10 and 11, the smaller litters displaying the effects of VPA-exposure more than the larger litters.

When comparing the within-litter variation between the sexes of each litter, there was no significant difference between the groups [$F_{(7, 24)} = 2.34$, $p = 0.0570$) (Fig. 22B). This result indicates no difference in response to VPA for males and females in each litter.

Percentage of spontaneous alternations: when comparing the percentage of spontaneous alternations for litter-to-litter variation, there was found to be no significant differences

between the litter sizes [$F_{(3, 28)} = 1.14$, $p = 0.2594$] (Figure 23A). There was also no significant difference in spontaneous alternations when comparing within-litter when separated by sex [$F_{(7, 24)} = 1.6$, $p = 0.1826$] (Figure 23B).

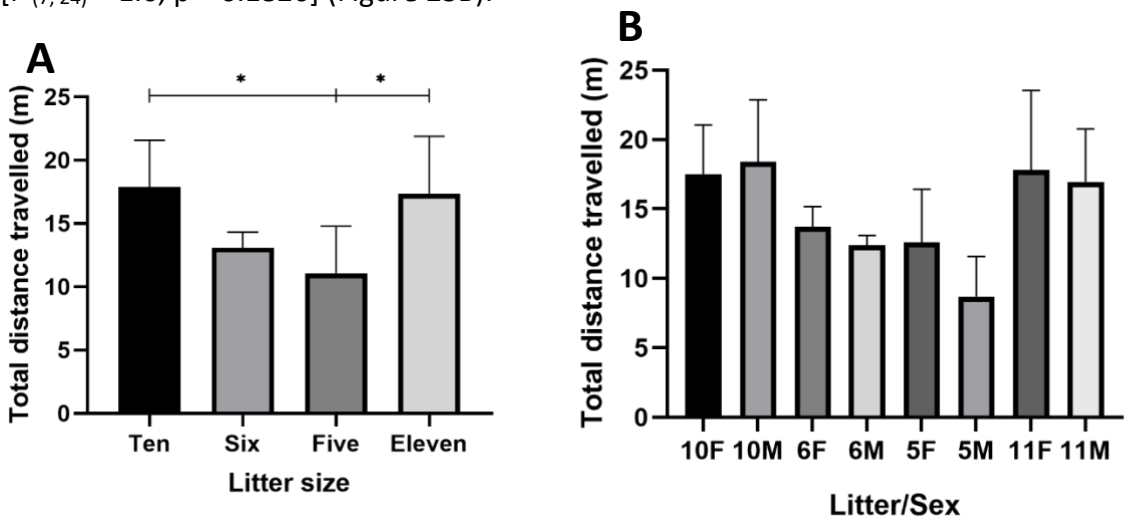


Figure 22: **Y-maze assay: total distance (m)**. The total distance travelled during the y-maze test for the VPA-exposed autism model. The x-axis represents the size of the litter, and the M and F indicates the sex, male and female respectively. **A**, the total distance travelled comparing the litter sizes with litter size 5 travelling the shortest distance, compared with litter 10 and 11. **B**, the total distance travelled comparing within-litter variation between the sexes, and litter-to-litter comparisons by sex, with no significant differences between groups. Error bars indicate standard deviation. Data is presented as mean ± standard deviation where * indicates $p < 0.05$.

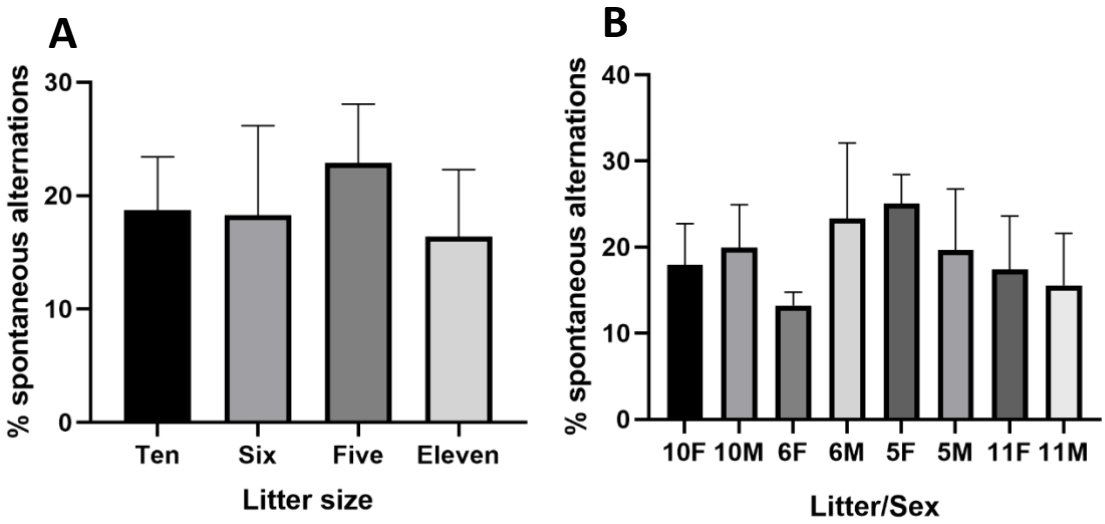


Figure 23: **Y-maze: percentage of spontaneous alternations**. The percentage of spontaneous alternations amongst litter sizes for the VPA-exposed autism model. The x-axis represents the size of the litter, and the M and F indicates the sex, male and female respectively. **A**, the percentage of spontaneous alternations between litter sizes of VPA-exposed model showing no significant differences between groups. **B**, the percentage of spontaneous alternations comparing within-litter variation between the sexes, and litter-to-litter comparisons by sex, with no significant differences between sexes within each litter. Error bars indicate standard deviation. All data is presented as mean ± standard deviation.

3.3. LEVELS OF VPA IN BLOOD SERUM

The concentration of VPA (ng/ml) in the blood serum samples of the VPA-exposed litters was calculated from the standard curve of the standard VPA samples, including the serum of the control group. There was a significant difference in VPA serum concentrations between the litter sizes showing a litter-to-litter variation [$F_{(4, 29)} = 18.17$, $p = 0.0000$]. The *post hoc* comparisons between groups showed that there was a significant difference between litter size 10 and 5 ($p < 0.001$) and 10 and 6 ($p < 0.001$), with litter size 10 having the highest concentration of VPA. There was also a significant difference was found between litters 11 and 5 ($p = 0.001$), with litter 11 having the highest concentration of VPA, but no significant difference between 11 and 6 ($p = 1$). When comparing litter size 10 and 11, there was a significant difference between these groups ($p = 0.003$), with litter 10 having the highest concentration of serum VPA. When looking at the control group, there was a significant difference between the controls and litter size 11 ($p < 0.001$), litter size 5 ($p < 0.001$) and litter 6 ($p < 0.001$), where the controls seemingly had the highest concentration of VPA. When comparing the sexes for litter-to-litter variations, there was a significant difference between litter 10M and 6M ($p = 0.036$), litters 10M and 5M ($p = 0.003$) and litter 10F and 5F ($p = 0.009$). However, there were no significant differences between 10F and 6F ($p = 0.418$), litter 11M and 5M ($p = 0.532$), litter 5M and 6M ($p = 1$) and litter 5F and 6F ($p = 1$).

For within litter comparisons, there was a significant difference between all groups, VPA-exposed and control [$F_{(8, 25)} = 9.05$, $p < 0.001$]. However, when looking at the *post hoc* comparisons, there were no significant differences between the males and females of each litter size ($p > 0.05$).

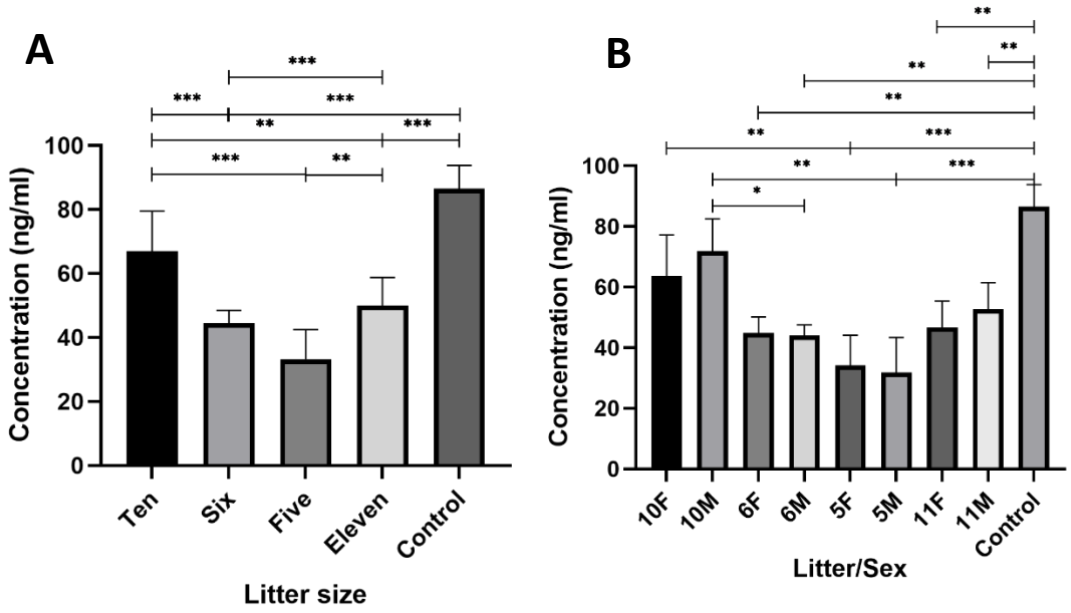


Figure 24: VPA concentrations in blood serum for the VPA-exposed rats with comparison amongst litter size. The x-axis represents the size of the litter, and the M and F indicates the sex, male and female respectively. **A**, the VPA concentration comparing litter-to-litter variation of the VPA-exposed group and control. **B**, the VPA concentration within-litter variation between the sexes, and litter-to-litter comparisons by sex against the control. Error bars indicate standard deviation. All data is presented as mean ± standard deviation where * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$.

CHAPTER FOUR - DISCUSSION

The valproic acid (VPA) autism model is widely used due to its association with many of the developmental delays and cognitive impairments seen in children diagnosed with autism spectrum disorder (ASD). Exposure to VPA during pregnancy is not only associated with neural tube defects such as exencephaly, anencephaly and spina bifida (Menegola et al., 2005; Vajda et al., 2004); but also, behavioural, and cognitive deficits (Taleb et al., 2021). In this study, we compared the behaviour and morphology of VPA exposed rat pups to control pups reared in similar conditions. We further undertook within-group comparisons to identify the effect of sex, and litter size on the morphological and behavioural changes associated with VPA exposure. Clear morphological differences were identified between VPA-exposed and control groups, with VPA treated pups showing shorter tails and more marked tail deformities, such angular distortions herein referred to as “kinks”, and localised enlargement herein referred to as “bumps”. These differences appeared to be dependent on the relative level of VPA exposure, with individuals from the smaller litter sizes having seemingly the most severe effects, i.e. the shortest tails compared to VPA exposed individuals from larger litter sizes. Behavioural testing analysing repetitive behaviour, learning and memory, exploratory and locomotor behaviour, mostly showed no significant differences between VPA and control groups (with the exception of VPA exposed female pups which showed greater locomotor activity). Nevertheless, within group comparisons of the VPA-exposed group provided valuable insight into the utility of including litter sizes in analyses of behavioural effects of teratogens such as VPA, especially with respect to the reproducibility of research findings.

Overall, it is clear from this study that there is variation in the behavioural deficits associated with autism, in males and females, when examining litter size effects, which are not present when the behavioural assay methods are analysed with a large, pooled sample group. The results of the litter-to-litter comparisons show significant differences in the effects of VPA on small and large litter sizes as well as differences in the males and females from the same litter. Therefore, the examination and statistical analysis of behaviour and morphology of each litter size should be considered along with the results of individuals of different litter sizes as suggested by Lazic and Essioux (2013). It is the opinion from this study that individuals of a small litter sizes would be best suited when compared to a small litter size of the control

counterpart, when looking at both morphology and behaviour. The VPA-model can be examined further by looking into individuals, which may result in a more accurate representation of the varied effects that individuals can display in the VPA-model of autism.

4.1. BEHAVIOURAL AND MORPHOLOGICAL FEATURES OF ASD COMPARING VPA VS CONTROL

The effects of in-utero exposure on morphological features

Both the male and female VPA-exposed rats had shorter tails than their control counterparts, indicating that both sexes were physically affected by VPA exposure during development. Moreover, both sexes displayed the kink tail deformity, which has also been reported in the work of Choi et al. (2014) as the "*crooked tail phenotype*". Both the length and crookedness of the tail are evidence of neural tube defects, which not only results from genetic mutations but also exposure to teratogens such as VPA (Choi et al., 2014). In other previous reports, exposure to VPA in zebrafish and *Xenopus* developing embryos also resulted in developmental anomalies such as tail defects (Gurvich et al., 2005; Tung & Winn, 2010), findings which are consistent with the VPA-exposed males and females in the current study. This result is consistent with findings that VPA exerts epigenetic effects as a histone deacetylase inhibitor (HDACs) (Gurvich et al., 2005; Tung & Winn, 2010). Menegola and colleagues (2005) previously found evidence of effects on the axial skeleton at term, as a result of hyperacetylation on embryos, such as extra vertebrae and fused vertebrae. Importantly, Choi and colleagues (2014) further demonstrate that the F2 and F3 generations were not directly affected by VPA in these ways, which indicated that the specificity of transgenerational transfer and that congenital defects involve other mechanisms unrelated to transmissible epigenetic changes (Choi et al., 2016).

Further morphological effects were seen in the relationship between brain and body size. This study quantified the brain mass, expressed as a percentage of body mass, when examining the brain mass as a percentage of body mass in 52 – 54-day old rats, it was found that females in both the VPA-exposed and control groups possessed a larger relative brain mass compared with their respective male counterparts. The pooling of both paraformaldehyde (PFA) fixed brain masses, as well as non-perfused brain masses can be seen as a potential confounding

variable to these findings. However, Favre and colleagues (2013) completed a study on the general health of VPA exposed Wistar rats and followed the same procedure for their analysis (use of both fixed and fresh brain tissue). This study found evidence of lower relative brain mass compared with body mass on postnatal day (PND) 1 and PND14 for both males and females with the effects of brain growth being more apparent in PND14 brain masses (Favre et al., 2013). Another study had a similar outcome in terms of a reduced brain mass in both male and female offspring compared with their control counterparts (Mychasiuk et al., 2012). At embryonic days 14 (E14) and E16, brain mass of the VPA-exposed groups was smaller than that of the control groups (Go et al., 2012). However, the brain mass became larger than the controls from postnatal day 2 (PND2) (Go et al., 2012), it is important to note that this study did not express brain mass as a percentage of total body mass. There is evidence that brain overgrowth and accelerated neuronal growth occurs in both males and females affected with autism in early childhood (Favre et al., 2013; Mychasiuk et al., 2012) followed by normal or slower growth in brain size during later childhood through to adolescence (Casanova, 2007; Chomiak et al., 2010; Chomiak & Hu, 2013; Courchesne et al., 2011). The effects of VPA appear to be more evident in the smaller brain mass of the males at PND52-54 in the current study due to altered brain development during the prenatal neurodevelopmental stages. Although altered brain development is considered the underlying neuropathological cause of ASD-like behaviours, studies have noted increased number of neurons and cortical enlargement in VPA treated rats but does not necessarily equate to a larger brain mass (Chomiak et al., 2010; Chomiak & Hu, 2013).

Does in utero exposure to VPA alter behavioural patterns?

Though it is difficult to perfectly recreate human behaviour in animal models, behavioural assays allow for the assessment of rodent behaviour that is consistent with that seen in humans. The behavioural assays used in this study assessed many of the behaviours typically seen in individuals with autism, including repetitive and restrictive, lack of interest in a new environment, hyper-/hypo reactivity as well as short-term memory. One of the hallmark symptoms of autism is repetitive behaviour (American Psychiatric Association, 2022a). The marble burying test has been known to produce good conditions to examine repetitive behaviour in rats, with the motor response, i.e. digging, being one example of the stereotypical behaviour that can be linked to similar behaviours in human autism (Angoa-

Pérez et al., 2013; Baronio et al., 2015; Choi et al., 2016). No changes in the drug-exposed rats' relative repetitive behaviour within the marble burying test was observed in the current study, consideration by sex also did not change this outcome which was consistent with such outcomes previously reported by Kang and Kim (2015) and Thomas et al (2009). It is important to note that the current assay allowed for acclimatization before commencement, whereas some studies previously did not include acclimatization. The aspect of prior acclimatization for this assay is important to consider when reading its outcomes in literature as it is somewhat controversial, with authors arguing that without prior acclimatization, the resulting digging behaviour should not be concluded as repetitive behaviour, but rather a natural response in a novel context (Kim et al., 2016). To account for this reasoning, the current study acclimatized all study subjects to ensure that the observed behaviour, i.e. digging in the test could safely be categorized as repetitive behaviour rather than a fearful response to a novel environment, which is what would be expected for all rats entering a new or unknown environment.

Furthermore, the marble-burying appears to be an assay that is used less frequently as most repetitive behaviour commonly recorded in rats involves self-grooming, jumping and digging in bedding. However, the use of this assay allows for analysis of a specific repetitive behaviour, rather than requiring distinguishing between 'normal' grooming and 'excessive' grooming (Lewis et al., 2007; Pearson et al., 2011; Ryan et al., 2010). Abnormal repetitive behaviour is also commonly seen in animals housed individually and as a result of early deprivation from social interaction (Lewis et al., 2007). All animals in this study were housed individually, but they were provided with environmental enrichment provided by colour and tunnels, which potentially counteracted the lack of social interaction and may account for the lack of increased repetitive behaviour and lack of stereotypy. The marble burying appears to be complex and various factors need to be taken into account when observing repetitive behaviour using this particular assay. This study removed novelty to the environment, however, as noted above, being exposed to a novel or known environment does not affect the digging response of subjects.

Though they are not typically used to test it, the results of the Y-maze and hole board behavioural assays further point to the lack of repetitive behaviour in this study. The Y-maze is another assay that provides an environment to test repetitive behaviour by assessing

repeated consecutive entries into the same arms i.e. a reduction in percentage of spontaneous alternations. There was no significant difference in the percentage of spontaneous alternations between the VPA-exposed rats and the controls. The expected result was that there would have been a decrease in the spontaneous alternations, indicating insistence on sameness and repetitive behaviour, which had been previously reported by Kumar and Sharma (2016b), however, this is not the case in the current cohort. Therefore, it is concluded that there was no effect on repetitive behaviour.

The primary purpose of the hole board assay is to allow rats to freely explore a novel environment and requires the active extension of a rat's neck into the bottom holes in order to explore; it thus indicates interest or lack-thereof (Kumar & Sharma, 2016b). In the current study, the VPA-exposed females showed an increased propensity to dip their heads into the holes relative to their control counterparts in the test, an indication of heightened exploratory behaviour. This finding is consistent with that of Servadio et al. (2016), where VPA treated animals also showed increased head dipping. Various researchers reported decreases in the exploratory behaviour of the VPA-exposed animals (Bringas et al., 2013; Kumar & Sharma, 2016b; Mirza & Sharma, 2019; Sandhya et al., 2012), a finding that is in direct contrast with the current work and that of Servadio et al. (2016) . Interestingly, Servadio et al. (2016) suggest that head dipping indicates repetitive behaviours, as opposed to exploratory behaviours, which was the conclusion from others (Bringas et al., 2013; Kumar & Sharma, 2016b; Mirza & Sharma, 2019; Sandhya et al., 2012). In this study, the conclusion made from the head dipping behaviour is that of exploration as the study examined only the total number of investigations rather that the repetition of head-dipping in the same hole, i.e. all head dips, regardless of which hole they were done, were quantified and summed up together rather than only those done in the same hole, i.e. repetitive movement.

Locomotor activity is typically quantified using the open field test (Bringas et al., 2013; Fereshetyan et al., 2021; Merali et al., 2014; Zerrate et al., 2007), however, in the current study, the hole board test, was employed to also assess locomotor behaviour. When compared to all other groups, the VPA-exposed females in the current study had a significant increase in their locomotor activity, both in speed and distance travelled. This finding is reminiscent with reports by Bringas et al., 2013 and Kumar & Sharma, 2016b, who reported elevated locomotor activity in their VPA-exposed cohort, albeit they had only used VPA-

exposed males. Moreover, they quantified generalized locomotor behaviour based on the crossing of a stationary beam (Bringas et al., 2013; Kumar & Sharma, 2016b), as opposed to the distance and speed quantifications conducted herein. Once again, the females in the current study showed a significant degree of hyperlocomotion, accompanied by increased exploratory behaviour, evidenced by repetitive head dips in this assay.

Taken together, the observations made in the hole board and Y-maze behavioural tests are that VPA altered primarily locomotor functions on exposed, given that exposed animals exhibited increased distances travelled and changes in average speed of travel. Further, no significant differences were seen in the latency to react in the hot plate thermal assay, suggesting no differences in sensory processing between VPA-exposed and control individuals. This is in contrast to previous studies that showed a delayed response to the thermal stimulus, hyposensitivity (Fereshetyan et al., 2021), or an increase in the latency to respond (hypersensitivity) (Kerr et al., 2013).

Thus, in the present study, the main differences seen between VPA-exposed and control groups were morphological and locomotor.

4.2. BEHAVIOURAL AND MORPHOLOGICAL FEATURES OF ASD COMPARING VPA LITTER SIZES

Both morphology and behavioural characteristics were observed for VPA litter sizes, comprised of four litters consisting of 5, 6, 10 and 11 individuals. These four litters were compared to each other for any similarities or differences for tail length, brain mass, repetitive and restrictive behaviours, hyper-reactivity to sensory stimuli, and locomotor behaviour. Following which each litter size was further divided into male and female individuals to determine sex differences in the above-mentioned characteristics.

Does litter size affect the morphological impacts of VPA?

Due to genetic similarity, two animals from the same litter tend to be more similar than two animals from different litters, which can be evaluated using between-litter heterogeneity and within-litter homogeneity (Lazic & Essioux, 2013). Taking into account the advice to consider

litter-to-litter variation from Lazic and Essioux (2013) for tail length, the current study revealed that VPA exposure affected all the individuals in a litter, however, the larger litter sizes, consisting of ten and eleven litter mates, showed less prominent effects on tail length than the smaller litter sizes, of five and six litter mates. This is an indication that the severity of abnormalities may ultimately be dependent on the amount of VPA that individual pups had been exposed to *in utero*. In human studies conducted to assess the rates of foetal malformation resulting from VPA use in pregnant women, Vajda and colleagues (2004), found varying morphological anomalies in the offspring, associated with different daily doses of medication, in the offspring including spina bifida (> 1000 mg dose), cleft palate (800 and 1700 mg dose) and congenital heart disease (400, 500, 1500 and 2000 mg dose) (Vajda et al., 2004). The frequency of foetal malformations was shown to be higher in women using valproate treatment when compared to controls after exclusion of genetic or cytogenetic anomalies (Diav-Citrin et al., 2008). The rate of malformations also increased further when pregnant women were specifically treated for epilepsy with does higher that 1000 mg. However, this same study by Diav-Citrin et al., (2008) had no cases of neural tube defects (NTDs), an inconsistent finding to the current study, which found the “crooked tail phenotype”, which is indeed an indication of neural tube defects. Other malformations present in offspring of VPA treated mothers can include congenital heart disease, ventricular septal defects and pulmonary hypertension (Vajda et al., 2004).

When looking at the litter-to-litter variation for brain mass as a percentage of body mass there was a significant difference between the relative brain mass in individuals of litter 10 and those of litter 11, with litter 11 having relatively larger brains. There was no significant difference in relative brain mass between litter 5 and 6, between litters 10 and 6, and lastly between litters 11 and 6, indicating a lack of variability in the effects of VPA on brain mass between the small and larger litter sizes. Additionally, there was no significant difference between the males and females of each litter suggesting that both males and females were affected in a similar way by VPA-exposure. The dearth of knowledge and literature as it pertains to the litter-to-litter variation and within-litter variation using the VPA-induced autism model may be related to the low reproducibility of studies making use of the VPA autism model (Lazic & Essioux, 2013). Though there is some evidence that males are more likely to be affected than females in human autism cases (Blaylock, 2008; Geier & Geier, 2006),

this study found both male and females of each litter showed similar effects in terms of tail length and brain mass.

Does litter size affect behavioural outcomes of VPA exposure?

Behavioural assays completed were to establish any discrepancies in the behavioural repertoire observed between different litters, i.e. different VPA exposure levels, as well as between the sexes. The marble burying test showed no significant differences when comparing the different litter sizes as well as no significant differences between the sexes within each litter size. This was also true when analysing the number of spontaneous alterations in the Y-maze assay and the total number of investigations in the hole board assay, for sensory processing, the hotplate thermal assay showed no significant differences between the different litter sizes of the VPA-exposed groups, as well as no difference within the respective litters when compared by sex. The most significant distances were seen in assessing the total distance travelled in the hole board and Y-maze assays.

While the hole board assay showed no significant differences for the total number of investigations when looking at the different litter sizes and within each litters' sex comparisons, significant differences with respect to the distance travelled were observed.

The shortest distance travelled during the hole board assay was recorded for individuals in litter size 6, suggesting a lack of interest to explore the unknown environment, which was not observed when only comparing the VPA-exposed and control groups. Even though the results indicated that litter 11 had a higher total distance travelled compared with the smaller litter size of 6, within litter variation between males and females of litter size 6 showed no differences, while those of litter size 11 did (with females of the litter travelling greater total distances than the males). A significant difference was also seen between males in litter size 6 and litter size 10. It is a well-established that autism is more prevalent in males compared with females, due to the early onset of puberty in males and a genetic predisposition to the development of autism (Bambini-Junior et al., 2011; Blaylock, 2008). These results do show a trend towards males being more vulnerable to VPA effects, however, it still not conclusive. The total distance travelled in the Y-maze assay further supports the effect of VPA exposure on locomotor activity, with litter size 5 individuals travelling the shortest distance compared to litter size 10 and 11 respectively. However, unlike in the case of the hole board assay, there

were no sex differences. Therefore, this assay indicates that males are not the only sex that show the effects of VPA exposure. This also confirms that there is heterogeneity in the diverse responses from one litter size to the next.

The results from Lazic and Essioux (2013) of litter differences showed that litter effects accounted for up to 61% of the previously unexplained variation in the data when comparing only VPA and the control (Lazic & Essioux, 2013). This, along with the findings of the current study strongly indicate that variation in litter and the analysis of more than one animal per litter should be taken into account when looking at behavioural variations within the VPA-induced autism model.

4.3. SERUM CONCENTRATION OF VPA

The serum concentrations of VPA were found to be lowest in the individuals from the smaller litter sizes, five and six, compared to the larger litter sizes, and the highest VPA concentration was in the control group. This brings into question the specificity of the VPA ELISA kit. However, it is noted that the levels of VPA in rat serum was conducted to further examine whether smaller litter sizes are more likely to receive higher concentrations of VPA per individual pup than larger litter sizes. Drugs, such as VPA, are normally taken up by cells via passive diffusion, moving from areas of high to low concentrations, with the rate of diffusion dependant on many factors such as protein binding, lipid solubility and molecular size (Chillistone & Hardman, 2014). Therefore, smaller litter sizes will receive a higher concentration of VPA per individual resulting in a higher concentration gradient and passive diffusion into cells. Observations in humans show that there was no correlation between maternal serum VPA concentration levels and infant serum levels, along with no correlation between maternal milk levels and maternal serum levels of VPA (Kacirova et al., 2019, 2021). A correlation was observed between infant serum levels and maternal milk VPA concentrations, however, these VPA concentration levels did not reach the lower limit of the reference range even when mothers continued their VPA dosage through the breastfeeding stage (Kacirova et al., 2021). This indicates that VPA concentrations are not highly detectable even when collected in infancy through continued exposure during pregnancy. In the current study, the rat pups naturally suckled however, due to the single dosage of VPA, the accumulation of VPA in their serum was unlikely to be detected after a few weeks. Thus in

order to get a clear idea of the relationship between the effects of VPA on 'severity' of behavioural phenotypes seen between litters (and individuals), the collection of serum must be done several times after birth, specifically immediately after birth, and every subsequent week up until sacrifice to determine the VPA concentration levels and the metabolism of VPA in the rat pups after the single maternal VPA dose. Furthermore, VPA can be given in more than one dose during pregnancy to imitate the effects of human dosage for treatment of epilepsy throughout the entire pregnancy. These dosages can be continued during natural suckling to determine the concentration of VPA in maternal milk and pup blood serum. The VPA ELISA results of this study are most likely an indication of nonspecific binding, and do not give a definitive answer on whether individuals in smaller litter sizes ultimately receive a greater relative dose of VPA in the model investigated. Although, given the clear consistent and greater severity of neural tube defects in smaller litters, it seems reasonable to infer that VPA does have greater effect on the smaller litters as opposed to larger ones. However, the results of the behavioural testing suggest that there is value in VPA ELISA test when blood is taken at various stages of the rat pups' life, early childhood through to adolescence. Future studies making use of the above-mentioned changes to the methodology, continued dosage or the collection of serum immediately after birth, may provide clearer evidence of this.

CHAPTER 5 - CONCLUSION

The assessment of the behavioural alterations in the VPA autism rat model undertaken in this study show that there is no clear pattern when observing behavioural changes in comparing VPA- exposed and control individuals. However, when the VPA-exposed groups were compared by litter size, the smaller litter sizes appeared to display the effects of VPA exposure in both behavioural and morphological alterations. In general, across the litters both males and females appeared to be similarly affected by exposure to valproic acid. When the litters were separated by sex, the change in tail length became even more evident, where smaller litter sizes had shorter tails. This pattern continued through to behavioural effects, where males and females of the same litter showed varied results in the majority of the behavioural assays, mainly with females showing more hyperlocomotion. This highlights the fact that females and males are both important when observing

behavioural and morphological effects of VPA exposure, and not just males, and thus very important to incorporate both sexes into studies in autism models, which much literature has thus far neglected in favour of males only. Due to litter size discrepancies observed, there is also importance in separating the VPA-exposed groups by litter size when examining the behavioural and morphological effects of VPA, as the current study provides evidence that when studying the VPA-exposed autism model, size really does matter.

Study Limitations

Rat breeding does create some limitations in terms of the uncertainty of pregnancies, with a total of 21 rats used for breeding purposes, only 8 dams were positive for pregnancy. This did affect the timing of the behavioural assays, start and end date, and to align with the next round of breeding rats. This resulted in delays in behavioural tests due to equipment failure or technological issues to be unforgiving and required everything to be prepared well in advance.

The large sample of rats meant that keeping and storing records were of the utmost importance so that data was not lost or mislabelled including making sure that each rat was properly checked and weighed, completed each behavioural assay, labelled correctly when saving video files and kept in order when animals were euthanised.

The design of the study required continuous testing of the animals, with some tests requiring nearly 11 hours of testing time, and the average testing time was 8 hours. The study design, which included four behavioural assays, and included a variety of behavioural alterations, this required each test to take a maximum of two days, with no room for error. This was also an exhaustive process with minimal resting intervals between subject testing and one person always monitoring the tests.

The VPA ELISA method was limited as we only had serum from rats at PND 52-54 and not at birth as seen in previous studies. This limited comparability to other studies and reduced chances of recording detectable levels of VPA. On further review, the results would have been more accurate if the ELISA method had been conducted on newborn pup serum, which would have allowed for more accurate inferences on VPA exposure between litter mates.

CHAPTER SIX - REFERENCES

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APPENDIX A

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)

STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: 2022/03/07/B

APPLICANT: Dr B Maseko
School: School of Anatomical Sciences; Department: N/A; Location: WRAF

PROJECT TITLE: Autism neuropathology: an investigation of the inflammatory and synaptopathological states in autistic brains using the Valproic Acid Sprague dawley rat model

Category: B; Species and Numbers involved: *72X (12 dams + 60 pups),dams at 10-12 weeks old nulliparous; pups from birth to 60 days, male or female, Sprague dawley (Rattus norvegicus)*

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 29 Mar 2022. This approval remains valid until 20 Apr 2024 and is conditional to the following (if blank there are no special conditions):

Condition 1	Condition 2	Condition 3	Condition 4

All material changes to the approved research must be reported to the AREC before they are implemented. Failure to do so will invalidate this clearance certificate.

An annual progress report must be provided to the AREC.

The use of these animals is subject to AREC guidelines on the use and care of laboratory animals, is limited to the procedures described in the application and is subject to additional conditions listed below:

I, the Chair of the AREC (or my designated representative) am satisfied that the proposed research is ethical as judged by local law, international standards and University policy.

Signed: _____ _____ Date: ___22/04/2022____

(Chairperson of the AREC)

I am satisfied that the persons listed in this application are competent to perform the procedures described in the application, in the context of Section 23 (1) (c) of the veterinary and Para-veterinary Professions Act (19 of 1982).

CC: Student supervisor: «Title1» «Initials1» «Supervisor_surname»
Director Wits Research Animal Facility (WRAF): Dr Kim Jardine

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)

Signed: _____ _____ Date: 22/04/2022_____

(Registered Veterinarian) CC: Student supervisor: «Title1» «Initials1» «Supervisor_surname» Director Wits Research Animal Facility (WRAF): Dr Kim Jardine

APPENDIX B

Goat Valproic Acid (VA) ELISA kit

(Competitive ELISA)

MyBioSource.com

96 Tests

Catalog Number: MBS7271085

Store all reagents at 2-8°C

Valid Period: six months

For samples:

Serum, plasma, cell culture supernatants, body fluid and tissue homogenate

Important Note!

Sample Preparation:
With respect to 6.2, we suggest pre-experimenting with neat (undiluted) samples, 1:2 or 1:4 dilutions. Please avoid diluting your samples more than
1:10 as it would exceed the dilution limit set for this kit. If the expected concentration of the target is beyond the detection range of the kit, please contact technical support.

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develop.

12) Incomplete washing will adversely affect the test outcome. All washing must be performed with Wash Solution provided. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells.

13) Because TMB is light sensitive, avoid prolonged exposure to light. Also avoid contact between TMB and metal, otherwise color may

12. QUALITY CONTROL

1) It is recommended that all standards, controls and samples be run in duplicate. Standards and samples must be assayed at the same time. 2) The coefficient of determination of the standard curve should be ≥
0.95.

3) Cover or cap all kit components and store at 2-8° C when not in use.
4) Microtiter plates should be allowed to come to room temperature before opening the foil bags. Once the desired number of strips has been removed, immediately reseal the bag with desiccants and store at 2-8°C to maintain plate integrity.
5) Samples should be collected in pyrogen/endotoxin-free tubes.
6) Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
7) When possible, avoid use of badly hemolyzed or lipemic serum. If large amounts of particulate matter are present, centrifuge or filter prior to analysis.
8) When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
9) Do not mix or interchange different reagent lots from various kit lots.
10) Do not use reagents after the kit expiration date.
11) Read absorbance immediately after adding the stop solution.

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1. INTENDED USE

This VA ELISA kit is a 1.5 hour solid-phase ELISA designed for the quantitative determination of Goat VA. This ELISA kit for research use only, not for therapeutic or diagnostic applications!

MyBioSource.com

2. PRINCIPLE OF THE ASSAY

VA ELISA kit applies the competitive enzyme immunoassay technique utilizing a polyclonal anti-VA antibody and an VA-HRP conjugate. The assay sample and buffer are incubated together with VA-HRP conjugate in pre-coated plate for one hour. After the incubation period, the wells are decanted and washed five times. The wells are then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction forms a blue colored complex. Finally, a stop solution is added to stop the reaction, which will then turn the solution yellow. The intensity of color is measured spectrophotometrically at 450nm in a microplate reader. The intensity of the color is inversely proportional to the VA concentration since VA from samples and VA-HRP conjugate compete for the anti-VA antibody binding site. Since the number of sites is limited, as more sites are occupied by VA from the sample, fewer sites are left to bind VA-HRP conjugate. A standard curve is plotted relating

the intensity of the color (O.D.) to the concentration of standards. The VA concentration in each sample is interpolated from this standard curve.

3. MATERIALS

All reagents provided are stored at 2-8° C. Refer to the expiration date on the label.

	MATERIALS	SPECIFICATION	QUANTITY
1	MICROTITER PLATE	96 wells	stripwell
2	ENZYME CONJUGATE	6.0 mL	1 vial
3	STANDARD A (0.5mL)	0 μmol/L	1 vial
4	STANDARD B (0.5mL)	25 μmol/L	1 vial
5	STANDARD C (0.5mL)	50 μmol/L	1 vial
6	STANDARD D (0.5mL)	100 μmol/L	1 vial
7	STANDARD E (0.5mL)	250 μmol/L	1 vial
8	STANDARD F (0.5mL)	500 μmol/L	1 vial
9	SUBSTRATE A	6 mL	1 vial
10	SUBSTRATE B	6 mL	1 vial
11	STOP SOLUTION	6 mL	1 vial
12	WASH SOLUTION (100 x)	10 mL	1 vial
13	BALANCE SOLUTION	3 mL	1 vial
14	INSTRUCTION	1	

NOTE: The BALANCE SOLUTION is used only when the sample is **cell culture supernatants, body fluid and tissue homogenate**; if the sample is serum or plasma, then the BALANCE SOLUTION is a superfluous reagent.

cross-reactivity detection between VA and all the analogues, therefore, cross reaction may still exist in some cases.

11. SAFETY NOTES

This kit contains small amount of 3, 3', 5, 5'-Tetramethylbenzidine (TMB) in Substrate B. TMB is non-carcinogenic but it is hhazardous in case of skin contact, eye contact, ingestion and inhalation. In case of contact, rinse affected area with plenty of water. The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, and face protection. Care should be taken when handling the Standard because of the known and unknown effects of it.

MyBioSource.com

Care should also be taken to avoid contact of skin or eyes with other kit reagents or specimens. In the case of contact, wash immediately with water.
Do not pipette by mouth.

Avoid generation of aerosols.

Waste must be disposed of in accordance with federal, state and local environmental control regulations.
All blood components and biological materials should be handled as potentially hazardous. Decontaminate and dispose specimens and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour a 121.5°C.

NOTE:

1) Any variation in operator, pipetting and washing technique, incubation time or temperature, and kit age can cause variation in result. Each user should obtain their own standard curve.
2) If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
3) If specimens generate values higher than the highest standard, dilute the specimens and repeat the assay.

10. CERTIFICATE OF ANALYSIS

1) In the same lot CV%:<10 2) Different lot CV%: <10 3)
Spike Recovery: 92-101% 4) Linearity:

	Range %
1:1	94 – 102
1:2	92 - 104
1:4	98 - 105
1:8	94 - 109

5) Sensitivity: The sensitivity in this assay is 1.0 μmol/L.
6) Specificity: This assay has high sensitivity and excellent specificity for detection of VA. No significant cross-reactivity or interference between VA and analogues was observed. **NOTE**: Limited by current skills and knowledge, it is impossible for us to complete the

The types of sample:	
Sample I:	serum or plasma
Sample II:	cell culture supernatants, body fluid and tissue homogenate

4. SPECIMEN COLLECTION AND STORAGE

MyBioSource.com

Serum - Use a serum separator tube and allow samples to clot for 2 hours at room temperature or overnight at 2-8°C. Centrifuge at approximately 1000 × g (or 3000 rpm) for 15 minutes. Collect serum and assay immediately or aliquot and store samples at -20°C or -80°C.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000 × g (or 3000 rpm) at 2 - 8°C within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C or -80°C.

Tissue homogenates - The preparation of tissue homogenates will vary depending upon tissue type. For this assay, tissues were rinsed in ice-cold PBS (0.02mol/L, pH 7.0-7.2) to remove excess blood thoroughly and weighed 300-500mg before homogenization. Minced the tissues to small pieces and homogenized them in 500ul of PBS with a glass homogenizer on ice. The resulting suspension was subjected to ultrasonication or to two freeze-thaw cycles to further break the cell membranes. After that, the homogenates were centrifugated for 15 minutes at 1500×g (or 5000 rpm).

Collect the supernate and assay immediately or aliquot and store samples at -20°C or -80°C.

Cell lysates - Cells should be lysed according to the following directions.

1) Adherent cells should be detached with trypsin and then collected by centrifugation. Suspension cells can be collected by centrifugation directly.
2) Wash cells three times in PBS.
3) Cells were resuspended in PBS and subjected to ultrasonication for 3 times. Alternatively, freeze cells at -20 °C. Thaw cells with gentle mixing. Repeat the freeze/thaw cycle for 3 times.
4) Centrifuge at 1000×g (or 3000 rpm) for 15 minutes at 2-8 °C to remove cellular debris.
5) Assay immediately or store samples at -20°C or -80°C.

Cell culture supernatants and other body fluids - Centrifuge cell culture media at 1000 × g (or 3000 rpm) for 15 minutes to remove debris. Assay immediately or store samples at -20°C or -80°C.

NOTE:

1) Samples should be aliquoted and must be stored at -20°C (less than 3 months) or -80°C (less than 6 months) to avoid loss of bioactivity and contamination. If samples are to be run within 24 hours, they may be stored at 2- 8°C. Avoid repeated freeze-thaw cycles. Fresh samples without long time storage are recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples

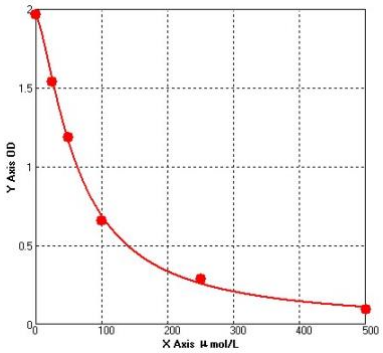
1) The standard curve is used to determine the amount of samples.

2) First, average the duplicate readings for each standard and sample. All O.D. values are subtracted by the mean value of blank control before result interpretation. DO NOT subtract the O.D of standard zero.

3) Construct a standard curve by plotting the concentration on the horizontal (X) axis against the average O.D. for each standard on the vertical (Y) axis, and draw a best fit curve e using graph paper or statistical software to generate a four paramater logistic (4-PL) curve-fit or logit-log linear regression curve. An x-axis for the optical density and a y-axis for the concentration is also a choice. The data may be linearized by plotting the log of the concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis.

4) Calculate the concentration of samples corresponding to the mean absorbance from the standard curve.

5) Standard curve for demonstration only.



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4) Wash the microtiter plate using one of the specified methods indicated below:

a) Manual Washing: Remove incubation mixture by aspirating contents of the plate into a sink or proper waste container. Fill in each well completely with 1× wash solution, and then aspirate contents of the plate into a sink or proper waste container. Repeat this procedure five times for a total of FIVE washes. Take care not to scratch the surface of the wells.

b) Automated Washing: Wash plate FIVE times with diluted wash solution (350-400 μL/well/wash) using an auto washer. It is recommended that the washer be set for a soaking time of 10 seconds and shaking time of 5 seconds between each wash.

5) After washing, invert plate, and blot it against absorbent paper or paper towels until no moisture appears. Complete removal of liquid at each step is essential to good performance.

6) Add 50 μL **Substrate A** and 50 μL **Substrate B** to each well including blank control well, subsequently. Cover and incubate for 15-20 minutes at 37°C. (Avoid sunlight If the color is not dark, please prolong the incubation time. But the longest time is 30min).

7) Add 50 μL of **Stop Solution** to each well including blank control well. Mix well.

8) Determine the Optical Density (O.D.) at 450 nm using a microplate reader immediately.

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9) Incubator (37°C).

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9. CALCULATION OF RESULTS

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2) Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts of certain chemicals.

3) Samples containing a visible precipitate must be clarified prior to use in the assay. Care should be taken to minimize hemolysis. Do not use grossly hemolyzed or lipemic specimens.
Do not use heat-treated specimens.

5. MATERIALS AND EQUIPMENT REQUIRED BUT NOT

Precision pipettors and disposable tips to deliver 10-1000 □l. A multi-channel pipette is desirable for large assays.
100 mL and 1 liter graduated cylinders.

Distilled or deionized water.

Tubes to prepare sample dilutions.

Absorbent paper.

Microplate reader capable of measuring absorbance at 450 nm.

Centrifuge capable of 3000 × g.

Microplate washer or washing bottle.

10) Data analysis and graphing software.

6. SAMPLE PREPARATION

1) We are only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient amount of samples in advance.

2) Please predict the concentration before assaying. If values for these are not within the range of the standard curve, **users must determine the optimal sample dilutions for their particular experiments**. We suggest pre-experimenting with neat (undiluted) samples, 1:2 or 1:4 dilutions. Please avoid diluting your samples more than 1:10 as it would exceed the dilution limit set for this kit. If the expected concentration of the target is beyond the detection range of the kit, please contact technical support.

3) If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.

4)

6. ASSAY PROCEDURE

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4) Owing to the possibility of mismatching between antigen from other resource and antibody used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.
5) Influenced by the factors including cell viability, cell number and also sampling time, samples from cell culture supernatant may not be detected by the kit.

7. REAGENTS PREPARATION

1) Bring all kit components and samples to room temperature (20-25 °C) before use.
2) Samples - Please predict the concentration before assaying. If concentrations are unknown or not within the detection range, a preliminary experiment is recommended to determine the optimal dilution. PBS (pH 7.0-7.2) or 0.9% physiological saline can be used as dilution buffer.
3) Wash Solution - Dilute 10 mL of Wash Solution concentrate (100×) with 990 mL of deionized or distilled water to prepare 1000 mL of Wash Solution (1×). If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. The 1× wash solution is stable for 2 weeks at 2-8°C.
   Do not dilute the other components which are ready- to-use.

Please read Reagents Preparation before starting assay procedure. It is recommended that all Standards and Samples be assayed in duplicate. It is strongly recommended to do a preliminary experiment before measuring all samples.

Secure the desired numbers of coated wells in the holder then add 100 □L of **Standards (**Shake the bottle of each standard gently by hand and Pipette up and down the solution of standard for 3 times before adding**)** or **Samples** to the appropriate well. Add 100 □L of PBS (pH 7.0-7.2) in the blank control well.

Dispense 10□□L of **Balance Solution** into 100□□L samples only, mix well. **(NOTE**: This step is required when the sample is cell culture supernatants, body fluid and tissue homogenate; if the sample is serum or plasma, then this step should be skipped.)

3) Add 50 □L of **Conjugate** to each well (NOT blank control well). Mix well. Mixing well in this step is important. Cover and incubate the plate for 1 hour at 37°C.