Sleep disturbances and the orexinergic system in a valproic acid model of autism

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Declaration

I, Mokgatjane Gaffane, declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science (Medicine) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.

(Signature of candidate)

21st day of October 2024, in Pretoria

Abstract

Sleep disturbances are the most prevalent comorbidities in autistic individuals and have been implicated in the disorder's aetiology, however, their exact role is not fully understood. This study aimed to evaluate the sex-specific effect of sleep on autism symptomology. Pregnant rats were given a single intraperitoneal injection of VPA, and their pups subjected to a 16hr dark/ 8hr light cycle to induce sleep disturbance. Autism-like features were assessed with marble burying, balance beam (BB), y-maze, novel object recognition (NOR) and three-chambered sociability behavioural tests. Three-way factorial ANOVAs were used to determine the interaction between sex, sleep and autism. Significance was found for the main effect for sleep condition in the time spent investigating the novel object (NOR) and the interaction between sex and sleep condition in the number of foot slips (BB). Additionally, a preliminary investigation of the cellular diversity of the lateral hypothalamic area (LHA) was provided to elucidate the orexinergic regulation of sleep/wake cycles. From descriptions of astrocytes, oligodendrocytes, and neurons, sleep condition was presumed to affect orexinergic signalling through the indirect excitation by nearby astrocyte populations. The results of the behavioural study support the growing understanding of a sex-specific presentation of autism and highlight the need for greater inclusivity in diagnostic criteria which are currently biased towards male presentations. Additionally, the results of the cytological investigation support the suggestion of hyperactive orexinergic functioning in autism and warrant research efforts into the underlying orexinergic control of sleep/wake functioning as a potential mechanism underlying its pathogenesis.

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List of abbreviations and symbols

5-HT 5-hydroxytryptamine

ADOS autism diagnostic observation schedule

AP autism pathology

APA American Psychological Association

ASD autism spectrum disorder

ATEC autism treatment evaluation checklist

C control

CANTAB Cambridge Neuropsychological Test Automated Battery

CARS Childhood Autism Rating Scale

CBCL child behaviour checklist

CNS central nervous system

CNV copy number variant

CSHQ children's sleep habits questionnaire

DD constant darkness

DLAN dim light at night

DP3 developmental profile

DQ/IQ developmental quotient/intelligence quotient

DSM-IV-TR diagnostic and statistical manual of mental disorders

EEG electroencephalogram

EMB extreme male brain

FAP female autism phenotype

FMR1 fragile X messenger ribonucleoprotein 1

FMS fundamental movement skills

FPE female protective effect

FXS fragile X syndrome

GD gestational day

IGT Iowa gambling task

nSD non-sleep disturbed

L/D light/dark

PND postnatal day

PPVT peabody picture vocabulary test

PSG polysomnography

PSQ paediatric sleep questionnaire

RBS-R repetitive behaviour scale-revised

REM rapid eye movement

RGC retinal ganglionic cells

RHT retinohypothalamic tract

RNA ribonucleic acid

RRBI restricted, repetitive patterns of behaviours, interests, or activities

S sex

SAS spontaneous alternation score

SC sleep condition

SCN suprachiasmatic nucleus

SD sleep disturbed

SDSC sleep disturbance scale for children

TD typically developing

TH tryptophan hydroxylase

VPA valproic acid

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CHAPTER 1: Introduction

Autism is one of the disorders comprising a group of neurodevelopmental disorders called autism spectrum disorders (ASDs), diagnosed through the assessment of its core behavioural features, which are understood to occur as a result of impaired neurodevelopment (Geoffray *et al*., 2016). These impairments are the culmination of some combination of intrinsic and extrinsic factors, resulting in irregularities in the development of brain structures that ultimately hinder their ability to properly execute their respective functions (Geoffray *et al*., 2016). The mechanisms underlying these processes are poorly understood, partly due to the spectral nature of the disorder. Thus, resulting in great heterogeneity in the presence or absence of certain features, as well as the severity of those behaviours which are observed (Lorsung *et al*., 2021). As such, no single intrinsic factor (genetic, endocrine dysregulation, etc.) has been attributed to the emergence of the disorder. Instead, the behaviours observed to characterise the disorder have been used to direct efforts into understanding the disorders underlying aetiology (Lorsung *et al*., 2021). These behaviours include not only autism's diagnostic features but others that have been found to comorbid the disorder, including seizures, gastrointestinal disturbances, psychiatric disorders such as anxiety and depression as well as sleep disorders (Burman *et al*., 2023). Despite the heterogenous nature of autism presentation, sleep disorders are among the most reported comorbidities to the disorder (Fang *et al*., 2021; Lorsung *et al*., 2021; Burman *et al*. 2023).

Sleep dysregulation, although not a core behavioural symptom associated with autism, is the most frequently reported co-morbidity to the disorder and implicated in the daytime functioning and presentation of autistic behaviours (Fang *et al*., 2021; Lorsung *et al*., 2021). Further, given that autistic behavioural symptoms manifest as a result of neurodevelopmental irregularities, research efforts have shifted to encompass the various factors playing a role in brain development and maturation of neural circuitry (Lorsung *et al*., 2021; Barone *et al*., 2019). Paramount among these are the neurotransmitter systems. Neurotransmitter signalling systems have proven valuable in aiding the understanding of the neural systems underlying the behavioural symptoms of autism. These systems are responsible for critical processes such as neuroplasticity, movement and sleep (Marotta *et al*., 2020). Among those most documented are the serotonergic, dopaminergic, cholinergic and catecholaminergic systems (Marotta *et al*., 2020). However, less documented with regards to autism symptomatology is the orexinergic system, despite multiple lines of evidence implicating the system in autism pathology and the

commonly associated dysregulated sleep (Nambu *et al.,* 1999; Sakurai, 2007; Kohyama, 2016). Specifically, the role of the orexinergic system in maintaining wake and inhibiting sleep (Sakurai, 2007; Pizza *et al.,* 2022) has led to the hypothesis for its hyperactive functioning in autism (Piri *et al*., 2024). Further, the system's extensive neural circuitry (Nambu *et al.,* 1999; Peyron *et al.,* 1998; Sakurai *et al.,* 1998) and ability to modulate the activity and function of other neurotransmitter systems (Zhang *et al*., 2013; Yamashita & Yamanaka, 2016), identify it as an area of focus potentially aiding in the understanding of sleep disturbances in autism (Piri *et al*., 2024). It may also shed light on their role in worsening existing symptoms or resulting in their emergence, as previously suggested (Richdale *et al.,* 2009; Souders *et al*., 2009; Reynolds & Malow, 2011).

The use of environmental animal models (in other words, those utilising identified risk factors for autism during early development) for experimentation are favoured in autism research for their ability to mimic early developmental risk factors associated with the emergence of the disorder later in life (Missig *et al.,* 2020). Widely cited in translational autism research is the gestational administration of valproic acid (VPA), favoured for its face, construct, and predictive validity (Mabunga, 2015) and utilised in the present study.

It has been suggested that sleep disturbance plays a role in mediating autism-like behaviours (Missig *et al*., 2020). There is a wealth of literature documenting sleep disturbances in the disorder (Brooks & Canal, 2013; Miike *et al*., 2020; Missig *et al*., 2020; Lorsung *et al*., 2021; Burman *et al*., 2023). However, the relationship between sleep disturbance and existing autism pathology on the resulting behavioural symptoms remains unclear (Missig *et al*., 2020). Additionally, an evident diagnostic gap exists between sexes (Kanner, 1943), highlighting the female phenotype of autism. This gender-based difference is not adequately addressed by current diagnostic tools, leading to fewer diagnoses among females (Napolitano et al., 2022). Further, despite an increasing awareness of the importance of the inclusion of both males and females in preclinical research (Cahil, 2012), there are still significant gaps in the knowledge of female presentations of the disorder (Napolitano *et al*., 2022). This highlights the need for greater female representation in autism research to learn about sex differences, which may result in more accurate diagnoses and targeted therapeutic interventions (Lorsung *et al*., 2021).

Study aims and objectives

The aim of the present study was to investigate sleep as a comorbidity of autism in a valproic acid (VPA) Sprague Dawley rat (*Rattus norvegicus*) model, by observing the effects of sleep disturbance on autism-like behaviour in both males and females.

Objectives:

1. To determine the impact of sleep disturbance on the core autistic behavioural features in a valproic acid (VPA) model of autism spectrum disorder.
2. To determine whether sleep disruptions display sex-specific effects on the autism-like behaviours of the VPA rat model of autism.
3. To assess how the orexinergic system may be altered by sleep disturbance in the VPA rat model of autism.

CHAPTER 2: Sleep effects on autistic behavioural phenotypes

2.1 Literature Review

2.1.1 Autism and its animal models

Autism is one of the disorders comprising a group of neurodevelopmental disorders called autism spectrum disorders (ASDs) and is diagnosed according to the guidelines set out in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR, American Psychiatric Association (APA), 2013). The manual confirms an autism diagnosis through the assessment of behavioural symptoms considered to characterise the disorder. These include a range of behaviours which imply impaired social interaction and communication, as well as restricted, repetitive, or stereotyped movements (APA, 2013; Lorsung *et al.,* 2021; Katz-Zetler *et al.,* 2023).

The manner in which these behaviours present in individuals is altered but not uniform, but rather exists across a spectrum, varying in the presence or absence of certain features as well as the severity of those features which are observed (Lorsung *et al*., 2021). Further, the cause of these behavioural alterations is understood to lie in impaired neurodevelopment (Lorsung *et al*., 2021). As such, the paradigm underpinning research attempting to understand the disorder's aetiology tends to operate based on a developmental hypothesis (Geoffray *et al.,* 2016). That is, some combination of intrinsic (genetic, endocrine dysregulation etc.) and extrinsic (gestational exposure to risk factors, perinatal brain injury etc.) factors culminate in irregularities in the development of brain structures that ultimately hinder their ability to properly execute their respective functions. The resulting behavioural features that are observed to be characteristic of autism are then used to direct efforts in understanding the mechanisms underlying these developmental irregularities (Geoffray *et al.,* 2016).

This understanding often requires experimentation, which is difficult and often unethical to holistically conduct in humans (Missig *et al*., 2020). As such, animal models are commonly used to mimic the condition as it is seen in humans, for the ability to experiment (Missig *et al.*, 2020). Further, although autism is a uniquely human condition, many of its core deficits can be observed in animals through behavioural testing (Napolitano *et al*., 2022). These animal models can be characterised as focusing on either genetic or environmental factors, or sometimes a combination of both (Missig *et al.*, 2020).

2.1.1.1 Genetic animal models

Genetic models target specific mutations, deletions and risk genes associated with autism aetiologies (Napolitano *et al.,* 2022), thus imitating syndromic forms of the disorder (Missig *et al.*, 2020). Most common among these include *de novo* (non-inherited) copy number variants (CNVs) which predispose individuals to the disorder (Robinson *et al.*, 2013). However, because the specific gene abnormalities are largely unknown, modelling their complex interactions under experimental conditions remains a challenge (Missig *et al.*, 2020). Further, none of the known genetic risk factors are present consistently and uniformly in all known cases of autism, making it difficult to attribute the resulting symptoms to a single causal agent and wholly infer the combined effects of known genetic risk factors (Missig *et al.*, 2020). Still, several syndromic autism diagnoses can be attributed to specific genetic abnormalities or broader medical conditions through genetic testing (Napolitano *et al.,* 2022). These include but are not limited to the following syndromes: Fragile X, Rett's, Prada Willi, Angelman, Smith-Magenis, Potocki-Lupski, Timothy and Tuberous Sclerosis Complex (Napolitano *et al.,* 2022).

The most prevalent of these syndromic forms of autism is Fragile X Syndrome (FXS), estimated to occur in 1:5000 in males and between 1:4000 – 8000 in females (Napolitano *et al.,* 2022). FSX is the silencing of the fragile X messenger ribonucleoprotein 1 (FMR1) gene located on the X-chromosome (Missig *et al.,* 2020) as a result of the abnormal expansion of repeated chemical bases at the start of the gene (Napolitano *et al.*, 2022). As the FMR1 gene is responsible for coding for a key ribonucleic acid (RNA) binding protein involved in regulating synaptic plasticity and neuronal growth, the translational abnormalities resulting from its silencing provide a basis for the neurological deficits resulting in the observed behavioural features characteristic of autism (Napolitano *et al.,* 2022). As such, FSX has been modelled in animals, predominantly mice, through deletions of the species-specific genetic homolog of the FMR1-producing gene (Missig *et al.,* 2020; Napolitano *et al.,* 2022).

In addition to FSX, several chromosomal CNVs are associated with the disorder including Smith-Magenis Syndrome and Potocki-Lupski Syndromes related to the deletion and duplication of chromosomal region 17p11.2 respectively (Missig *et al.,* 2020). Most widely cited with regards to autism aetiology, however, is the hemi-deletion of the chromosomal region 16p11.2 (Missig *et al.,* 2020; Napolitano *et al.,* 2022), a commonly used genetic target for autism animal models evidenced to produce a range of the behavioural features reminiscent of the condition in humans. (Missig *et al.,* 2020).

2.1.1.2 Environmental animal models

As opposed to genetic models, environmental models are easier to use for experimentation as they mimic early developmental risk factors associated with autism emergence later in life (Missig *et al.*, 2020).

Perhaps most widely cited in literature is the valproic acid (VPA) model, which entails the prenatal administration of VPA salt, which is otherwise commonly used for the treatment of epilepsy and mood disorders in humans (Zhang *et al.*, 2018; Chaliha *et al*., 2020; Missig *et al.*, 2021; Napolitano *et al.*, 2022). Its adoption for use in animal models originates from human clinical (Napolitano *et al.*, 2022) and epidemiological studies, which previously provided evidence for the increased susceptibility of development of autism-like symptoms following exposure during pregnancy (Chaliha *et al.*, 2020). The VPA model's widespread use can be attributed to its validity amongst certain criteria (Mabunga, 2015) which are discussed below. These include face validity, in that it is able to mimic the core diagnostic features observed in human subjects which present themselves unambiguously, allowing for observation through extensively documented behavioural tests (Chaliha *et al.*, 2020). Secondly, its use as a model entails the prenatal administration of valproic acid (2-propylpentanoic acid) during critical times in neurodevelopment, suggested to be related to the processes surrounding neural tube closure (Zhang *et al.*, 2018; Chaliha *et al.*, 2020). This occurs around gestational day 11-12 in rats (Zhang *et al.*, 2018). The effects of its administration during foetal development have been evidenced to not only mimic autism-like symptoms in resulting offspring, but also some aspects of the neurological bases of these symptoms (i.e. its aetiology). In particular, hypoplasia of the pons, occurring soon after neural tube closure (Chaliha *et al.*, 2020), and thus giving the model construct validity for its homology between species. Thirdly, various drugs have been tested on the model to develop drug interventions, the most notable of which is Risperidone (Zhang *et al.*, 2018). Its use in the treatment of autism symptoms was eventually adapted for humans, making the model valuable for its translational relevance (that is, deriving use from animal studies for clinical use in humans) and giving it predictive validity (Zhang *et al*., 2018).

2.1.2 Sex differences

Early population prevalence studies have resulted in the broadly cited four-to-one (4:1) sex ratio of ASD (Kanner, 1943), indicating that males are four times more likely than females to be diagnosed with the disorder according to the criteria of the DSM (APA, 2013). Interestingly, Napolitano and colleagues (2022) suggest that this disparity is reflective of the

female phenotype of autism, which is unaccounted for by the current diagnostic tools, thus resulting in fewer diagnoses among females. Over the years, multiple theories have arisen in attempts to account for the apparent diagnostic disparity between the two sexes. These include the extreme male brain (EMB) theory, the female protective effect (FPE) and female autism phenotype (FAP) model (Napolitano *et al.,* 2022). Considered collectively, the theories that have been put forward to explain the diagnostic disparity between the sexes, highlight the need to include females in autism studies in order to learn the sex differences which may result in more accurate diagnoses and targeted therapeutic interventions.

2.1.2.1 Extreme Male Brain (EMB) Theory

The Extreme Male Brain (EMB) Theory asserts that the structural (morphological) and related functional differences in the brains of males and females give rise to the observed differences in autism susceptibility between the sexes (Napolitano *et al.,* 2022). Specifically, attributing distinct neuro-morphological and neuro-functional profile to autistic individuals based on sex (Crespi *et al.,* 2019). The theory asserts that autistic individuals exhibit a tendency to possess an extreme male profile which is biassed towards systemising, with deficits in the ability to empathise (Richards, 2022). The theory establishes an empathising-systemising axis onto which the human population can be classified, using the capacity for both to establish five brain types along the axis (Richards, 2022). Despite asserting that males and females may assume any point on this axis, Baron-Cohen (2002, 2004, 2005) also attributes the extreme brain types, namely, hyper-systemising/hypo-empathising and hypo-systemising/hyper-empathising, to males and females respectively. As such, the EMB theory, as it is currently understood, posits a role for human sexual dimorphism in various aspects in predicting the risk for the development of autism (Crespi *et al.,* 2019).

Of the three theories discussed herewith, the EMB has lost credibility over the years, suggesting a larger role for the FPE and FAP theories in accounting for the diagnostic disparity between the sexes (Richards, 2022).

2.1.2.2 Female Protective Effect (FPE) Theory

The Female Protective Effect (FPE) theory was first proposed in the late 20th century by a study that found that autistic females had a larger number of relatives with autism or certain language impairments than their male counterparts (Tsai *et al.,* 1981). Their findings have since been confirmed in multiple studies assessing both behavioural and genetic concordance among

siblings with at least one autism diagnosis (Robinson *et al.*, 2013). This increased familial genetic load suggests that females require an increased burden of genetic abnormalities for behavioural impairments to be observed (Napolitano *et al*., 2022). This is in accordance with several studies which have found that females with diagnosed autism have more *de novo* CNVs in their genome compared to males (Levy *et al.*, 2011; Robinson *et al.*, 2013), as well as more severe ASD behavioural phenotypes when they do present, presumably due to the increased genetic abnormalities (Napolitano *et al*., 2022).

Further, despite the decreased familial concordance in males, their genetic risk is higher due to the increased penetrance of the genes responsible for the disorder's onset (Barret & Tsien, 2008; Kumar *et al*., 2015). That is, the incidence of genetic abnormalities resulting in ASD outcomes is higher in males for a lesser number of affected genes as compared to females (Napolitano *et al*., 2022). For instance, despite having fewer CNVs in their genome overall, males have an increased incidence of occurrence at loci which have been linked to ASD outcomes (Levy *et al.*, 2011). This has been demonstrated and most widely cited to occur at locus 16p11.2 (Weiss *et al.*, 2008; Mellford *et al.*, 2009; Pinto *et al.*, 2010; Levy *et al.*, 2011). Further, the chromosomal differences between males and females have possibly enabled a protective factor on the female X-chromosomes. That is, that the paternal X-chromosome may compensate for any abnormalities that may occur on the maternal side (Hull *et al*., 2020; Napolitano *et al.*, 2022) such as FXS, the most prevalent form of syndromic autism, occurring almost exclusively in males (Missig *et al.*, 2020; Napolitano *et al*., 2022).

2.1.2.3 Female Autism Phenotype (FAP) Theory

The apparent diagnostic disparity cannot be explained solely through the presence of various biological shields inherent in the female genome as proposed by the FPE theory (Levy *et al*., 2011; Napolitano *et al*., 2022). The FAP has been proposed in addition to the FPE to provide a more holistic understanding of sex-differences within the disorder and asserts that females are underdiagnosed due to a difference in the presentation of behaviours between the sexes (Hull *et al.*, 2020). It further highlights the possibility that the FPE, however accurate in some cases, may operate based on inaccurate estimates from population prevalence studies (Hull *et al.*, 2020), inherently biassing the diagnostic criteria resulting in fewer female diagnoses. Given that the behavioural diagnostic criteria were based on observed male behaviours (Kanner, 1943), these processes are likely less effective in identifying the disorder in females, which may present differently and instead receive diagnoses of unspecified

developmental disorders (Wilson *et al.*, 2016). Evidence for this assertion exists mainly in animal models of the disorder which have assessed males and females simultaneously for the presence of autism-like behaviours (Napolitano *et al*., 2021). For instance, to date, only a few studies have studied both sexes in FXS models of the disorder in rodents (Loesch *et al*., 2004; Baker *et al*., 2010; Ding *et al.*, 2014; Nolan *et al*., 2017).

The assertions of the FAP theory highlight the need for studies inclusive of both sexes across an array of contexts to learn the presentation of autism across the full spectrum in which it exists (Hull *et al.*, 2020). The importance of both male and female representation in autism studies has been evidenced in literature. For instance, Loomes and colleagues (2017) found that studies conducted in clinical settings underestimated the number of autistic females when compared to population-based studies. Thus, suggesting that the nature of the study being conducted may inaccurately represent the ratio in that given population and fail to accurately represent populations across various contexts worldwide (Hull *et al.*, 2020).

Generally, there exists an under-representation of females in autism studies across a range of disciplines. For instance, Napolitano and colleagues (2022) attributed the lack of studies comparing sex differences in structural and functional brain characteristics from magnetic resonance imaging (MRI) as being due to the relatively few females developing ASD when compared to males, a pattern likely due to the discrepancy between diagnosed male and female cases.

2.1.3 Sleep and autism

In addition to the core autistic diagnostic behavioural characteristics, a range of other behaviours have proven relevant in understanding the mechanisms underlying its development and appearance. These behaviours comprise a number of comorbidities which are associated with the disorder, perhaps the most prevalent of which is sleep disturbance problems (Fang *et al*., 2021; Lorsung *et al*., 2021).

2.1.3.1 Sleep as a circadian behaviour

Sleep typically occurs habitually at specific times of the day according to the time cues provided by light, in particular, the distribution of light and darkness over a 24-hour period (Lorsung *et al*., 2021). As such, its regulation is often studied in relation to a branch of science called chronobiology (Vitaterna *et al*., 2001). Central to this type of research is the understanding of circadian rhythms, which describe the co-ordination or alignment of

behaviours and their underlying biological processes to the external environment (Ross, 2015) according to near-24-hour cycles (Halberg, 1960). These rhythms exist at all levels of biological organisation and are endogenously generated, allowing them to be conserved within individual cells (Vitaterna *et al.*, 2001). Intracellular rhythms are regulated by transcriptional-translational feedback loops (Barone *et al.*, 2019; Lorsung *et al.*, 2021; Pandi-Perumal *et al.*, 2022) as driven by clock genes and their protein products (Lorsung *et al.*, 2021). However, in the absence of a central pacemaker, the maintenance of these rhythms is short-lived (Hall & Hall, 2020) and synchronisation amongst various cycles in the body is near impossible (Lorsung *et al*., 2021)

Enabling both the maintenance of endogenous body clocks and their global synchronisation is the suprachiasmatic nucleus (SCN), a collection of roughly 20 000 neurons positioned adjacent to the optic chiasm in the hypothalamus of the brain (Hall & Hall, 2020; Ross, 2015). The SCN serves as an internal "master clock" or pacemaker responsible for regulating circadian rhythmicity (Goeffray *et al*., 2016). It is entrained by light, its primary time cue (Lorsung *et al*., 2021), and processes photic information received by photosensitive ganglion cells in the retina (RGCs) along the retinohypothalamic tract (RHT) (Hall & Hall, 2020). This signalling pathway regulates the expression of the neurotransmitters responsible for initiating gene expression which resets the SCN according to the time cues received (Lorsung *et al*., 2021). In turn, the SCN is able to communicate with internal peripheral tissues through neural and endocrine signals (neurotransmitters, hormones etc.), allowing them to regulate local physiology contributing to specific behaviours and facilitate organismal function by ensuring synchrony amongst the various body clocks (Lorsung *et al.*, 2021).

In addition to circadian rhythms (Process C), sleep is influenced by homeostatic pressure as determined by an individual's preceding sleep/wake history (Tam *et al.*, 2020). This sleep homeostatic pressure, termed Process S, is increased during wake and dissipated when asleep (Geoffray *et al*., 2017) as evidenced by measured by slow wave activities (SWA) during non-REM sleep recorded by EEGs (Tam *et al.*, 2020).

2.1.3.2 Nature of sleep disturbance in autism

The presence of sleep disturbance in children with autism is commonly reported from parental or caregivers' subjective accounts of children's sleeping patterns, often entailing the use of sleep diaries and questionnaires that measure the presence and severity of a number of sleeping behaviours through a scoring system (Maurer *et al.*, 2023). Most utilised in studies of

this nature is the Children's Sleep Habits Questionnaire (CSHQ) (Maurer *et al.,* 2023), although other standardised questionnaires include the Sleep Disturbance Scale for Children (SDSC) and Paediatric Sleep Questionnaire (PSQ) (Kim *et al*., 2023). The CSHQ characterises the nature of reported sleep disturbances according to bedtime resistance, sleep onset delay, sleep anxiety, nighttime sleeplessness, nighttime wakings, and daytime sleepiness (Kim *et al*., 2023; Maurer *et al.,* 2023). Further, including accounts for parasomnias, defined by Missig and colleagues (2020) as unusual physical or experiential events accompanying sleep. These may include, but are not limited to sleep talking, sleepwalking, and night terrors (Wiggs & Stores, 2004; Maurer *et al,* 2023).

Characterising the nature of SDs in ASD is limited by methodological inconsistencies, small sample sizes and the heterogeneity of sleep behaviours parallelling those of the ASD symptoms themselves (Missig *et al*., 2020). Further, screening tools such as the CSHQ are useful only in identifying the presence of sleep problems and not discriminating between its various types (Owens *et al.,* 2000). Parental reports of this nature have indicated that autistic children are more likely to have bedtime resistance increasing sleep onset time, as well as frequent awakenings and decreases in total sleep time (Couturier *et al*., 2005; Krakowiak *et al*., 2008). Additionally, two meta-analyses have provided a summary of the findings of studies utilising more objective measures such as polysomnography (PSG) and actigraphy (Elrod & Hood, 2015; Diaz-Roman *et al*., 2018). Those studies including actigraphy measurements could only find significant differences in the increased sleep latency between autistic and typically developing individuals (Diaz-Roman *et al*., 2018). Conversely, more consistent differences in the total sleep time, sleep latency and time in REM sleep could be detected from those studies utilising PSG (Elrod & Hood, 2015).

No single form of sleep disturbance presents consistently among all individuals with ASD (Missig *et al.,* 2020), and indeed not all present with sleep disturbance (Misisg *et al*., 2020). However, the most frequently reported across studies of parental reports, PSG and actigraphy are sleep onset and maintenance insomnia (Cohen *et al.,* 2014; Kohyama, 2016). These specifically relate to the increased latency to fall asleep, decreased proportion spent asleep while in bed, evidenced by decreased time in REM sleep from PSG studies and decreased total sleep duration (Cohen *et al.,* 2014).

Further, studies assessing the daytime functioning of sleep disturbed individuals have aided in identifying, not only the presence of the SDs in ASD, but also their impact on autistic

and other behavioural features (Maurer *et al*., 2023). For instance, Aathira and colleagues (2017) assessed the severity of the behavioural co-morbidities of autistic individuals classified as either good or poor sleepers by the CSHQ. The results of their study confirmed the presence of the frequently reported SDs in the poor sleeping group, further identifying the presence of daytime sleepiness following SD. Additionally, Goldman and colleagues (2009), were able to show evidence for the increased irritability and hyperactivity in poor sleeping groups despite failing to show statistical significance for differences for most of the sleep parameters assessed. Furthermore, identifying significant correlations between fragmented sleep (i.e. more frequent arousals) and repetitive behaviours assessed using the Repetitive Behaviour Scale (RBS-R). Taken together, these findings suggest an increase in core as well as affective or co-morbid behaviours according to the extent of SD in ASD (Malow *et al*., 2006; Goldman *et al*., 2009; Aathira *et al*., 2017).

2.1.3.3 Sleep disturbance in VPA models of autism

Consistent with human studies showing abnormal sleep in autistic individuals, various animal studies have found evidence for abnormal sleep/wake behaviours in VPA treated rats. These behaviours have typically been determined using some combination of objective measures of sleep states or behavioural characteristics such as locomotor activity, arousal thresholds, and feeding (Allada & Siegel, 2008).

For instance, Cusmano and Mong (2014) monitored the EEG and electromyogram (EMG) activity of rats housed under reversed 12hr/12hr L/D cycles. The authors found that VPA rats experienced bouts of wakefulness in both light and dark phases, contrary to the expected behaviour of Sprague Dawley rats that are nocturnal and typically quiescent during the light phase (Missig *et al*., 2019). Despite similar periods of wakefulness during the dark phase between the groups, this increased light-phase wakefulness of the VPA rats decreased their total sleep time across both phases. Furthermore, the decreased total sleep time is likely the result of the decreased non-REM sleep of VPA rats in the light phase (Cusmano & Mong, 2014). The authors attributed these abnormal sleep/wake behaviours to dysfunction in the gamma-aminobutyric acid (GABA)ergic system which plays an important role in initiating and maintaining sleep (Saper *et al*.., 2010; Hou *et al*., 2017).

Additionally, under the same reversed 12hr/12hr L/D cycles, Tsujino and colleagues (2007) found that VPA rats exhibited more frequent arousals in the light or sleep phase when compared to controls. Arousals have been detected using various PSG indicators such as

accelerated heart rate and increased variability between beats from electrocardiograms or gross body movements lasting between three and 60 seconds from electromyograms (Mirmiran *et al*., 2003). These frequent arousals in the light phase are contrary to what is expected for nocturnal rodents which tend to show increased locomotor activity in the dark phase (Hunt *et al*., 2022). The authors also found that VPA rats exhibited increased feeding bouts during the light phase (Tsujino *et al*., 2007). These results suggest circadian dysfunction in the VPA rats which is likely the result of the inability to effectively entrain to the reversed L/D cycles (Vitaterna *et al*., 2001). This dysfunction was confirmed by physiological and histological measures of the serotonergic system (Tsujino *et al*., 2007). The former was evidenced by the increased baseline measures of extracellular 5-HT levels in the frontal cortices of VPA rats as measured through microdialysis probing (Tsujino *et al*., 2007). Further, immunohistochemistry (IHC) revealed a caudal shift of 5-HT-positive neurons in the dorsal raphe nuclei of the VPA rats (Tsujino *et al*., 2007), a finding consistent with a previous study (Miyazaki *et al*., 2005).

2.1.3.4 Relationship between sleep disturbances and autism symptomology

The increased prevalence of SDs in autistic individuals compared to those typically developing (Richdale *et al*., 2009; Hodge *et al*., 2014) suggests an increased vulnerability of developing SDs in autism (Galli *et al*., 2022). However, the mechanisms underlying the development of SDs is not fully understood, especially as disorders comorbid to existing autism pathology (Verhoeff *et al*., 2018; MacDuffle *et al*., 2020; Galli *et al*., 2022). A number of hypotheses have arisen in attempts to understand the relationship between sleep and autism pathology as well as the mechanisms potentially underlying their interaction. Two of these hypotheses pertain to existing autism pathology resulting in sleep problems: the first suggests that there are mechanisms underlying autism impairment which also implicate sleep, primarily as it relates to the functioning of various neurotransmitter systems (Mazzone *et al*., 2018; Burman *et al*., 2023). For instance, the serotonergic and GABAergic systems which are crucial for promoting and maintaining sleep have been shown to be dysfunctional in ASD (Burman *et al*., 2023). Devnani and Hedge (2015) found that sleep-promoting preoptic areas containing dense populations of GABAergic neurons are underdeveloped in autistic children. Further, hyperserotonemia or the elevated plasma levels of serotonin, a biological deficit commonly presenting in the disorder (Melke *et al*., 2008; Tordjman *et al*., 2013; Janušonis, 2014), is representative of the system's abnormal peripheral functioning (Lorsung *et al*., 2021).

The second hypothesis attributes sleep problems in ASD to manifestations of its core behavioural symptoms (Mazzone *et al*., 2018). For instance, Wiggs and Stores (2004) have suggested that individuals with excessive or repetitive routines before bedtime may suffer delays in sleep onset. Alternatively, those with problems related to sensory stimulation may struggle with maintaining sleep due to frequent arousals (Mazzone *et al*., 2018). These two hypotheses support the possibility that the sleep problems associated with autism pathology may worsen existing symptoms as suggested by multiple authors (Cohen *et al*., 2014). For instance, Schreck and colleagues (2004) have shown that total sleep time is predictive of the severity of deficits in the social domain in ASD children. Further, suggesting a bidirectional relationship between the two (Richdale *et al*., 2009; Souders *et al*., 2009; Reynolds & Malow, 2011), such that autism pathology causes SD, which in turn worsens existing autism symptoms and potentially leads to additional maladaptive behaviours resulting from the SD (Mazzone *et al*., 2018). These maladaptive behaviours may adopt the form of externalising (for example, aggression and impulsivity) and internalising (for example, anxiety and withdrawal) behaviours (Missig *et al*., 2020), which do not constitute the disorder's diagnostic symptoms but may worsen them, continuing a cycle of impairment (Mazzone *et al*., 2018).

2.2 Materials and Methods

2.2.1 Ethics

Ethical clearance was obtained through the University of the Witwatersrand Animal Research Ethics Committee (AREC; Johannesburg, South Africa) (Clearance certificate number 2022/10/03/B – Appendix A) whose guidelines are in accordance with those of the National Health Research Ethics Council of South Africa.

2.2.2 Study subjects

Eighteen female Sprague Dawley *(Rattus norvegicus)* rats were purchased from the Wits Research Animal Facility (WRAF) and allocated to two sample groups, namely: Autism (VPA; n=14) and Control (C; n=4). This unbalanced allocation of the female rats was due to small litter sizes from the VPA females (Appendix B). All rats were housed individually in polycarbonate cages (L - 46cm; W - 21cm; H - 14cm) lined with aspen bedding and various environmental enrichments (shredded paper, plastic ball & tunnel). The rats were allowed free access to food and water and their housing room maintained at constant humidity with temperatures between 24-26℃. All rats were subjected to welfare checks in accordance with WRAF guidelines. These included daily checks by the attending nurse or veterinarian, daily handling to maintain sociability (less frequently for pregnant females to minimise stress), as well as weekly weight monitoring and welfare scoring (Appendix C).

For experimentation purposes, light/dark cycles were altered during the various study phases. These alterations are described in detail below.

2.2.3 Autism induction

During this first phase of the study, all the female rats were housed under a 12-hour light/12-hour dark cycle. The females were mated overnight and for each individual, day one of pregnancy was noted the day after the presence of a fallen vaginal plug as determined by observation. On day 12 of pregnancy, the dams in the Autism (VPA) group received a single subcutaneous injection of 600 mg/kg valproic acid VPA (250 mg/ml in saline, pH 7.3. Sigma, Oakville, CA) (Zhang *et al.,* 2018) in saline. The dams in the Control (C) sample group were instead treated with 2 ml of isotonic (0.9%) saline in the same mode. All saline used was sterilised by autoclave (SA-300VF, Sturdy, New Taipei City). All dams were then allowed to complete their pregnancies which lasted 21 days on average.

Upon birth, the resulting offspring (pups) were kept in the same cages as their mothers for the duration of the lactating period (21 days) and allowed to suckle freely until they were weaned on postnatal day (PND) 21.

2.2.4 Sleep disturbance

On PND21, the pups were separated by sex and randomly allocated to two further sample groups, namely, sleep disturbed (SD) and non-sleep disturbed (nSD), resulting in a total of four sample groups at this stage:

Table 2.1: Group sample sizes.

Sample group	Sample size (n)	
	Females	Males
VPA Sleep Disrupted (VPA-SD)	4	6
VPA Non-Sleep Disrupted (VPA-nSD)	4	5
Control Sleep Disrupted (C-SD)	11	11
Control Non-Sleep Disrupted (C-nSD)	11	10

Pups in the sleep disturbed sample groups (VPA-SD; C-SD) were kept under irregular light/dark cycles. Particularly, an 8-hour light/16-hour dark cycle (lights on at 6pm and off at 10am the following morning) (Franket *et al.*, 1999). For logistical reasons and as an additional form of sleep disturbance, the rats were kept in complete darkness over weekends (Lights off from 10am on Friday morning until 6pm the following Monday). The VPA-nSD and C-nSD groups were kept under normal 12-hour light/12-hour dark cycles in a separate room.

2.2.5 Behavioural Testing

All pups were housed in individual cages and allowed an acclimatisation period of seven days (PND 21-28) before behavioural testing took place from PND29 to PND55, during their respective light phases. This testing was conducted to assess the impact of light-induced sleep disturbance on autism-like behaviours. Using the Logitech C270 HD webcam software system, rats were recorded undergoing testing. These recordings were then analysed using the

ANY-Maze video-tracking software (Version 7.32, Stoelting Co., Illinois, United States of America) and the data obtained used in subsequent statistical analyses.

To minimise the risk of stress from testing, only one test was conducted per individual per day and at least 24 hours allowed between subsequent tests. Additionally, as testing took place in a different room to where the rats were being housed in, they were allowed at least an hour to acclimatize to test room conditions (24-26℃ and constant humidity). Further, all experimental apparatus were cleaned with 70% ethanol between training and testing of different pups to minimise the effects of olfactory cues on the behaviours being assessed (Jin *et al.*, 2018; Chang *et al.*, 2017). Rats were also given food rewards after training and test days.

2.2.5.1 Marble Burying

This test was used to assess repetitive behaviour from the number of marbles buried. Each rat was placed in a clean cage with fresh aspen bedding and allowed to explore freely for 30 minutes. Thereafter, an image of the marble arrangement was taken to later assess the number of marbles buried (Figure 2.1).

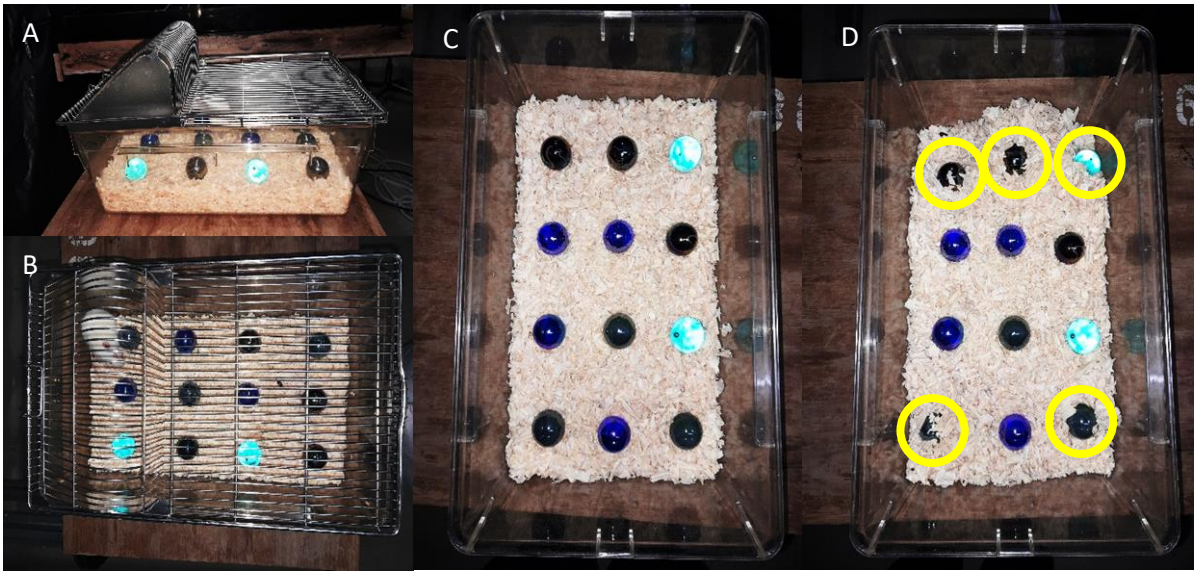


Figure 2.1: Lateral (A) and aerial (B) views of the marble burying experimental set-up consisting of 6cm of flattened aspen bedding in a clean polycarbonate cage with the feeding tray turned upside down to prevent rats from climbing out during testing. A 3X4 arrangement of twelve marbles was adopted showing the before (C) and after (D) of a male rat belonging to the VPA-SD group. Buried marbles are encircled in yellow.

2.2.5.2 Balance Beam

This test was used to assess motor balance and co-ordination from the number of foot

(hind paw) slips (Figure 2.2). The test consisted of two training days and a test day. For two consecutive days prior to test day, each rat was trained by allowing them to traverse a beam measuring a width of 4cm (two days prior) and 2cm (one day prior) respectively. This was done to familiarise rats with the testing procedure and minimise stress-related reactions to the test apparatus. On test day, rats were allowed up to five minutes to traverse a 1.5.cm beam four times (two from the left and right sides respectively). Failure to traverse in the allotted time or falling off the beam while traversing was considered a failed trial.

During both trial and test days, various means were employed to encourage the rats' movement across the beam: (1) a lamp was shone on the side of the starting platform to create an aversion, (2) a peanut was placed in the wooden box as a reward for traversing successfully, and (3) bedding from each rat's cage was placed inside to provide olfactory cues to direct movement. Additionally, tapping on the beam was done to encourage hesitant rats on training days.

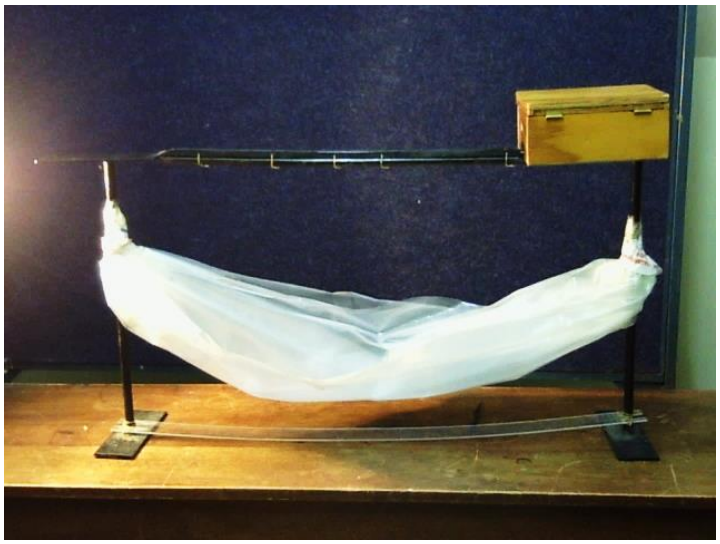


Figure 2.2: Balance beam experimental set-up consisting of a metal rod held up at 43.5cm by two metal poles at both ends. On one end was a metal platform (L - 19.5cm, W – 7cm) which served as the starting point. On the opposite end was a wooden box (L – 22cm, W- 18cm, H – 8cm) large enough to hold the test rats upon traversing the beam. Flat wooden beams of decreasing width were placed on top of the metal rod. Beneath the rod, a plastic was tied to both poles to catch any fallen rats.

2.2.5.3 Y-Maze

This test was used to assess spatial working memory based on the innate curiosity of rodents to explore previously unvisited areas (Kraeuter *et al.,* 2018). During testing, each rat was placed in the centre of the maze and allowed to choose an arm. When a choice had been

made, the rat was constricted to that arm for 15 seconds using a black Perspex partition. An entry into an arm was considered a "choice" when the rat's entire body (excluding the tail) was in an arm for more than 2 seconds) as determined by observation. Thereafter, the rat was briefly removed from the maze and placed in the centre. This was repeated 15 times.

Figure 2.3: Y-Maze test apparatus consisting of three transparent Perspex arms (L – 29cm, W-8.5cm, H – 15cm) joined at 120° & attached to a Perspex base, lined with plywood painted black to contrast the white rats.

2.2.5.4 Novel Object Recognition

This test was used to assess working memory in rodents based on their natural tendency for exploring novelty over familiarity in their environment (Barbosa & Silva, 2018) and consisted of two training days and a test day. Two days prior to test day, rats were acclimatised to an empty open field arena for five minutes. The following day (one day prior to test day), rats were acclimatised to the arena, this time with two identical objects placed in opposite corners (Figure 2.4A). On test day, the testing procedure consisted of two phases – During phase 1, rats were placed in the arena with the same two familiar objects from the second training day and allowed to explore freely for three minutes). They were then returned to their home cages and allowed a 15-minute break. Thereafter, during phase 2, they were returned to the arena with one of the familiar objects replaced with a novel, dissimilar object (Figure 2.4B) and again allowed to explore freely for three minutes.

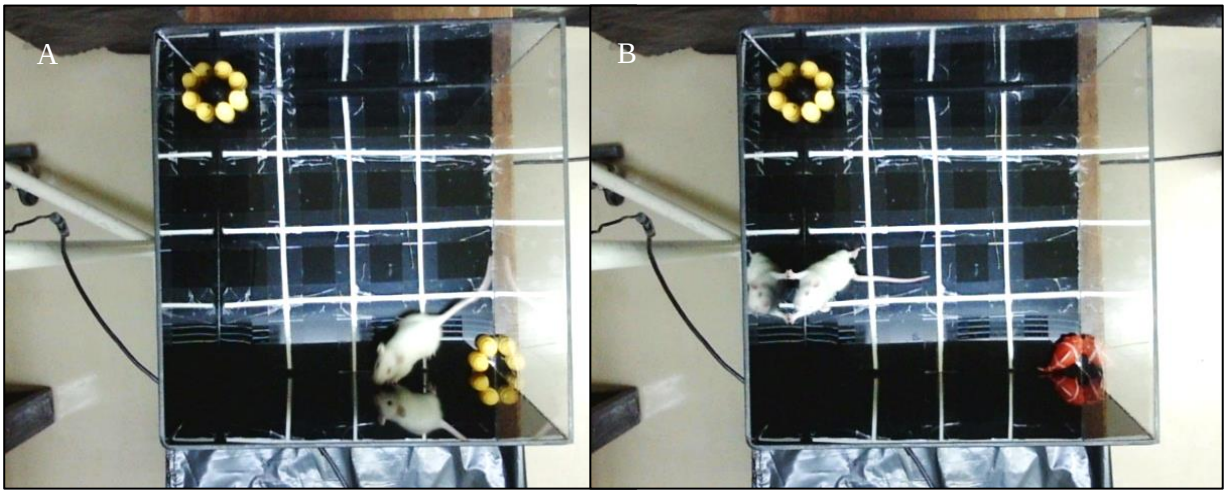


Figure 2.4: Aerial view of the open field arena with a rat undergoing novel object recognition testing in phase 1 (A) and phase 2 (B) where one of the familiar objects is replaced with a novel, dissimilar alternative. The test arena consists of a Plexi-glass box with an open top. The base (L – 40cm, W – 40cm) is marked with white tape to form a grid box of 16 squares ($10cm^2$ each). Attached to the base was four walls (W – 38cm, H – 41.5cm), three of which were black, and one transparent (right face).

2.2.5.5 Three-Chambered Sociability

This test was used to assess sociability in rodents based on their preference to interact with either social or non-social stimuli in their environment. The social stimuli used were age and sex-matched conspecifics removed from the sample. No specificity was observed between VPA and C individuals (i.e., controls were used as conspecifics for VPAs and vice versa). In the SD groups, four females (3 C & 1 VPA) and five males (3 C & 2 VPA) were removed. In the nSD groups, four females (3 C & 1 VPA) and four males (4C & 0 VPA). These individuals were, thus, not used for further analysis, reducing the group sample sizes (Table 2.2).

Table 2.2: Group sample sizes for the 3-chambered sociability behavioural test.

Sample group	Sample size (n)	
	Females	Males
VPA-SD	3	5
VPA-nSD	3	5
C-SD	8	7
C-nSD	8	7

The rats used as social stimuli were trained a day before the sample underwent testing. Training involved placing the rats underneath a metal cup in the 3-chamber apparatus for 15 minutes. This was done to acclimatize the rats to the test apparatus and minimize any stress which could have negatively impacted the willingness of test rats to interact with them.

On test day, the test rat was acclimatized to the 3-chamber apparatus first, by placing it in the centre chamber for 5 minutes with the coverings over the openings to restrict movement. The coverings were then removed, and the rat allowed to a further 10 minutes to freely explore all three chambers. After this 15-minute acclimatization period, the rat was returned to the centre chamber with both openings closed while the social (age and sec-matched conspecific) and non-social (inanimate object) stimuli were placed underneath metal cups in the two lateral chambers. Both partitions were then removed, and the rat allowed 10 minutes to freely explore all three chambers while recording.

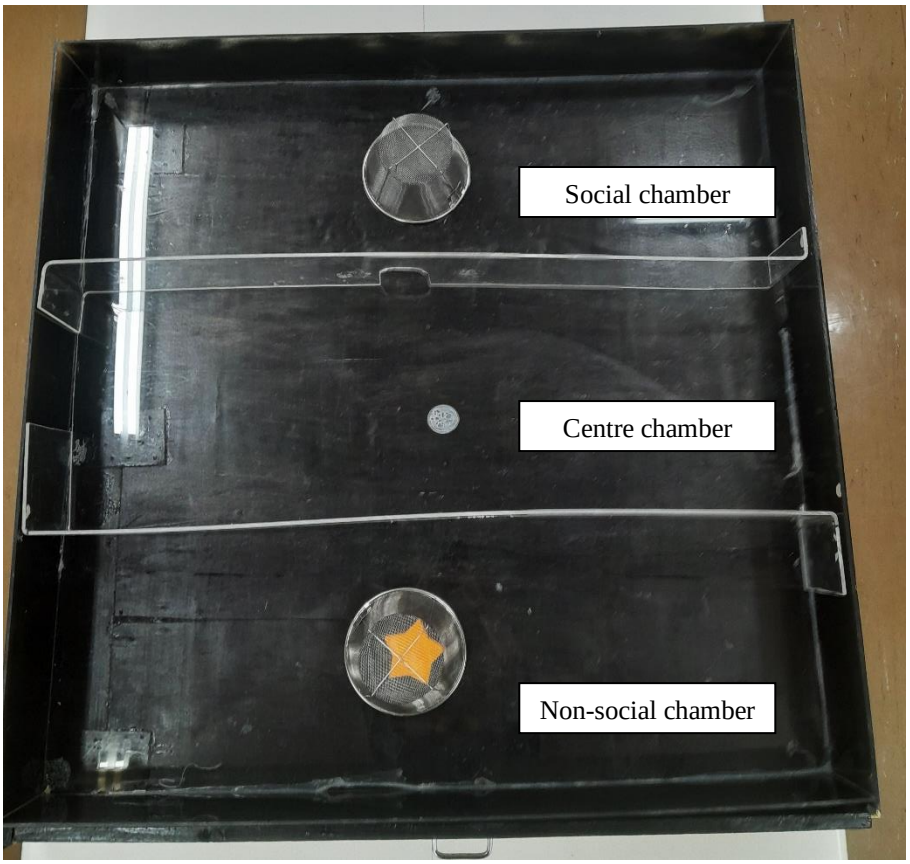


Figure 2.5: Aerial view of the 3-chambered sociability apparatus consisting of an open-top Perspex arena (L – 120cm, W – 120cm, H – 15cm) with removable partitions separating the apparatus into three chambers, a centre chamber and two lateral chambers (social and non-social shown here with metal cups under which social and non-social stimuli were placed during testing). Each partition has an opening to allow for movement of the rats between chambers.

2.2.6 Data Analysis

The videos recorded during behavioural testing (except for the marble burying test for which images were taken) were analysed using modified protocols from the video tracking software ANY-Maze (Version 7.32, Stoelting Co., IL, USA).

For each behavioural test, the variables to be used for further analysis (Table 2.3) were then assessed for group differences using estimated marginal means with significance accepted for p-values less than 0.05. Statistical analyses were run using the IBM Statistical Package for Social Sciences (SPSS: Versions 28.0.1.0 & 29.0.0.0). Firstly, multiple linear regressions (MLR) were employed to assess the predictive value of sex, autism pathology and sleep condition on the outcome variable(s) of each test. Secondly, a three-way (2x2x2 between subjects factorial) ANOVA was employed to determine the effect of sex on the interaction that

autism pathology (present/absent) and sleep condition (disturbed/non-disturbed) had on the outcome variables. In order to ensure reliability in the interpretations of the results obtained, the appropriate assumption tests were conducted for both tests (Appendix D and E).

Table 2.3: Behavioural test outcome variables.

Behavioural test	Description of outcome variable
Marble Burying	The **number of marbles buried** at least two thirds the depth of the bedding as determined by observation from images taken at various angles on test day.
Balance Beam	The **number of hind-paw foot slips** averaged across four trials (2x left and right respectively) as determined by ANY-Maze.
Y-Maze	The sequence of arm entries and subsequently, the number of alternations were determined by ANY-Maze. These were then used to calculate a **spontaneous alternation score (SAS)** for each individual, as defined by Bak *et al.* (2017) according to the following equation: $$SAS = \frac{Number\ of\ alternations}{Total\ number\ of\ arm\ entries - 2}$$
Novel Object Recognition	The **time spent investigating the novel object** during the second phase of testing was determined by ANY-Maze and the variable used for analysis was presented as a proportion of the total test time (three minutes).
3-Chambered Sociability	The **time spent investigating the social stimulus** was determined by ANY-Maze and used as a proportion of the total test time (final 10 minutes).

KEY: bolded entries indicate outcome variable for each test.

2.3 Results

2.3.1 Descriptive statistics

Following assumptions testing (Appendix D and E), the data used for each behavioural test underwent the following transformations: BB and NOR data were used as square root values and YM and 3C were reflected (1-SAS) and ranked respectively. The data of the MB test were used under logarithmic transformation with a consonant added as the dataset included zero values ($Log_{10}(marbles\ buried + 1)$). This transformation returned the least skewed data; however, the assumption of normality was still not satisfied (Table 2.6). Despite this, no outliers were present and the assumption of homogeneity in the variance was satisfied (Appendix E, Table 3).

Table 2.4: Descriptive statistics for each behavioural test.

Behavioural test	Outcome variable	n	Minimum	Maximum	Mean	SD (+/-)
MB	Ranked number of marbles buried	62	-28.50	36.00	3.89	15.31
BB	Square root number of foot slips	62	-1.25	0.99	0.11	0.44
YM	Reflected spontaneous alternation score (%)	62	-0.27	0.33	0.00	0.13
NOR	Square root time investigating novel object (%)	62	-2.54	1.78	3.25	0.95
3C	Ranked time investigating social stimulus (s)	45	-22.00	22.00	0.00	13.31

Table 2.5: Skewness and kurtosis scales.

	Negative	Normal	Positive
Skewness	-1.0 to -0.5	-0.5 to 0.5	0.5 to 1.0
Kurtosis	<-2	-2 to 2	>2

Table 2.6: Normality characteristics of the standard residuals of the outcome variable for each behavioural test.

Behavioural test	Outcome variable	W (*p*)	Skewness	Kurtosis
MB	Ranked number of marbles buried	0.92 **(<0.001)**	0.41	0.71
BB	Square root number of foot slips	0.95 (0.65)	0.02	0.25
YM	Reflected spontaneous alternation score (%)	0.89 (0.10)	0.37	0.57
NOR	Square root time investigating novel object (%)	0.97 (0.14)	0.07	-0.42
3C	Ranked time investigating social stimulus (s)	0.96 (0.085)	0.00	-1.20

KEY: "W" = Shapiro Wilk's test statistic; normality accepted at $p > 0.05$ (i.e. bolded table entries indicate non-normal values).

2.3.1.1 Marble Burying

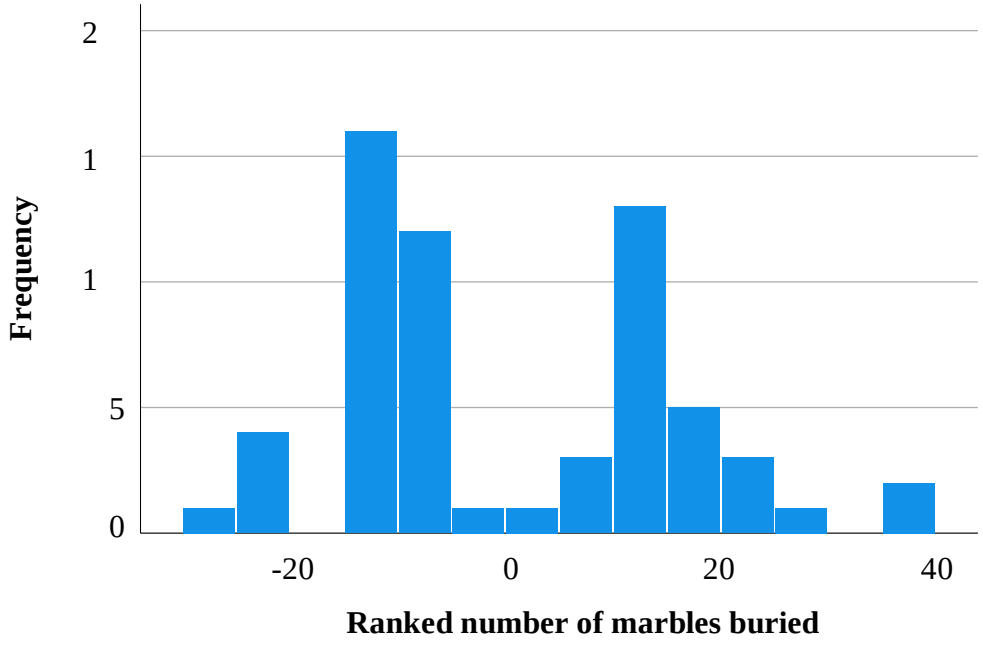


Figure 2.6: Histogram showing the ranked number of marbles buried obtained from the marble burying behavioural test.

2.3.1.2 Balance Beam

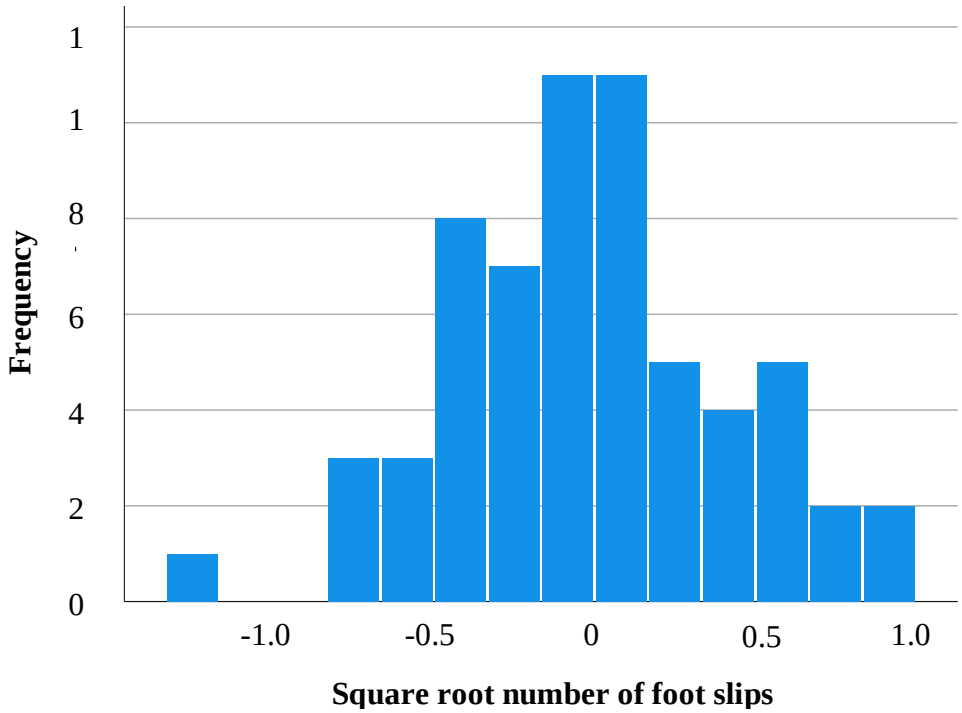


Figure 2.7: Histogram showing the spread of the square root number of foot slips obtained from the balance beam behavioural test.

2.3.1.3 Y-Maze

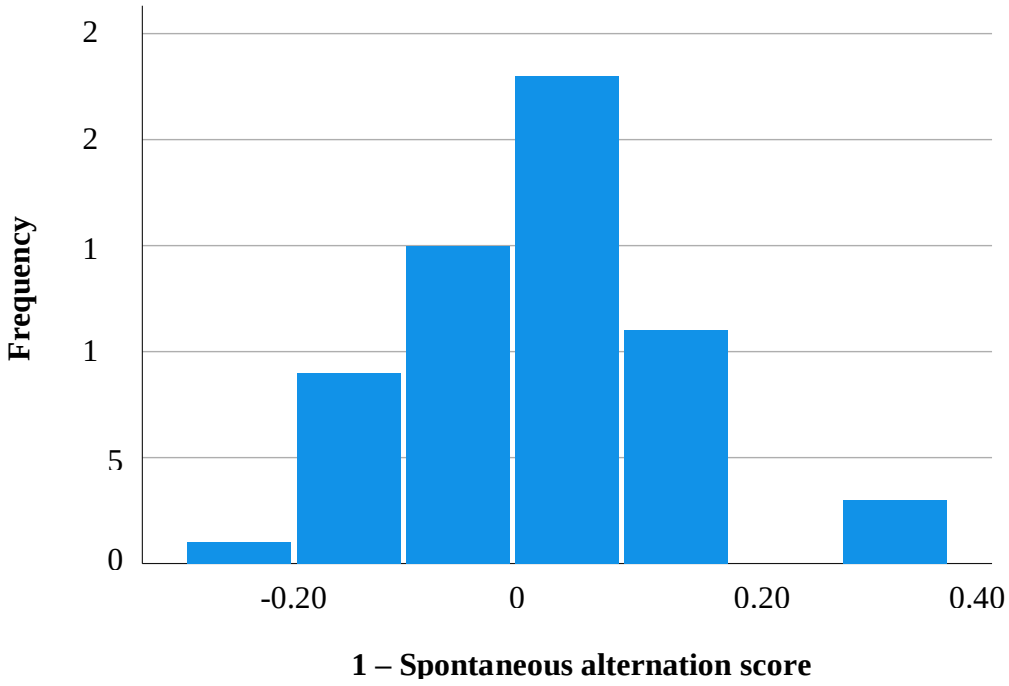


Figure 2.8: Histogram showing the spread of the reflected spontaneous alternation scores obtained from the y-maze behavioural test.

2.3.1.4 Novel Object Recognition

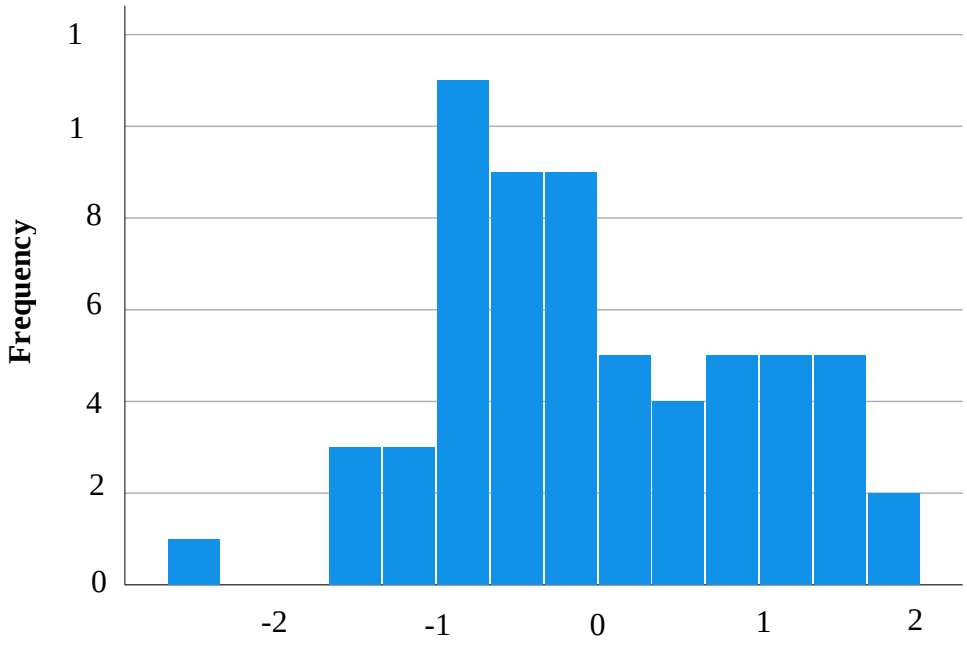


Figure 2.9: Histogram showing the spread of the square root time spent investigating the novel object obtained from the novel object recognition behavioural test.

2.3.1.5 Three-Chambered Sociability

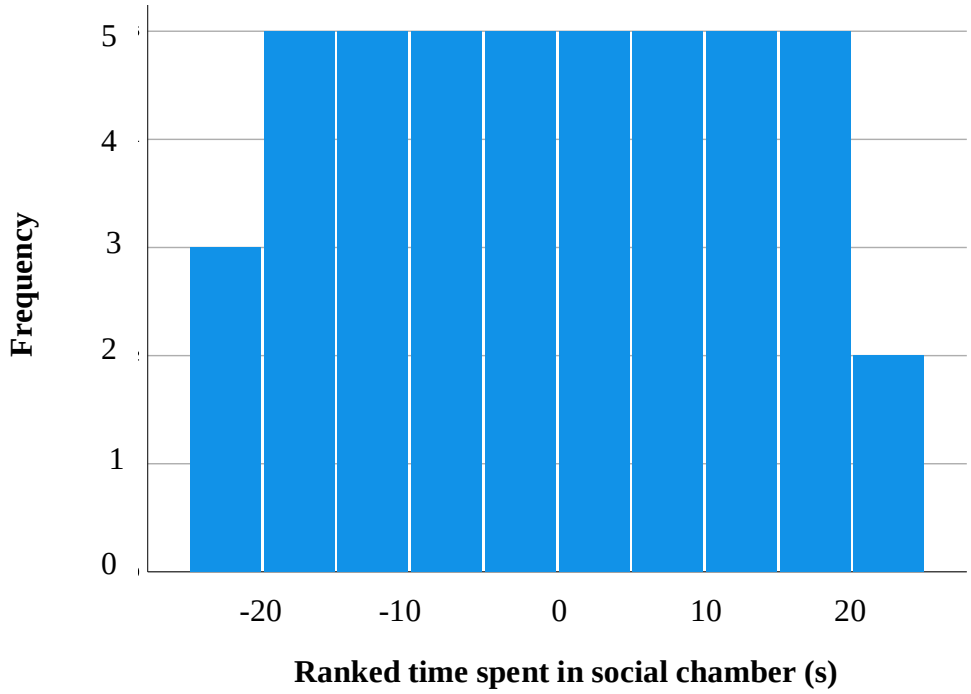


Figure 2.10: Histogram showing the spread of the time spent in the social chamber obtained from the three-chambered sociability behavioural test.

2.3.2 MLR prediction models

Graphical representations of the regressions are presented as scatterplots showing the relationship between each test's respective dependent variable and its unstandardized predicted value. This value represents the predictor variable generated by the model and is used to interpret the effects of each independent variable on the outcome. Additionally, the semi-partial correlations are included to show the contribution of each independent variable to these prediction models (Table 2.7).

Table 2.7: Semi-partial correlations of independent variables and outcomes variables of each behavioural test.

Behavioural test	Outcome variable	Semi-partial correlation (p-value)		
		Autism pathology	Sleep condition	Sex
MB	Ranked number of marbles buried	0.063 (0.629)	0.113 (0.391)	-0.048 (0.716)
BB	Square root number of foot slips	0.403 **(0.001)**	0.138 (0.249)	-0.021 (0.863)
YM	Spontaneous alternation score (%)	0.089 (0.496)	0.044 (0.734)	-0.056 (0.672)
NOR	Time investigating novel object (%)	-0.256 **(0.024)**	0.439 **(<0.001)**	0.185 (0.101)
3C	Time investigating social stimulus (s)	0.013 (0.931)	0.167 (0.281)	0.117 (0.449)

KEY: bolded entries represent significant p-values ($p < 0.05$).

Table 2.8: Results of the Fischer's F test for the MLR analysis of each behavioural test.

Behavioural test	df $(regression, residual)$	$F\ (p-value)$
MB	3,58	0.372 (0.773)
BB	3,58	4.330 (**0.008**)
YM	3,58	0.251 (0.860)
NOR	3,58	7.806 (**0.001**)
3C	3,43	0.835 (0.835)

KEY: bolded entries represent significant p-values ($p < 0.05$).

Table 2.9: MLR goodness of fit measures for each behavioural test.

Behavioural test	R	R^2	SEE
MB	0.137	0.019	16.763
BB	0.428	0.183	0.408
YM	0.113	0.013	0.126
NOR	0.536	0.288	5.482
3C	0.207	0.043	85.704

KEY: "R" = multiple correlation coefficient; "R^2" = co-efficient of determination; SEE = standard error of the estimate.

2.3.2.1 Marble Burying

The rank-transformed data was used in a multiple linear regression to predict the number of marbles buried from autism pathology, sleep condition and sex. Despite the assumption of normality not being satisfied (Appendix D, Figure 2), this violation was non-substantial due to the absence of any significant outliers (Appendix D, Table 4) as well as an acceptable number of data observations. This is according to the general rule that a minimum of 20 data points is required for each independent variable. In this case, a sample of 62 was used in accordance with the required minimum of 60 for this study design.

The results of the F-test failed to show evidence for the predictive value of the three independent variables [F (3,58) = 0.372; p = 0.773]. In addition, none of the three independent variables contributed significantly to the model (Table 2.7) which is observed to explain 1.9% of the variance in the number of marbles buried (R^2 = 0.019).

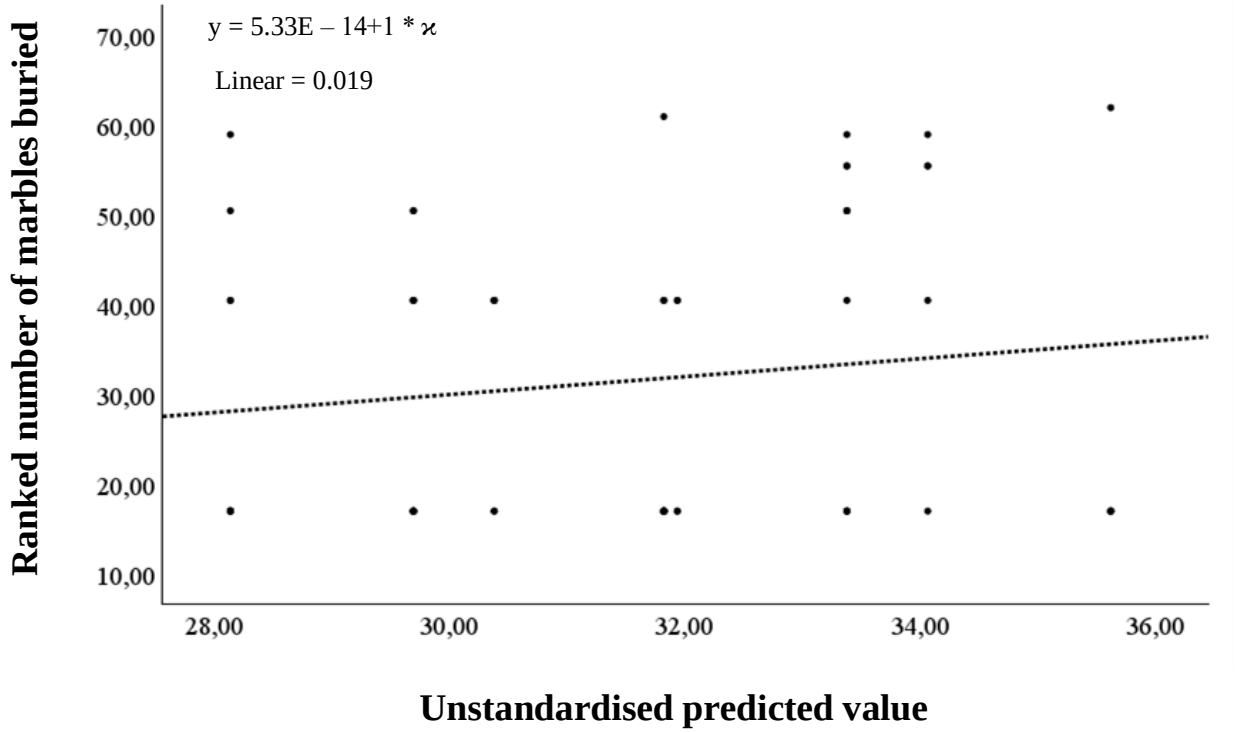


Figure 2.11: Scatterplot showing the predictive relationship between the predictor variable (generated by the model to represent the effect of the independent variables autism pathology, sleep condition and sex) and the ranked number of marbles buried.

2.3.2.2 Balance Beam

After employing a square root transformation, the data were used in a multiple linear regression to predict the number of foot slips from autism pathology, sleep condition and sex. The F-test showed evidence that these variables statistically significantly predicted the number of slips (F (3,62) = 4.330, p = 0.008). However, only autism pathology contributed significantly to the prediction value of this model ($p<0.05$) which is observed to explain 18.3% of the variance in the number of slips ($R^2 = 0.183$).

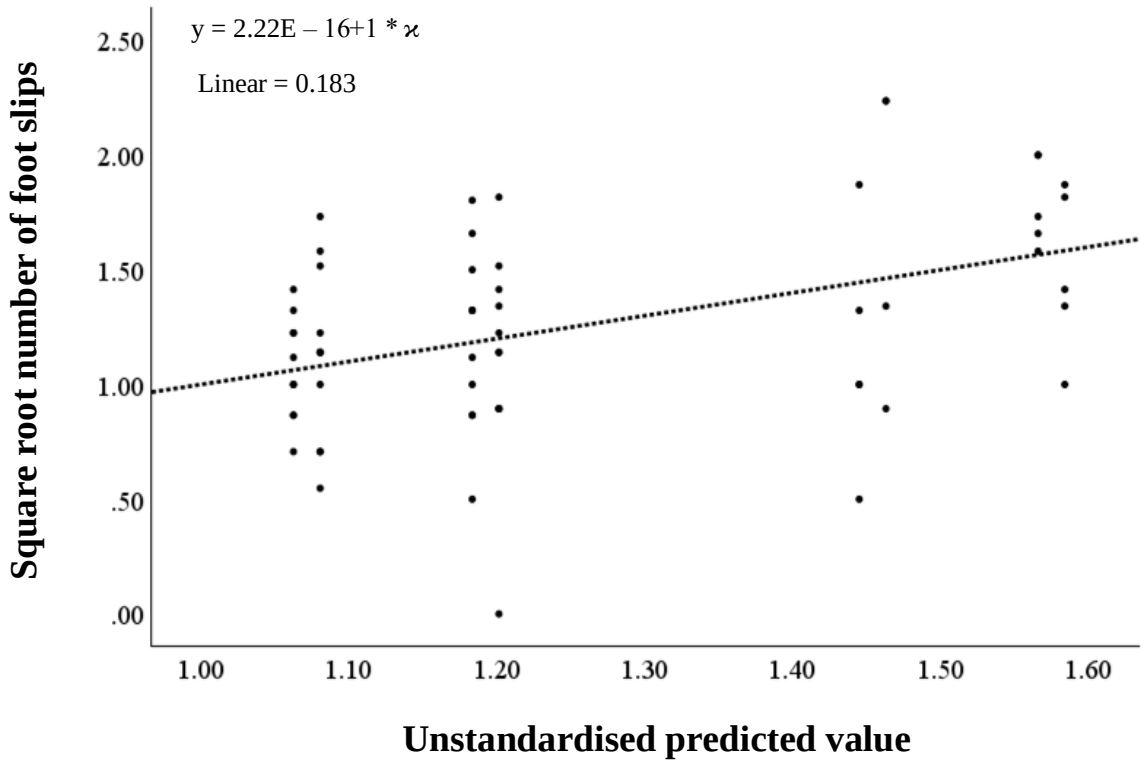


Figure 2.12: Scatterplot showing the predictive relationship between the predictor variable (generated by the model to represent the effect of the independent variables autism pathology, sleep condition and sex) and square root number of foot slips.

2.3.2.3 Y-Maze

A multiple linear regression was run to predict the spontaneous alternation score (%) from autism pathology, sleep condition and sex. The F-test failed to show statistical evidence for their predictive value (F (3,58) = 0.251, p = 0.860 > 0.05) with none of the variables

contributing significantly to the model. This model is observed to explain 1.3% of the variation in the spontaneous alternation score (R^2 = 0.013).

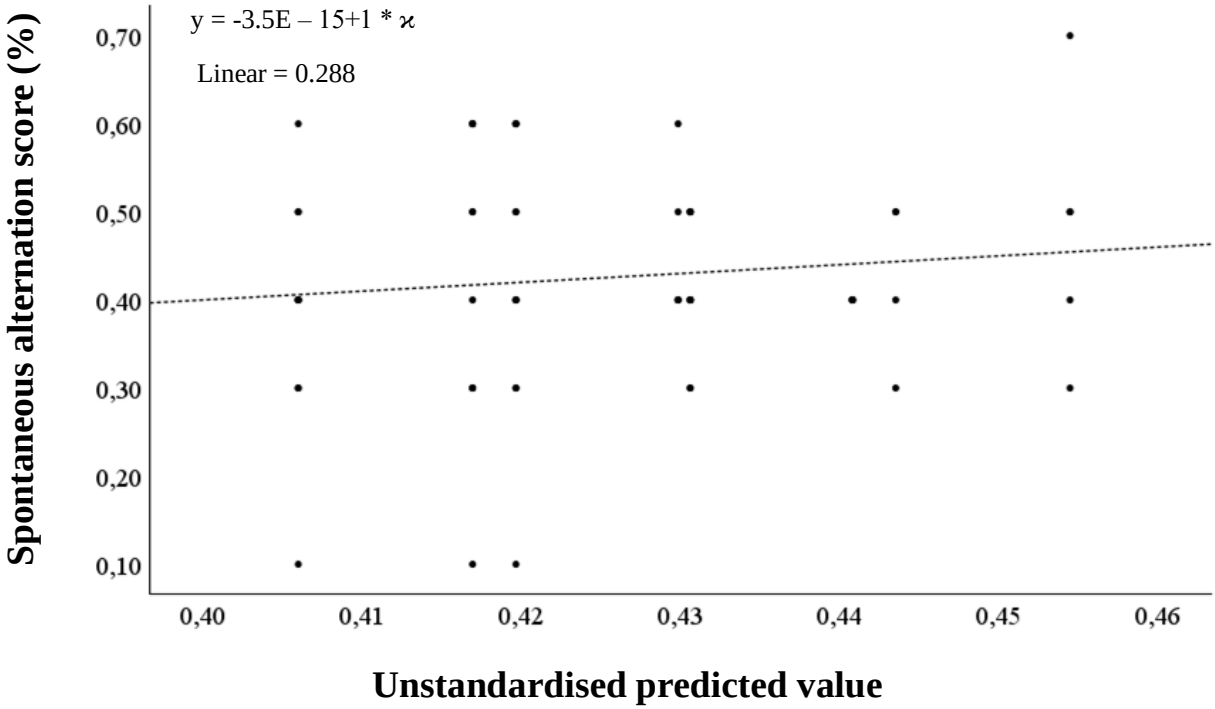


Figure 2.13: Scatterplot showing the predictive relationship between the predictor variable (generated by the model to represent the effect of the independent variables autism pathology, sleep condition and sex) and spontaneous alternation score (%).

2.3.2.4 Novel Object Recognition

A multiple linear regression was run to predict the time spent investigating the novel object (s) from autism pathology, sleep condition and sex. The F-test showed evidence that these variables statistically significantly predicted the time the rats spent investigating the novel object (F (3,58) = 7.806, p = 0.001 < 0.05). Further, autism pathology and sleep contributed significantly to the prediction model which is observed to explain 28.8% of the variation in the outcome observed (R^2 = 0.288).

Using Mahalanobi's distance, eight observations were identified to be outliers (Appendix D, Table 4), although only four were extreme outliers and subsequently excluded from the analysis. This remaining sample size does not deviate too far from the 60 observations

that are required for a study design assessing the prediction value of three independent variables.

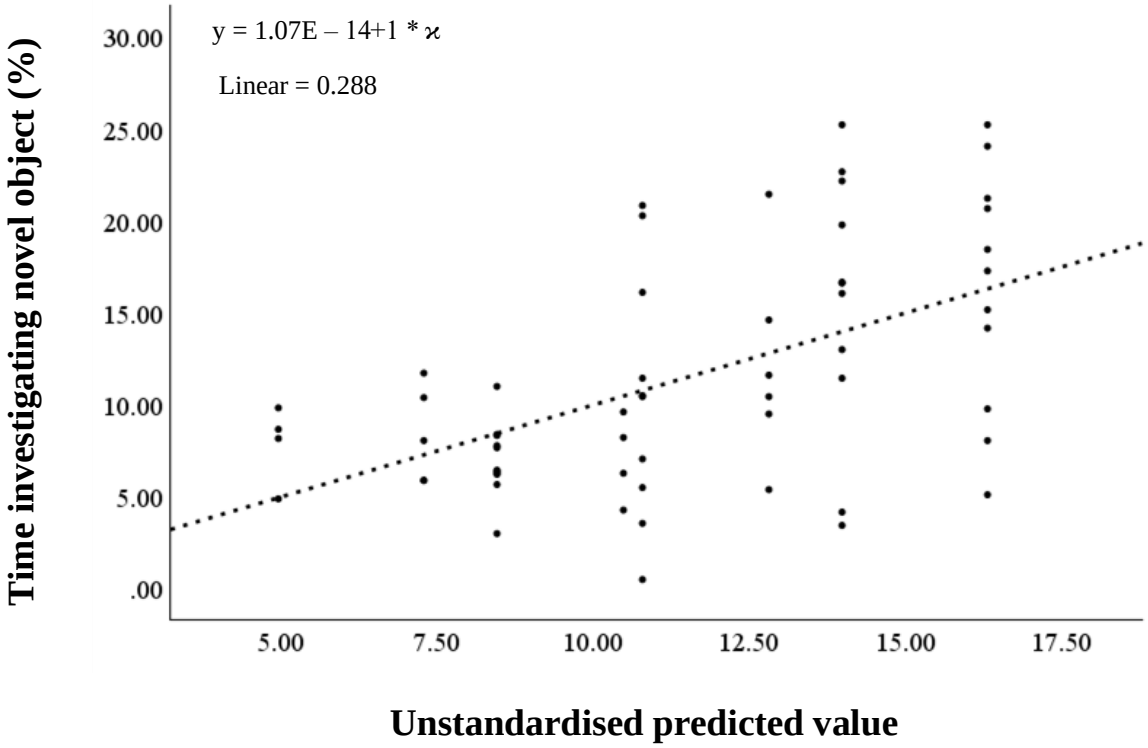


Figure 2.14: Scatterplot showing the predictive relationship between the predictor variable (generated by the model to represent the effect of the independent variables autism pathology, sleep condition and sex) and time spent investigating the novel object (%).

2.3.2.5 Three-Chambered Sociability

A multiple linear regression was run to predict the time spent in the social chamber (%, used as a measure of the rats' sociability) from autism pathology, sleep condition and sex. The F-test failed to show statistical evidence that these variables significantly predicted the outcome ($F(3,43) = 0.835$, $p = 0.835 > 0.05$). Further, none of the independent variables contributed significantly to the prediction model which is observed to explain 4.3% of the variation in the time spent in the social chamber ($R^2 = 0.043$).

The regression was run despite not all the assumptions being satisfied. Of these assumption violations, the first was the presence of eight outliers (Appendix D, Table 4). Unlike the other tests (n = 62 for MB & BB; n = 57 for YM), the three-chamber test removed study subjects for use as novel conspecifics during testing. These individuals were not assessed and thus excluded from the sample. The second violation was the presence of outliers detected using Mahalanobi's distance (Appendix D, Table 4). Only one of these observations was

significant and, thus, automatically excluded from the analysis. As such, the other seven likely did not contribute significantly to the non-normality of the residuals, maintaining the test's robusticity against the normality violation.

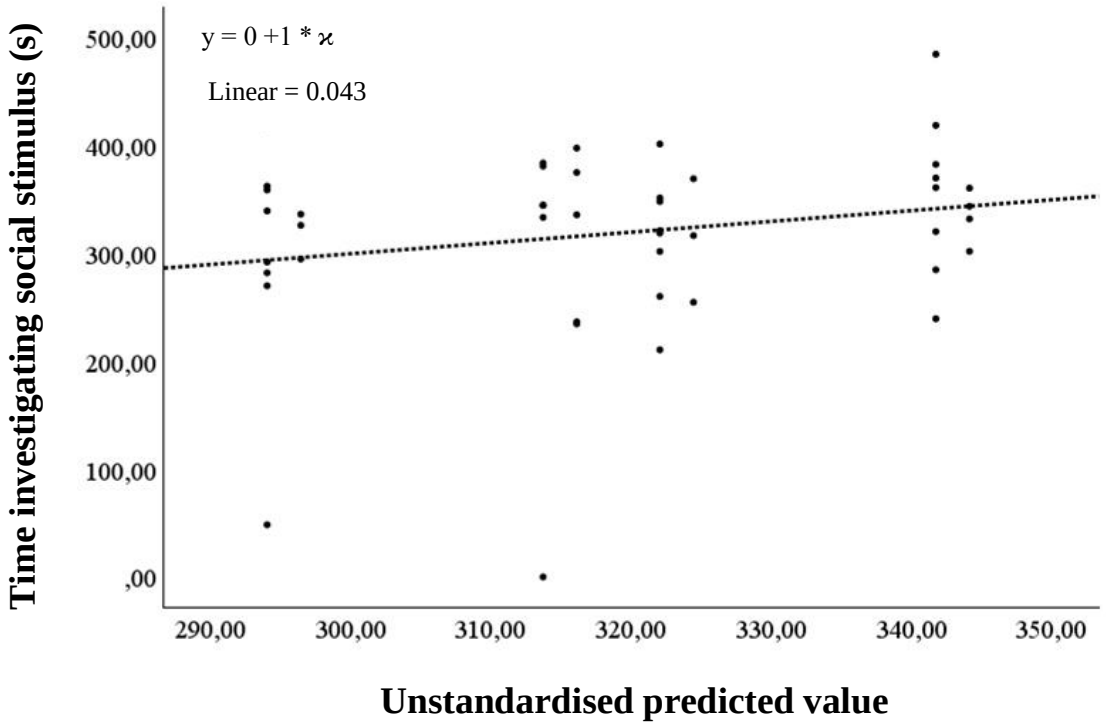


Figure 2.15: Scatterplot showing the predictive relationship between the predictor variable (generated by the model to represent the effect of the independent variables autism pathology, sleep condition and sex) and time spent in the social chamber (%).

2.3.3 Interaction effects

Following assumption testing (Appendix E), the data were assessed for group differences using the Fisher's (F) test which assumes equality in the variance of the dependent variable amongst groups across all levels of the independent variables. As an unbalanced study design was utilised (i.e. unequal sample sizes across groups), group differences were assessed from estimated marginal means (EMMs). Significance in the main and interaction effects accepted for p-values less than 0.05. Further, data are presented with interaction plots illustrating the effects of sex on the interaction that autism pathology and sleep condition have on the respective outcome variables.

Table 2.10: Results of the Fischer's F test for the main and interaction effects of each behavioural test.

Behavioural test	Outcome variable	F-statistic (p-value)						
		Three-way interaction	Two-way interactions			Main effects		
		AP*SC*S	AP*SC	AP*S	SC*S	AP	SC	S
MB	Log marbles buried	2.622 (0.111)	0.362 (0.550)	3.131 (0.082)	0.010 (0.992)	0.242 (0.625)	1.901 (0.174)	0.141 (0.708)
BB	Square root number of foot slips	2.492 (0.120)	0.625 (0.433)	0.368 (0.547)	5.048 **(0.029)**	12.666 **(<0.001)**	1.761 (0.190)	0.201 (0.656)
YM	Reflected (1 -) spontaneous alternation score	0.011 (0.917)	0.079 (0.780)	0.011 (0.917)	0.403 (0.529)	0.607 (0.440)	0.062 (0.805)	0.141 (0.708)
NOR	Square root time investigating novel object (%)	1.444 (0.290)	3.266 (0.076)	0.103 (0.750)	0.181 (0.673)	3.419 (0.070)	7.042 **(0.010)**	2.305 (0.135)
3C	Ranked time investigating social stimulus (s)	3.283 (0.896)	0.001 (0.981)	0.137 (0.714)	0.069 (0.795)	0.467 (0.499)	0.053 (0.820)	1.381 (0.247)

KEY: bolded entries represent significant p-values ($p < 0.05$).

2.3.4.1 Marble Burying

The results of the F-test failed to find a significant interaction amongst the three independent variables [F (1,54) = 2.622, p = 0.11]. Likewise, no significant effect was found for the AP or SC main effects. In addition to this lack of significance, no main effect for SC was found, evident from the lower number of marbles buried in nSD sample groups compared to SD in the females and VPA males, but not C males where SD subjects buried less marbles than their nSD counterparts [F (1,54) = 1.901, p = 0.174]. Similarly, no main effect for AP was found as neither the VPA nor C groups performed similarly in males and females. Instead, C females buried more marbles than their VPA counterparts, whereas the opposite was observed in males. These results suggest a two-way interaction between AP and S, however this effect was not significant [F (1,54) = 3.131, p = 0.082]. Likewise, no significant two-way interactions were found between AP and SC [F (1,54) = 0.362, p = 0.550] and SC and S [F (1,54) = 0.010, p = 0.922].

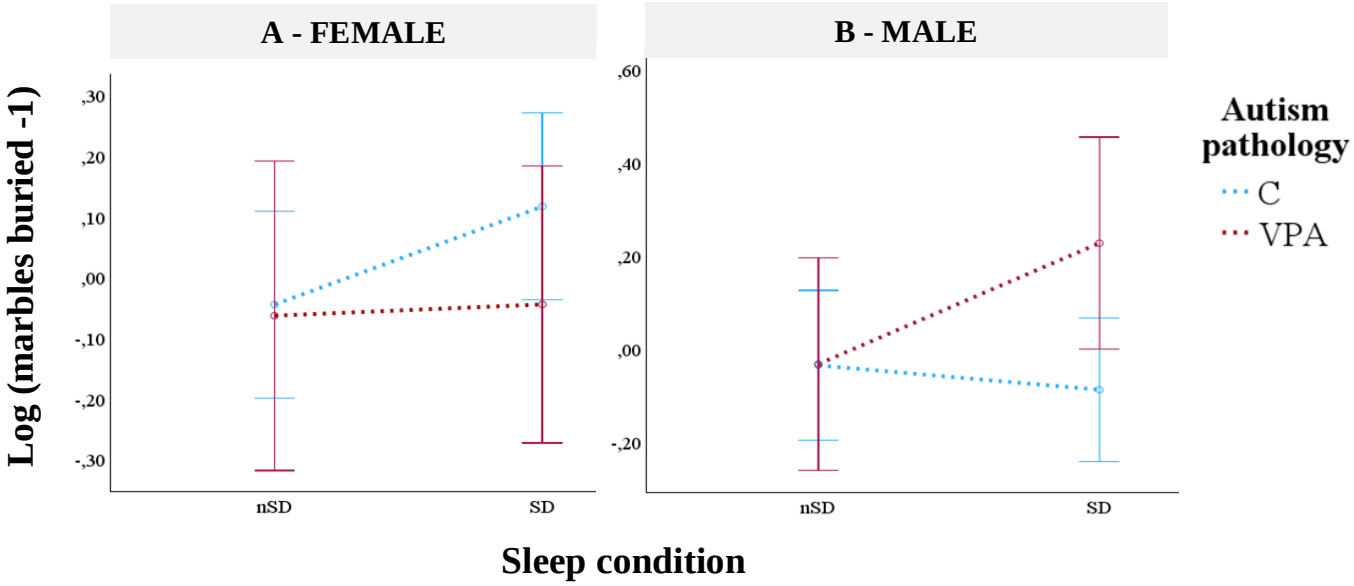


Figure 2.16: Interaction between sleep condition and autism pathology on the log number of marbles buried in female (A) and male (B) subjects obtained from the marble burying behavioural test. Error bars shown are according to a 95% confidence interval (CI); p>0.05 for the main effects of autism pathology (AP), sleep condition (SC) and sex (S), the two-way interactions between autism pathology and sleep condition (AP*SC) and sex (AP*S), sleep condition and sex (SC*S) as well as the three-way interaction amongst all independent variables (AP*SC*S).

2.3.4.2 Balance Beam

The results of the F-test failed to find a significant interaction amongst the three independent variables [F (1,54) = 2.492, p = 0.120]. However, a significant main effect for autism pathology was observed [F (1,54) = 12.666, p < 0.001] evident from the higher number of foot slips in VPA compared to C subjects observed regardless of sex. Conversely, no main effect was present for sleep condition [F (1,54) = 1.761, p = 0.190]. Instead, the effect of sleep condition on the number of foot slips was found to be mediated by sex, evidenced by the significant two-way interaction (SC*S) between the two [F (1,54) = 5.048, p = 0.029]. Specifically, in that male SD subjects performed significantly worse on the balance beam compared to their nSD counterparts. For the females, the differences in the performances were less pronounced, with almost no effect observed in the C group and nSD subjects performing worse than their SD counterparts in the VPA group.

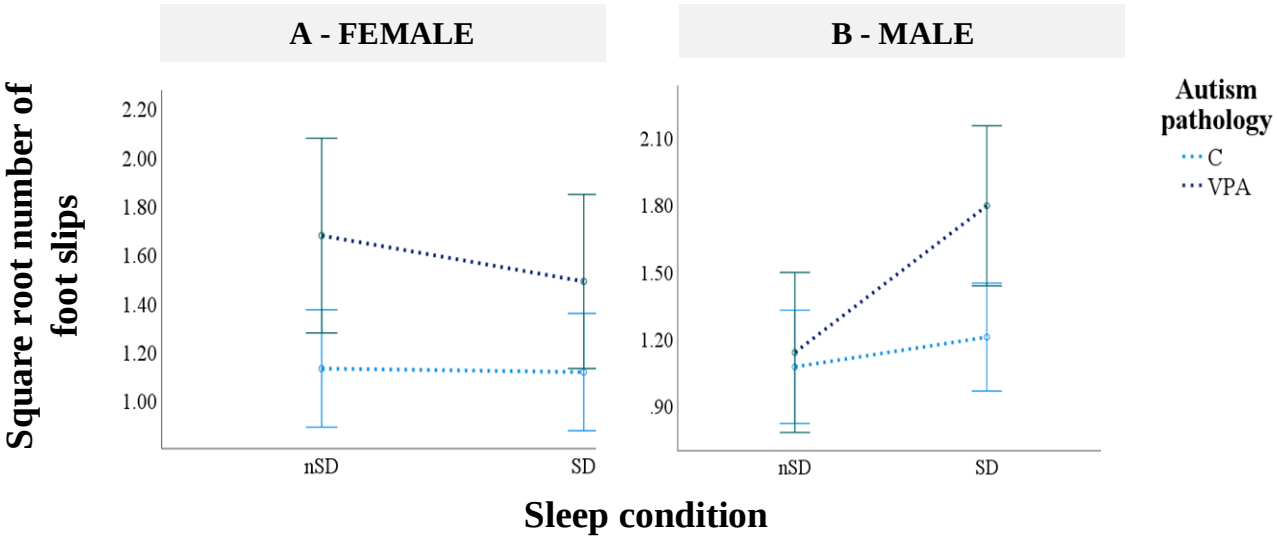


Figure 2.17: Interaction between sleep condition and autism pathology on the square root number of foot slips in female (A) and male (B) subjects obtained from the balance beam behavioural test. Error bars shown are according to a 95% confidence interval (CI); p< 0.05 for the autism pathology main effect (AP) and the two-way interaction between sleep condition and sex (SC*S); p > 0.05 for the main effects of sleep condition (SC) and sex (S), the two-way interactions between autism pathology and sleep condition (AP*SC) and sex (AP*S) as well as the three-way interaction amongst all independent variables (AP*SC*S).

2.3.4.3 Y-Maze

The results of the F-test failed to find a significant interaction amongst the three independent variables [F (1,54) = 0.011, p = 0.917]. A main effect for AP was found, evident from the higher scores obtained by C subjects when compared to VPA for both males and females, however, this main effect was not significant [F (1,54) = 0.607, p = 0.440]. Conversely, no main effect was found for SC in that nSD and SD sample groups did not perform consistently in males and females. Instead, nSD males had lower scores than SD subjects and no clear effect was found in females as C-SD subjects had lower scores than C-nSD and SD appeared to have no effect in VPA subjects, evident from the fairly equal scores across groups. These results suggest an interaction between SC and S, however this interaction was not significant [F (1,54) = 0.403, p = 0.529].

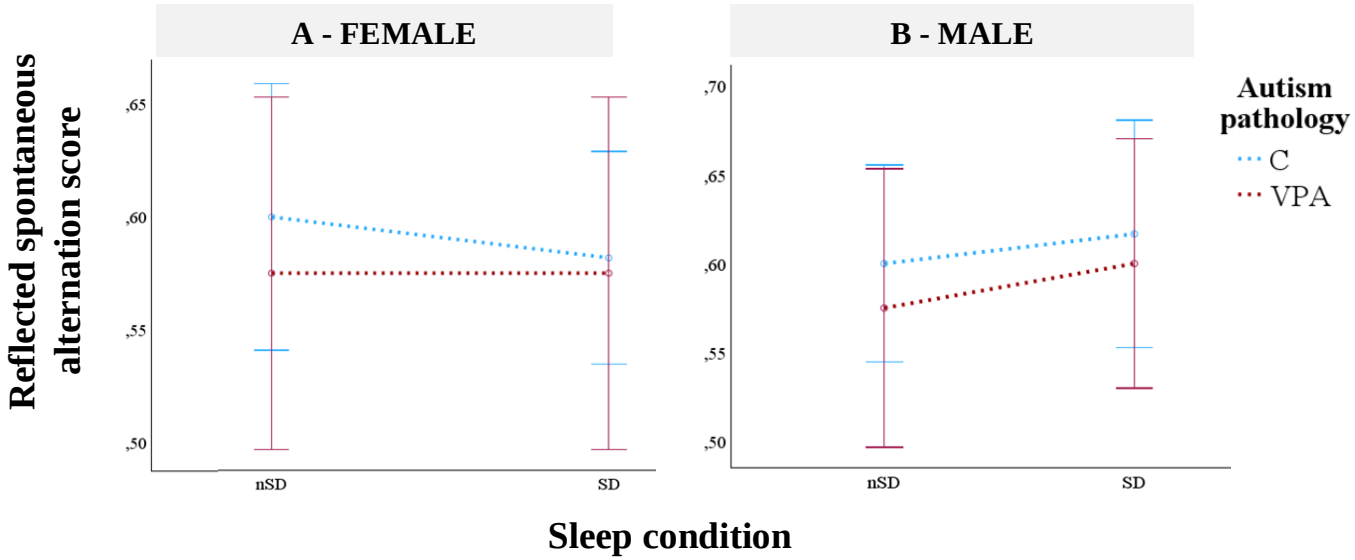


Figure 2.18: Interaction between sleep condition and autism pathology on the reflected spontaneous alternation score in female (A) and male (B) subjects obtained from the balance beam behavioural test. Error bars shown are according to a 95% confidence interval (CI); p>0.05 for the main effects of autism pathology (AP), sleep condition (SC) and sex (S), the two-way interactions between autism pathology and sleep condition (AP*SC) and sex (AP*S), sleep condition and sex (SC*S) as well as the three-way interaction amongst all independent variables (AP*SC*S).

2.3.4.4 Novel Object Recognition

The results of the F-test failed to show evidence a significant interaction amongst the three independent variables [F (1,54) = 1.444, p = 0.290]. A main significant main effect was observed for SC [F (1,54) = 7.042, p = 0.010] indicating that for both males and females, the time spent investigating the novel object differed significantly between nSD and SD subjects. Specifically, in that nSD subjects spent less time investigating the novel object compared to the SD counterparts. However, the manner in which AP affected this finding seemed to differ between the sexes despite no significance being found for its interaction with SC [F (1,54) = 3.266, p = 0.076] or S [F (1,54) = 0.103, p = 0.750]. For the females, no consistent effect for AP could be observed as C- nSD subjects performed worse than their SD counterparts, whereas the opposite was observed for the VPA subjects. However, for the males, nSD subjects performed consistently worse than their SD counterparts in both C and VPA groups. This indicates an absence of a main effect for both AP [F (1,54) = 3.419, p = 0.070] and S [F (1,54) = 2.305, p = 0.135], both for which no significance was found.

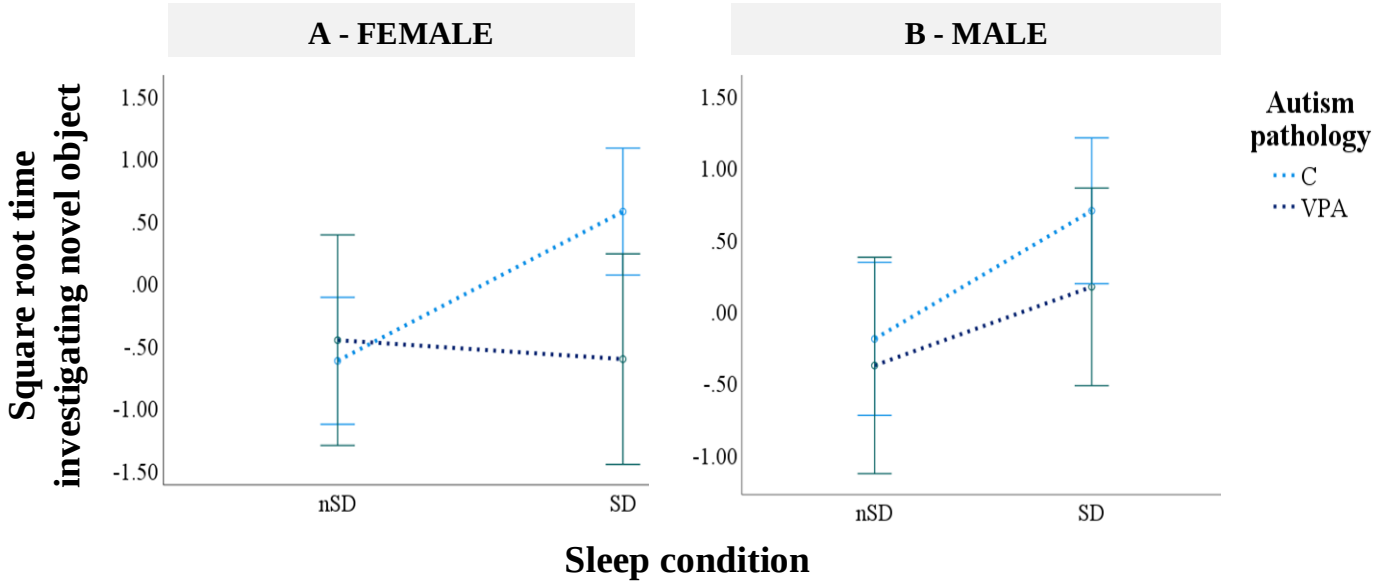


Figure 2.19: Interaction between sleep condition and autism pathology on time spent at the novel object in female (A) and male (B) subjects obtained from the novel object recognition behavioural test. Error bars shown are according to a 95% confidence interval (CI); $p < 0.05$ for the sleep condition main effect (SC); $p > 0.05$ for the main effects of autism pathology (AP) and sex (S), the two-way interactions between autism pathology and sleep condition (AP*SC) and sex (AP*S), sleep condition and sex (SC*S) as well as the three-way interaction amongst all independent variables (AP*SC*S).

2.3.4.5 Three-Chambered Sociability

The results of the F-test failed to show evidence for a significant interaction amongst the three independent variables [F (1,54) = 3.283, p = 0.896]. A main effect for AP can be observed, evidenced by the longer times C subjects spent investigating the social stimulus compared to VPA subjects regardless of sex. However, it was not significant [F (1,54) = 0.467, p = 0.433]. Conversely, no main effect was observed for SC in that neither nSD nor SD subjects performed in a consistent manner for both sexes. The SC main effect was also not significant [F (1,54) = 0.053, p = 0.820]. Instead, nSD spent less time at the social stimulus than SD subjects for the males whereas no clear effect could be observed for the females. That is due to the greater amount of time spent at the social stimulus for C-nSD subjects than their SD counterparts and seemingly no difference across both VPA groups. These results indicate that the effect of SC on the outcome was mediated by sex, despite no significant effect found for their interaction [F (1,54) = 0.069, p = 0.795]. Further, no significant interactions were found between AP and SC [F (1,54) = 0.001, p = 0.981] and S [F (1,54) = 0.137; p = 0.714] respectively.

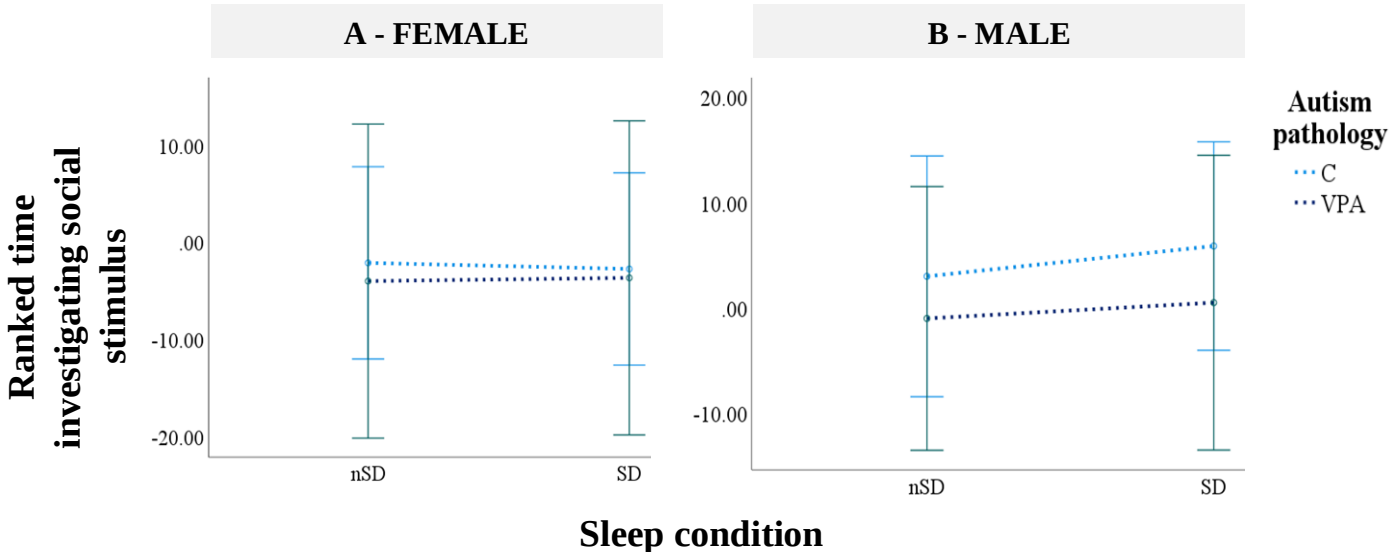


Figure 2.20: Interaction between sleep condition and autism pathology on time investigating the social stimulus in female (A) and male (B) subjects obtained from the three-chambered sociability behavioural test. Error bars shown are according to a 95% confidence interval (CI); p>0.05 for the main effects of autism pathology (AP), sleep condition (SC) and sex (S), the two-way interactions between autism pathology and sleep condition (AP*SC) and sex (AP*S), sleep condition and sex (SC*S) as well as the three-way interaction amongst all independent variables (AP*SC*S).

2.4 Discussion

The present study was aimed at determining the impact of light-induced sleep disturbance on autistic behaviours. Sleep dysregulations, although not diagnostic behaviours associated with autism, are the most frequently reported co-occurring conditions (Fang *et al*., 2021; Lorsung *et al*., 2021). As such, in recent years, autism research has shifted to encompass sleep and its potential role in the disorder's aetiology. In so doing, the relationship between sleep and autism pathology has been called into question, and still remains largely unclear (Burman *et al*., 2023; Maurer *et al*., 2023). Some authors have suggested a bidirectional relationship between the two, whereby existing autism pathology may cause sleep dysregulations and, in turn, a worsening of existing or appearance of additional deficits (Richdale *et al*., 2009; Souders *et al*., 2009; Reynolds & Malow, 2011). Still, there exists a vast account in literature of the autistic phenotypes in VPA rodent models as well as an increasing account of the nature of sleep in autistic individuals. Taken together, this extant literature will be discussed here in the context of each of the behaviours assessed in attempts to better understand this relationship.

2.4.1 Explanatory and predictive value of autism pathology, sleep condition, and sex

MLRs were run to determine the explanatory and predictive value of the three independent variables across the behaviours assessed. The usefulness of predictive models such as the MLR in studies of human behaviour (and by extension, animal models of human behaviour) has been called into question due to the frequency of these studies reporting R^2 values less than 0.50 (Gulati, 2024). This has led to these findings being considered unreliable due to the relatively high predictive capacity of empirical studies (Peterson, 2023). However, behaviour is influenced by a wide range of variables which cannot wholly be accounted for during experimentation and, thus, during statistical testing (Peterson, 2023). This may limit the predictive capacity of the models themselves, however, considerations beyond the R^2 alone have been shown to be useful in assessing goodness of fit in these models in order to infer a more informed understanding of the variables being used to explain and predict behavioural outcomes.

In terms of their explanatory nature, semi-partial correlations were used to determine the proportion of the model explained by each variable. The results showed statistical significance for the contributions of autism pathology in the square root number of foot slips (obtained from the BB test) ($p = 0.001$; Table 2.7) and both AP ($p = 0.024$; Table 2.7) and SC

($p < 0.001$; Table 4.6) in the time investigating the novel object (obtained from the NOR test). No statistically significant contributions by sex were found for any of the tests (Table 2.7). In terms of the predictive value of each test's model, statistical significance was found for BB and NOR (Table 2.8). Notably, all of the test's R^2 values were relatively low (less than 1% predictive value for MB, YM and 3C) including those where statistical significance was found (greater than 10% predictive value for BB and NOR) (Table 2.9).

It is currently understood that studies of human behaviour (and by extension, animal models of human behaviour) tend to have R^2 values less than 50% due to the limited capacity to predict outcomes of this nature (Gulati, 2024).

2.4.2 Repetitive behaviours

Restricted, repetitive patterns of behaviours, interests, or activities (RRBIs) are a core component in the presentation of autism and comprise one of the diagnostic criteria of the disorder (Edwards *et al*., 2024). According to the DSM-IV-TR, diagnostic criteria may be classified in terms of four narrow domains (APA, 2013; Edwards *et al*., 2024).

The current study focussed on the domain related to stereotyped or repetitive motor movements and/or in the use of objects or speech (APA, 2013). In autistic humans these may include, for example, lining up toys, echolalia, or the consistent use of idiosyncratic phrases (Edwards *et al*., 2024). In the present study, behaviour in this domain was assessed from the number of marbles buried, obtained from the marble burying test. The results failed to show evidence for a significant three-way interaction among the independent variables [$F(1,54) = 2.622$, $p = 0.11$], as well as any significant main effects for these variables separately or their two-way interactions (Table 2.10). Evidently, the lack of main effects revealed a sex-dependent role for autism pathology and sleep condition in the number of marbles buried across groups (Figure 2.16).

For the males, VPA subjects buried more marbles than their control counterparts, irrespective of sleep condition (Figure 2.16B), consistent with a wealth of literature attributing similar RRBI deficits in this domain in both humans (Kumar *et al*., 2022) and animal models (Baronio *et al*., 2015; Gonzales *et al*., 2016; Wu *et al*., 2017; Eissa *et al*., 2018; Wang *et al*., 2018). Further, both VPA-nSD and C-nSD subjects performed similarly, indicating that in the absence of sleep disturbance, autism pathology had minimal effect on the outcome. However, the effect of sleep disturbance differed between VPA and control subjects in that it worsened performance in VPA subjects (VPA-SD buried more marbles than VPA- nSD) and improved

it in controls (C-SD buried fewer marbles than C-nSD) (Figure 2.16B), highlighting a role for sleep in the regulation of this behavioural domain with regards to autism.

Evidence for the role of sleep in this regard relates to the neurotransmitter systems underlying the regulation of sleep/wake cycles (Lorsung *et al*., 2021). Specifically, multiple lines of evidence have implicated the serotonergic system as a potential factor underlying dysfunction in autism in general and as it relates to RRBIs (Soorya *et al*., 2008; Lorsung *et al*., 2021). Serotonin (5-hydroxytryptamine or 5-HT) is a neurotransmitter produced in the CNS and duodenum of the large intestine, comprising the functionally distinct central and peripheral serotonergic systems respectively (Lorsung *et al*., 2021). With regards to the peripheral system, an early study found increased serum levels (hyperserotonemia) in autism-diagnosed children (Schain & Freedman, 1961). Since then, several studies have confirmed hyperserotonemia as a biological deficit commonly presenting in the disorder (Melke *et al*., 2008; Tordjman *et al*., 2013; Janušonis, 2014), with an animal study correlating this finding to autistic-like behaviour in rats (Kahne *et al*., 2002).

Centrally, 5-HT synthesis occurs in serotonergic neurons primarily located in the raphe nuclei of the brainstem (Janušonis, 2014). This process occurs in a stepwise manner from two enzymes, namely tryptophan hydroxylase (TH) and aromatic amino acid decarboxylase (AAAD) and is said to play an integral role in the regulation and development of neural systems in the brain (Lorsung *et al*., 2021). This role is evident in the varying levels of its synthesis during various developmental stages, particularly late development, where elevated levels have been correlated with severity in the autism behaviours observed (Chugani *et al*., 1997). Further, the disruption of this synthesis likely manifests in deficits in behavioural features typical to autism (Lorsung *et al*., 2023). The manner in which these disruptions have been proposed to occur include but are not limited to (1) abnormal placental TH concentrations required for the perinatal development of the forebrain (Bonnin *et al*., 2011), (2) abnormality of 5-HT neuronal development, characterised by a caudal shift of the 5-HT-positive neuronal population in the dorsal raphe nucleus (Miyazaki *et al*., 2005), as well as (3) the mutual effects of abnormal postnatal synthesis and circadian disturbance (Takumi *et al*., 2020).

Anatomical abnormalities such as those observed by Miyazaki and colleagues (2005) have implications for the functional capacity of these serotonergic neurons which project to a number of brain regions and interact with a number of other neurotransmitter systems to influence a wide range of behaviours (Soorya *et al*., 2008; Lorsung *et al*., 2021). Further, the

diurnal pattern exhibited by 5-HT in both central and peripheral systems implicate the neurotransmitter in circadian function, by which sleep/wake rhythms are partly regulated (Lorsung *et al*., 2021). As such, disruptions in the functioning of the serotonergic system, by either its synthesis or circadian dysfunction, likely results in aberrant sleep functioning, for which 5-HT has a role in regulating, by promoting REM sleep (Lorsung *et al*., 2021).

These previously described serotonergic mechanisms may therefore also account for the effect that sleep disturbance played in the worsened performance of both VPA and control participants in the female groups (Figure 2.16). Further, similarly to the males, both VPA-nSD and C-nSD female subjects performed similarly, indicating that in the absence of sleep disturbance, autism pathology had minimal effect on the outcome. Additionally, control subjects performed worse than VPA, burying more marbles regardless of sleep condition, indicating a main effect for autism pathology, however, this effect was not significant [$F(1,54) = 0.242$, $p = 0.625$]. This finding is consistent with previous studies which fail to show evidence for the presence of motor stereotypies in females. In these studies, males are reported to show increased pre-occupations with object parts (Nicholas *et al*., 2008; Antezana *et al*., 2019), non-functional object arranging and manipulations (Hiller *et al*., 2014; Harrop *et al*., 2015) as well as repetitive finger, hand and arm mannerisms (Nicholas *et al*., 2008; Sipes *et al*., 2011; Antezana *et al*., 2019).

These results may be reflective of the representation of only one narrow domain throughout literature in which males are known to be deficient and primarily related to repetitive motor movement or in the use of objects or speech. As such, these results fail to account for the other domains in which female dysfunction may present (Edwards *et al*., 2024). Evidence for a Female Autism Phenotype (FAP) in RRBIs show more mixed results when all narrow domains are considered.

For instance, Antezana and colleagues (2019) used the RBS-R, which utilises a set of 43 questions related to five scales of RRBIs encompassing all its domains (Bodfish *et al*., 1999), to characterise the sex-specific nature of dysfunction in autistic adolescents. The authors found significant sex differences for 12 of the questions, out of which eight had higher scores for the females. These eight spanned four out of the five scales including: (1) self-injurious behaviour such as hair or skin pulling and self-rubbing or scratching, (2) compulsive behaviour in self-care and grooming habits, washing and cleaning as well as hoarding or saving, (3) ritualistic tendencies and the need for sameness in the insistence on sitting in one place or

adverse reactions to the changing behaviour or appearance of others and (4) restricted interests in showing strong attachments to a specific object. Comparatively, the four for which males scored higher spanned only two subscales including (1) stereotypic or repetitive behaviour in the use of objects and body movement of the hands and fingers as well as (2) restricted interests in the preoccupation or fascination with object parts and their movements.

Evidently, female RRBI deficits span a wider range of narrow domains than males. Further, the only common subscale for which deficits were present in both sexes was restricted interests. As such, it may be used to further support the FAP theory and illustrate how diagnostic criteria may be biased towards the male presentation of the disorder. For instance, despite being more inclusive of the various domains of RRBIs, it is known that the examples provided to help assessors best assign scores are focused more on typical interests of affected males such as dinosaurs, computers and weather, increasing the likelihood that these behaviours are not considered in affected females presenting with different interests (Antezana *et al*., 2019). As such, females are overlooked not due to the lack of intensity of observed behaviours but, rather, the nature of the behaviours themselves (Hiller *et al*., 2014; Antezana *et al*., 2019).

2.4.3 Social impairments

Broadly, social behaviour can be defined as any communication or interaction between two conspecifics of a given species (Jabarin *et al*., 2022), and in rodent models of autism, is most commonly assessed using the three-chamber (3C) behavioural test, as was utilised in the present study. This test is favoured for its simple methodological design and in the present study, sociability is represented by the increased time spent interacting with a social stimulus as opposed to a non-social one (Jabarin *et al*., 2022).

The results suggested the presence of a main effect for autism pathology; however, it was not significant [$F(1,54) = 0.467$, $p = 0.433$]. Nonetheless, the trend for VPA subjects being less sociable than their non-treated counterparts is in accordance with many studies reporting VPA rodents having a tendency for interacting with non-social stimuli in the presence of social stimulus. Thus, the lack of significance may be attributed to insufficient power due to the small sample size. The sample size for this test was decreased as a result of the exclusion of some individuals for their use as social stimuli during testing (n=54 for 3C as opposed to total n=62; Table 2.2). Hence, the recommended minimum sample size to ensure the robustness of the results of ANOVA was somewhat compromised. However, the decreased sociability of VPA

subjects has been significantly evidenced by studies utilising a range of sample sizes. Notably, only a relatively small number of studies used larger sample sizes (greater than 40 corresponding to one dependent and independent variable) (Kim *et al*., 2013b; De Theije *et al*., 2014; Cho *et al*., 2017; Campolongo *et al*., 2018; Hou *et al*., 2018; Melancia *et al*., 2018; Schiavi *et al*., 2019). Further, these studies were consistently able to detect significant differences in the sociability of VPA and untreated subjects. Conversely, studies with much smaller sample sizes presented with more varied results, also showing evidence for comparable levels of sociability between VPA treated and untreated groups (Baronio *et al.*, 2015; Dai *et al*., 2017; Eissa *et al.*, 2018; Wang *et al*., 2019). Thus, calling into question the reliability of smaller sample sizes in showing evidence for the widely cited autism-like phenotype of sociability in rodent models.

Additionally, the relatively small sample size used in the present study may also account for the lack of significance in the main effect observed for SC as well as the three two-way interactions (AP*SC; AP*S; SC*S). Despite this lack of significance, the effect that sleep condition had on the time spent investigating the social stimulus appeared to be influenced by sex, identifying a trend for the interaction of SC and S, even though no significance was found ($p = 0.795$; Table 2.15). For the males, nSD subjects performed worse than their SD counterparts in both C and VPA groups. Further, no consistent effect could be observed for the females, with C-SD subjects performing slightly worse than their C-nSD counterparts, whereas performance in VPA subjects appeared to be unaffected by sleep condition.

The results of studies utilising the Autism Treatment Evaluation Checklist (ATEC) have consistently evidenced the worsening of sociability measures in autistic children with moderate to severe sleep disturbances when compared to those with none (Cohen *et al*., 2014; Levin *et al*., 2022), consistent with the results of the present study where sociability was worsened in control females and contrary to what was found in the males (Figure 2.17). However, the discrepancy in the percentage of children included as exhibiting moderate to severe sleep problems, specifically 13% (Levin *et al*., 2022) and 40-80% (Cohen *et al*., 2014), show discrepancies in the outcomes observed. As such, the worsened performance of nSD male subjects (i.e. decreased time spent investigating the social stimulus) may be attributed to the varying extents to which SD affected each rat individually, resulting in the average decrease in time spent investigating the social stimulus contrary to what was expected. Still, these results (Cohen *et al*., 2014; Levin *et al*., 2022) suggest an association between sleep disturbance and

developmental trajectories in autism, particularly in the social domain, consistent with some of the results of the present study.

Further, the inconsistencies in the results obtained in the present study may be attributed to the unsuitability of the measure used to characterise sociability (Jabarin *et al*., 2022). Some researchers have asserted that the use of a single outcome variable reduces the complexity of social interactions which are better reflected by multiple variables more representative of the dynamic nature of the behaviour (Nester *et al*., 2017; Jabarin *et al*., 2022). These encompass a range of pro- (for example, mounting, play fighting, sniffing, etc) and antisocial (aggressive grooming, immobility, hiding, etc) behaviours which may better represent the nature of the rodents' social interactions (Chaliha *et al*., 2020). However, even in those studies which have included multiple variables as measures of sociability (Nester *et al*., 2017), inconsistency in the variables used and subsequent reporting of the measured variables across studies has made comparisons among them challenging (Chaliha *et al*., 2020). These inferential challenges are worsened by the lack of standardised protocols for the use of sociability tests in both human and animal studies (Chaliha *et al*., 2020; Jabarin *et al*., 2022).

Additionally, the use of multiple variables has implications beyond the accurate representation of a behavioural phenotype. With regards to the statistical analysis of these measured outcome variables, the issue of multiplicity has been raised (Hughes, 2005). Multiplicity in biostatistics has typically been raised with regards to clinical trials and primarily concerns the interpretation of a multitude of hypothesis tests related to a single outcome (Hughes, 2005). The basis of hypothesis testing describes the observed differences between groups, for each measured outcome variable, by generating a p-value that attributes some percentage of the occurrence of that outcome to chance. As such, complex behaviours such as sociability, which are suggested to be best represented by a multitude of measured outcome variables (Nester *et al*., 2017; Jabarin *et al*., 2022), may introduce the possibility of drawing inaccurate conclusions from the results of statistical analyses by increasing the role of chance in the observation of significant findings (i.e., increasing the likelihood of making a Type I error) (Proschan & Waclawiw, 2000).

Still, significant progress has been made in the understanding of sociability in rodents and consequently, their assessment using behavioural tests. With regards to the 3C test specifically, Nester and colleagues (2017) have enabled a path towards the development of protocols that include the set of behaviours nuanced to the test used to measure them. This was

based on their observations of mice behaviour under chambered sociability test conditions that revealed an initial exploratory phase during testing, driven by innate curiosity and not necessarily by the propensity or aversion for various stimuli (Nester *et al.*, 2017). This was followed by an increased tendency for interaction with stimuli, during which the propensity for sociability would be more accurately assessed (Nester *et al.*, 2017). For instance, employing precise measures of body posture could offer a more objective distinction between the currently measured pro- and antisocial behaviours (Nester *et al.*, 2017). Such consideration for the type of outcome variables used in the assessment of sociability coupled with multiplicity adjustments may prove to add value to behavioural neuroscience by improving the translational value in the use of animal models.

2.4.4 Cognitive rigidity

Cognitive flexibility, as defined by Dajani and Uddin (2015), is the ability of an individual to appropriately adjust their behaviour in response to a changing environment. The inability to do so effectively is one of the diagnostic criteria for autism (Chaliha *et al.*, 2020), manifesting behaviourally as deficits in cognitive functions such as working memory and attention (Dajani & Uddin, 2015). This cognitive rigidity, characteristic of the autistic behavioural phenotype, is frequently assessed in rodent models of the disorder using tasks involving spatial navigation and working memory (Jankínová, 2023).

2.4.4.1 Y-Maze

One such task is spontaneous alternation, based on the innate curiosity of rodents to explore previously unvisited areas (Lalonde, 2002; Kraeuter *et al.,* 2018), which was herein assessed using the Y-maze behavioural test.

The results of the present study failed to show significance for the main effect of autism pathology as modelled through gestational VPA exposure [$F(1,54) = 0.607$, $p = 0.440$]. Despite no significance being observed, the trends in the results were consistent with findings in multiple previous studies (Edalatmanesh *et al.*, 2013; Kumar & Sharma, 2016a, 2016b; Campolongo *et al.*, 2018; Cezar *et al.*, 2018; Mahmood *et al.*, 2018) showing a pattern of lower spontaneous alternation scores in VPA subjects compared to controls. As such, the trends in the current study are consistent with the cognitive rigidity characteristic of autism-like phenotypes in such animal models. Notably, a lot more studies have reported this trend in males than in females, representative of the disparity in the number of studies assessing autism-like

features in female animal models. The results of the present study are, therefore, interpreted with caution, acknowledging that the phenotype of autism presentation may be different in females, as suggested previously by Rivet and Matson (2011). Additionally, the disparity in the representation of female subjects in animal models of the disorder has limited test-specific comparisons in females.

Nevertheless, spatial learning and working memory assessments follow similar paradigms, despite differences in the actual tests utilised. For females, these behaviours are most frequently assessed using the Morris Water Maze test, which utilises reversal learning rather than spontaneous alternation to assess working memory and learning (Janíková, 2023; Murta *et al.*, 2023). Reversal learning tasks assess the ability to adapt behaviour based on changing rewards in the environment (Janíková, 2023). To do this, subjects must first learn to discriminate between various cues and associate their relevance to a reward (Janíková, 2023). During testing, subjects' intact learning abilities are evaluated based on their capability to adjust their associations with changing reward cues (Janíková, 2023). Using this test, a number of studies have identified a sex-dependent disparity in the performance of the subjects (Safari *et al.*, 2021; Ghahremani *et al.*, 2022). Specifically, VPA-treated females perform better than males in acquiring or learning the task during training (i.e. discriminating between cues) but perform similarly in their spatial retention when tested on subsequent days (i.e. altering association between cues when reward contingencies have changed) (Safari *et al.*, 2021; Ghahremani *et al.*, 2022). These results support the findings of the present study (Figure 2.18), suggesting that the effect of VPA treatment may be similar in males and females regarding working memory. However, the variations observed in the acquisition or learning process imply the necessity for further research to clarify the differences in learning mechanisms between the sexes.

The repetitive choosing of the same arm leading to these low spontaneous alternation scores (SAS) in VPA subjects indicates the presence of stereotyped behaviour (Stewart *et al.*, 2011; Murta *et al.*, 2023), implicating dysfunction in an additional diagnostic feature, namely repetitive behaviours. However, as the y-maze is not typically considered the appropriate test for this behaviour, interpretations of its results in this manner have raised criticisms in its operational paradigm (Stewart *et al.*, 2011) due to the potential for confusion and inconsistencies in reporting results across studies (Cash-Padgett *et al.*, 2016; Miedel *et al.*, 2017). Furthermore, spontaneous alternation, although not a direct measure of cognitive

rigidity, represents the willingness for exploration which may aid in identifying rigid and pervasive tendencies indicative of underlying dysfunction in cognitive flexibility (Jankínová, 2023). As such, its widespread adoption is justified in lieu of its simple methodology (Cleal *et al*., 2021), minimising excessive animal handling and manipulation of test apparatus (Heredia-López *et al*., 2016).

In the present study, no main effect for sleep condition was observed, evidenced by the inconsistent performance between males and females in the SAS scores of SD and nSD groups (Figure 2.18). These results suggest an interaction between sleep condition and sex, in that the effect of sleep condition on the spontaneous alternation scores is sex dependent. Despite this, no significant three-way interaction was found [$F(1,54) = 0.011$, $p = 0.917$]. For the males, nSD groups had lower spontaneous alternation scores than their SD counterparts (Figure 2.18B). These results are contrary to what was expected, failing to show evidence for the worsening of pre-existing cognitive rigidity as a result of dysregulated sleep, an effect that has been hypothesised in literature. Notably, this effect is not well-established among autistic children in literature (Al Backer *et al*., 2018; McArthur *et al*., 2023). Further, as cognitive flexibility is a complex, higher order skill related mainly to executive functions (Jankínová, 2023), the behaviours constituting deficits in this area are influenced by a wide range of cognitive functions such as planning, organizing, working memory, attention and inhibition (Dajani & Uddin, 2015). Additionally, literature presents evidence for heterogeneity in the presentation of these behaviours, however it is unclear whether this is due to the varied phenotype of sleep disturbance in autism individuals (similarly to the presentation of autism symptoms themselves) or due to the heterogeneity of methodologies used to test for cognitive function (Al Backer *et al*., 2018; Lage *et al*., 2023).

The heterogeneity in the presentation of behaviours related to cognitive flexibility as a result of SD are mimicked in the present study with regards to sex. Unlike the males, where a clear effect for SD was observed, no such effect was found for the females (Figure 2.18A). Female VPA subjects were seemingly unaffected by SD, evident from the similar spontaneous alternation scores between nSD and SD groups. In contrast, female control subjects exhibited signs of decreased cognitive flexibility after SD, as SD subjects had lower scores compared to their nSD counterparts. This effect observed in the C subjects is in accordance with the observations in literature and has been tested in human studies assessing the daytime functioning of so called “poor-sleeping” typically developing children as determined mainly

by actigraphy data and parental questionnaires (Al Backer *et al*., 2018; Deliens & Peigneux, 2019; McArthur *et al.*, 2019). The results of these studies show that in the absence of existing autism pathology, poor sleep can induce autism-like behaviours in this domain (Al Backer *et al*., 2018; Deliens & Peigneux, 2019; McArthur *et al.*, 2019). Further, these findings are not completely contrary to the results of the present study which fail to show the worsening of SASs following SD but suggest the persistence of autism-like behaviours in the presence of existing pathology (Figure 2.18). The worsening of cognitive rigidity may thus be limited to other areas of executive function as previously suggested (Al Backer *et al*., 2018). This is somewhat in accordance with the studies of poor sleeping autistic children where daytime functioning is intact in some respects, for instance, the ability to maintain attention (Al Backer *et al*., 2018), but not others. In these studies, poor sleeping habits in autistic children were characterised as consisting mainly of sleep onset and maintenance insomnia (Goldstein *et al*., 2001), possibly suggesting that the type of sleep disturbance may influence the resulting deficits in cognitive flexibility.

Neuroanatomical studies have helped identify brain areas involved in cognitive functioning, such as the fronto-striatal regions of the prefrontal cortex (Al Backer *et al*., 2018; Jankínová, 2023). These studies have shown evidence for similar deficits between males and females in this region such as decreased cell numbers (Hara *et al*., 2012; Kataoka *et al*., 2013). As such, Jeon and colleagues (2018) suggest that the impact of autism-inducing stimuli (for example, VPA exposure) is generalised across both sexes in their ability to produce similar cellular dysfunction. This suggests that observed sex differences are, instead, due to the mechanisms underlying neural differentiation during development (Jeon *et al*., 2018) which have been found to differ between the two (Kim *et al*., 2013a; Cho *et al*., 2017). For instance, the prefrontal cortical areas of VPA-treated male rats have been found to have deficits in the expression of methyl CpG binding protein 2 (Kim *et al*., 2013a; Cho *et al*., 2017), which is an X-linked transcriptional regulator strongly implicated in the development of another ASD, namely, Rett Syndrome (Neul, 2012). However, it has been linked to autism pathogenesis, specifically in males, due to its role in regulating the expression of various other neurotransmitters which aid in maintaining the brain's excitatory-inhibitory balance (Jeon *et al*., 2018). These and other lines of evidence which hinge on the absence of similar mechanisms in females when compared to males, reinforce the female protective effect (FPE) theory which may be relevant for X-linked dysfunction but still fail to provide an account for observed autism

pathology in females, highlighting the need for research efforts more targeted towards this cause.

2.4.4.2 Novel Object Recognition

The novel object recognition test is utilised to assess learning and memory and has been adapted for use in numerous species including humans, non-human primates and, most commonly, rodents (Lueptow, 2017; Haratizadeh *et al*., 2021). The NOR test assesses whether deficits in sociability, related to the preference for novelty, can be extended to the non-social domain (Chaliha *et al*., 2020). Further, the versatility in the use of this test lies not only in its application across a wide range of species but also in its ability to be modified to examine different types of memory (for instance, spatial) as well as the effects of various retention intervals related to short and long-term memory (Lueptow, 2017). In the present study, short-term memory and visual learning were assessed from the time spent investigating a novel object.

Deficits in working memory were initially considered to be limited to planning and problem-solving tasks, as a result of executive function deficits related to auditory and visuospatial information processing (Williams *et al*., 2005). Much of the literature assessing working memory in humans has been largely limited to include those with high-functioning presentations of the disorder, so as to minimise the confounding effects of intellectual and linguistic disability (Williams *et al*., 2005; Boucher *et al*., 2012). This was according to the assumption that verbal and visuospatial memory were inherently associated (McCann *et al*., 2018). These studies have predominantly shown evidence supporting the integrity or intactness of verbal working memory across various tasks. These, and other evidence in support for the dissociation of verbal and visuospatial working memory aided in the possibility for distinct neurobiological processes underlying their functioning and shifting of the research focus to encompass more visuospatial-based tasks (Williams *et al*., 2005). As a result, a range of behavioural tests have been utilised with mixed results relating to the deficits of various aspects of visuospatial processing and memory in autistic children compared to neurotypical children (Wang *et al*., 2017). Cardillo and colleagues (2020) have suggested that this is likely due to the varying demands of the tests used to assess the area of cognitive function. They further assert that the complete profile of cognitive impairment can only be represented fully using tests exercising a range of cognitive functions (Cardillo *et al*., 2020).

The results of the present study evidenced a trend for the worsened performance of VPA subjects when compared to controls (Figure 2.19). Although the main effect for autism pathology was not found to be significant [F (1,54) = 3.419, p = 0.070], it does suggest a reduced level of learning and memory regarding the familiar object presented during Trial one on the test day (Mathiasen & DiCamillo, 2010). This was evident in VPA subjects, who spent less time investigating the novel object. This result further indicates the need for sameness typical of autism-like phenotypes (Prior *et al.*, 1979; Kootz *et al*., 1982) and is contrary to the expected exploratory behaviour innate to rats (Barbosa & Silva, 2018). This is consistent with a number of studies with similar findings (Takuma *et al*., 2014; Faatehi *et al*., 2019; Shaaveisi *et al*., 2020). Further, similar autism-like deficits have been evidenced by studies which failed to find significant differences in the time spent investigating novel and familiar objects of VPA subjects (Campolongo *et al.*, 2018; Melancia *et al*., 2018) suggest a similar effect in the lack of recall for the familiar object.

Albeit fewer in number than those studies in support of the findings of the present study, evidence also exists for the increased exploration of the novel object for VPA subjects (Mychasiuk *et al*., 2012). In the study, the authors assessed active investigation according to the proximity of the rats' nose to the relevant object (Mychasiuk *et al*., 2012). As such, despite failing to evidence impairment of the VPA rats at this specific task, the authors found the rats had an increase in repetitive reaching attempts (unlike rearing) upon further discrimination of the nature of the rats' behaviour within the specified proximity (Mychasiuk *et al*., 2012). The repetitive reaching attempts were, therefore, attributed to dysfunction in the repetitive motor behaviour domain which is indicative of autism-like symptomatology. It is worth mentioning that the implications of multiplicity may extend to the inference of behaviours from tests not specific to the analysis of that behaviour. However, unlike sociability where the analysis of multiple variables is required to wholly represent the behaviour, repetitive behaviours constitute a number of motor stereotypes which when considered individually, may wholly provide merit for the inference of a repetitive action (Hughes, 2005). As such, increasing their reliability when inferred from other tests as was the case for Mychasiuk and colleagues (2012). Similarly to Mychasiuk and colleagues (2012), the present study found that in the absence of sleep disturbance, VPA- treated females spent more time investigating the novel object compared to their untreated counterparts. Despite failing to show any significant evidence for this occurrence [F (1,54) = 3.419, p = 0.070], these results may suggest the possibility for

behaviours representative of underlying autism-like dysfunction across a range of outcomes in the novel object recognition test.

It is suggested that this dysfunction may be attributed to central nervous system (CNS) developmental delays related to a number of processes including: (1) brain structures such as the prefrontal cortex and medial amygdala which have been implicated in the regulation of fear, (2) Purkinje cells in the cerebellum that play a role in the control of motor movements (Pierce & Courchesne, 2001) as well as (3) abnormalities in neural structures involved in regulating motivation which increases the propensity for exploration (Schneider & Przewłocki, 2005).

The role of the amygdala in the core behavioural deficits observed in autism (Bernier *et al*., 2005) have been explained, in part, by the Amygdala Theory of Autism (ATA). The amygdala is a collection of nuclei located bilaterally in the temporal lobes of the brain and the basis of the ATA is the observed abnormalities in the structure and functioning of these nuclei (Bernier *et al*., 2005). Structurally, anatomical abnormalities have been observed in the increased regional volume in children (Sparks *et al*, 2002; Schumann *et al*, 2004) as well as inconsistencies in the size and number of neurons in its various nuclei (Kemper and Bauman, 1998; Schumann and Amaral, 2006). Functionally, the amygdala was initially implicated in autism pathology for its role in moderating social behaviours (Bernier *et al*., 2005; Markram *et al*., 2008) due to early studies showing abnormalities in the ability of lesioned vervet monkeys to exhibit prosocial behaviours (Kling *et al*., 1970). The authors observed frequent withdrawals from group interactions as well as failure in the use of appropriate social cues to communicate with their conspecifics (Kling *et al*., 1970). These findings were evidenced in a number of other studies (Kling & Brothers, 1992). However, more recent studies have questioned its direct role in aberrant social functioning in autism (Amaral *et al*., 2003; Davis & Whalen, 2001), suggesting that the amygdala may instead impair other neuronal processes which, in turn, leads to dysfunction in various behavioural domains including sociability.

The mechanism by which this is thought to occur is through fear (Büchel & Dolan, 2000). Specifically, the processing of stimuli for the regulation of physiological fear responses and memories (Büchel & Dolan, 2000). These processes have been most frequently studied in autism through the use of fear conditioning paradigms which assess the synaptic plasticity of the amygdala (LeDoux, 2003). This plasticity is based on the ability of an inherently positive stimulus to produce a conditioned fear response following negative conditioning (Büchel &

Dolan, 2000) and then the ability to extinguish fear responses following positive conditioning (LeDoux, 2000; Rudy *et al*., 2004).

As such, abnormalities in the regulation of fear may occur with regards to the aforementioned processes in autism. Notably, unconditioned (inherently positive) stimuli are not the only mechanisms useful in assessing fear acquisition as this process is also influenced by perceived danger in an environment which may trigger similar unconditioned fear responses (LeDoux, 2000). As such, the worsened performance (i.e. decreased time spent investigating the novel object) of the VPA subjects across sample groups (with exception of the nSD females) (Figure 2.19) may be the result of heightened anxiety-like states due to the VPA treatment, and typical of some autistic individuals (Davis & Whalen, 2001; LeDoux, 2003). This increases their likelihood to perceive danger during behavioural testing and decreasing their propensity to explore the novel object, contrary to the inherent nature of most typically developing rodent species (Barbosa & Silva, 2018). This was the expected result for the VPA-treated subjects, potentially representative of their abnormal fear acquisition to the novel stimulus presented on test day. This suggestion for the altered perceptive ability in the VPA subjects is further supported by the testing procedures which (1) allowed frequent exposure to the apparatus and (2) enabled associative learning through the provision of food rewards following training and test days. The food rewards, in particular, are positive cues which, in typically developing subjects, are expected minimise or even alleviate fear responses. However, as the opposite was observed in VPA subjects, it is plausible that even these inherently positive cues could not extinguish the fear acquired from the exposure to the testing apparatus or testing itself. Further, identifying the potential for abnormal fear extinction.

Despite the opposite effect being observed in the nSD females (i.e. female control rats spending less time investigating the novel object than their VPA counterparts, opposite to the result for the male rats; Figure 2.19), similar impairments in perceptive ability (and thus fear acquisition) may be used to explain the results observed in the present study. It is thought that autistic individuals have impairments in perceptive ability that affect their ability to process environmental cues to inform decision making. However, more recently, studies have increasingly failed to show evidence for impairments when compared to typically developing individuals (Zeif & Yecheim, 2020). Perceptive ability has been most commonly assessed using the Iowa Gambling Task (IGT) which assesses impulsivity and the risk-taking approach based on responses to varying rewards.

Further, in male VPA and C subjects as well as female C subjects, SD increased the time spent exploring the novel object (Figure 2.19), indicating a main effect for SC that was also significant [$F(1,54) = 7.042$, $p = 0.010$]. Interestingly, these results are contrary to some studies that fail to show evidence for an association between performance in memory-related tasks and poor sleeping behaviour in autistic children (Boucher *et al.*, 2012; Maski *et al.*, 2015; McCann *et al.*, 2018; Desaunay *et al.*, 2020) as well as rodent models of autism comorbid with sleep disturbances (Scalise, 2023). Notably, the improvement in novel object recognition performance is unique to the present study as these studies do not show evidence for the improved working memory of autism individuals following sleep disturbance, but rather just the absence in the correlation between SD and worsened working memory performance. Further these results are said to support the notion that the deficits in working memory as a result of sleep disturbance in autism are restricted to the verbal domain (McCann *et al.*, 2018), calling into question the mechanisms through which sleep is understood to impact cognition. However, as verbal working memory was not assessed in the present study, it cannot be assumed that the results obtained nullify the role of sleep in the cognitive processes underlying visuospatial working memory.

Working memory deficits typical to autism are known related to dysfunctions in the pre-frontal and parietal cortices (Drummond *et al.*, 2000). However, the results of the present study suggest that this dysfunction may not be global, but rather localised to those areas responsible for verbal executive functions. Neuroimaging studies have provided evidence for the isolated activation of potentially dysregulated areas such as the left temporal lobe during verbal working memory tasks (Drummond *et al.*, 2000; Drummond *et al.*, 2001). However, activation in the right hemisphere with which spatial working memory is associated has failed to show similar dysregulation (Smith *et al.*, 1996). Unfortunately, many of these neuroimaging studies have focused on adults, with much of the dysregulation in autistic children unaccounted for. This highlights the need for studies more inclusive of a wider age range, especially due to the age-related changes in the neurological profiles and behavioural phenotypes associated with autism (Kouklari *et al.*, 2023) preventing the extrapolation of adult findings to younger individuals. Increased inclusivity in neuroimaging will provide clarity regarding the contrary evidence suggested by rodent animal models. Greater clarity may be enabled specifically as it relates to findings of studies utilising novel object recognition tests which suggest deficits in visuospatial working memory in VPA-treated subjects in general but fail to show evidence for the worsening of these outcomes as a result of sleep disturbance.

2.4.5 Motor co-ordination

Fundamental movement skills (FMSs) comprise the observable patterns of gross motor skills (Gandotra *et al*., 2020) including object control, locomotion and balance skills that form the basis for more advanced and fine motor skills (Gallahue *et al*., 2006). These FMSs first manifest as early childhood milestones said to emerge at various stages of development (Gandotra *et al*., 2020). For example, head lifting while prone at approximately two months, the ability to roll from back to belly at approximately five months and the maintenance of head position when sitting upright at approximately six months (Gandotra *et al*., 2020). Further, these FMSs develop in a fairly predictable manner until late childhood (Hardy *et al*., 2010), and have been considered a potential predictor of autism diagnosis at 24-26 months when the emergence of the disorders' diagnostic features become more apparent (Esposito *et al*., 2009; Karmel *et al.*, 2010; Landa *et al.*, 2013; LeBarton & Landa, 2019). However, the reliability of their use as early indicators of autism pathology has been criticised due to the shortcomings in literature in evidencing their widespread prevalence and specificity to autism (Ben-Sasson *et al*., 2009). This criticism is especially valid given that similar delays are commonly observed in other developmental disorders such as attention deficit hyperactivity disorder (ADHD) and Developmental Co-ordination Disorder (DCD) (Gandotra *et al*., 2020).

Despite this unclear specificity to autism, the frequently reported co-occurrence of motor impairments to the disorder have warranted research attention into their possible pathological relationship, specifically, as it relates to motor ability which was initially the most commonly used facet explored in human studies (Gandotra *et al*., 2020). Motor ability can be defined as the neuromuscular capacity underlying and predicting the performance of FMSs (Staples & Reid, 2010) and with regards to autism, has been evidenced to be altered in terms of postural instability, altered gait patterns and motor co-ordination (Fournier *et al*., 2010). In the present study, motor co-ordination and balance were assessed from the number of hind-paw slips while traversing a narrow beam.

The results obtained here showed evidence for the significant main effect for autism pathology [$F(1,54) = 12.666$, $p < 0.001$] in that VPA subjects had a higher number of foot slips when compared to C subjects (Figure 2.17), consistent with similar studies testing motor co-ordination on suspended beam or rotarod structures (Main & Khulesza, 2017; Al Sagheer *et al*., 2018). Further, evidence for the impairment of motor functioning in VPA subjects has been tested using rat developmental milestones such as eye opening and righting reflexes (time taken

to right after placed in a supine position) pre-weaning, found to be predictive of autism-like impairment of FMSs and sociability (Wagner *et al*., 2006; Al Sagheer *et al*., 2018). Additionally, grooming behaviour in rodents, considered to be a stereotypic motor behaviour, has been shown to be more frequent in autism rodent models (Gandal *et al*., 2010, Kaleuff *et al*., 2016; Castro *et al*., 2017).

Despite not being considered a core diagnostic feature of the disorder, motor impairments have been evidenced to occur more consistently alongside the disorder when compared to its core features in both human and animal studies (Gandotra *et al*., 2020). This has been attributed to the wide range of behaviours assessable in FMSs and the inability to infer the contribution of motor ability to the clinical presentation of the disorder. As such, brain regions implicated in autism pathology have been assessed to aid in establishing motor impairments as reliable predictors of autism pathology. The cerebellum is one such region which has been investigated due to its role in movement control and co-ordination as well as cognitive functions such as speech and language (Fatemi *et al*., 2012) and has been implicated in the worsening of social behaviours which are a hallmark for impairment in autistic individuals (Corchesne *et al*., 1994; Skefos *et al*., 2014; Al Sagheer *et al*., 2018).

Additionally, the present study failed to show evidence for a main effect for SC in the motor co-ordination of the rats [$F(1,54) = 1.761$, $p = 0.190$]. SC was instead mediated by sex, evidenced by the different results obtained between the sexes (Figure 2.17). For the males, SD was found to worsen motor co-oridnation across both VPA and C groups, evident from the higher number of hindpaw slips in these SD groups. Notably, the VPA-SD group was the worst performing, suggesting the worsening of existing autism-like pathology in this domain as a result of sleep disturbance (Figure 2.17B).

The worsening of motor function following sleep disturbance in autistic individuals is fairly well-documented in literature and has been attributed to the impact of sleep on memory consolidation (Kempler & Richmond, 2012), particularly, the consolidation-based enhancement and stabilisation effects of proper sleep (Walker, 2005). The former relates to the ability of an individual to show improvement in learned behaviour in the absence of further practice, whereas the latter describes the process by which the level of performance is maintained immediately after learning (Walker, 2005). Therefore, deficits in the motor performance of autistic individuals may be attributed to learning impairments caused by either (1) poor sleep resulting in poor enhancement of memory or (2) innate learning inefficiencies,

due to existing autism pathology that result in poor memory stablisation (Walker, 2005). In other words, autism pathology impairs the ability of an individual to effectively learn motor skills, thus accounting for the worsened performance of VPA groups when compared to controls in the present study. Further, these existing impairments are worsened in the presence of SD, further preventing the improvement of those learned behaviours as was observed in the present study with regards to the males. This is evidenced by the higher degree at which SD worsened outcomes in VPA subjects compared to C.

Notably, these effects are more commonly evidenced for fine motor skills (Philal & Born, 1997; Fischer *et al*., 2002; Walker *et al*., 2002), as it is suggested that sleep may have a greater influence on more complex skills, with less significant differences observable between autistic and typically developing individuals for more simple tasks (Kuriyama *et al*., 2012). There is less of an account on the effects of sleep on gross FMSs as well as more inconsistencies in the findings (Kempler & Richmond, 2012), highlighting the need for increased research attention into the effects of sleep on gross motor skill learning. This need is especially important because non-pharmacological interventions into the improvement of autism symptoms have been increasingly focused on motor behaviours for which there is potential for significant improvement following both sleep improvement and behavioural therapy (Brand *et al*., 2015; Lambert *et al*., 2016; Mohd Nordin *et al*., 2021).

Conversely, the females failed to show a consistent effect for the role of SD on motor co-ordination (Figure 2.17A). In the VPA groups, nSD females were observed to perform worse than their SD counterparts, contrary to what was expected. Further, SD appeared to have no effect in the motor co-ordination of female control subjects as both SD and nSD subjects performed similarly (Figure 2.17A).

2.4.6 Limitations

2.4.6.1 Sample size

18 female rats were mated over three separate rounds in order to facilitate a feasible workload for behavioural testing. Across all three rounds, VPA dams were observed to present with notably poorer outcomes related mainly to litter size (Appendix B) and pup wellbeing as monitored, in part, by weight as well as incidence of mortality. With regards to litter size, control dams were able to produce 43 pups in total from each of their four mated females in rounds one and three. Of these 43 pups, a fairly balanced distribution of 22 females and 21 males resulted, enabling a relatively even allocation to each of the four sample groups. Further,

only one mortality was documented post-weaning. This individual was considered to be the runt of the litter, observed to be severely emaciated compared to littermates. It is common for dams to abandon these individuals, resulting in poor feeding and, ultimately, death as was the case in the present study. It is also worth noting that this pup was from the first round of mated females who were initially housed in a room near ongoing construction, creating an unfavourable environment due to noise from construction tools and constant foot traffic as well as potentially compromised air quality from the dust. These adverse environmental conditions may have also contributed to the death of this individual, as no mortalities were observed for the control female mated in the third round. Further, despite being moved to more favourable conditions, all of the dams from the first round were notably stressed, as observed through their irritability and resistance to being handled during daily wellbeing checks.

Conversely, poorer outcomes were observed more often in VPA subjects. With regards to litter size, only 19 pups were obtained from three of the 14 mated females across all rounds. Further, the distribution of males and females were observed to be more unbalanced, with eight females and 11 males. These were distributed evenly across all sample groups, but with marked differences in the number allocated to each group compared to the control groups. Notably, much larger sample sizes were required for the study design which included a total of four sample groups with both males and females. However, as a result of these poor outcomes, mating could not be continued indefinitely in order to increase the sample. This was mainly due to time and monetary constraints. Specifically with regards to time, behavioural testing of all 3 rounds' pups was completed in June 2023, after which the study's additional objectives had to be completed. Further, the cost of housing each of the rats over this extended time period from birth to the end of testing was no longer feasible given the poor outcomes. These outcomes can most likely be attributed to the adverse effects of VPA administered gestationally evidenced extensively in literature. This evidence suggests that the degree of adversity in factors such as litter size are dose-dependent between 300 and 800 mg/kg, with doses exceeding 800 mg/kg resulting most commonly in maternal fatality (Komariah *et a*l., 2018).

2.4.6.2 Absence of circadian biomarkers

In the present study, sleep disturbance was inferred from altered behaviour during testing despite more direct measures existing to aid in more accurately inferring sleep disturbance from underlying circadian function (Lorsung *et al*., 2021). Although it was originally proposed to obtain biological samples such as urine, saliva or blood for the testing

of either melatonin or corticosterone, this was rejected for a number of ethical reasons. Blood samples are most commonly used to measure endocrine parameters, however, have been criticised due to their highly invasive nature (Touma *et al*., 2004) given that collection most commonly requires puncture of either the sublingual, facial or tail veins under light anaesthesia (Haarikrishnan *et al*., 2018). Further, in order to infer circadian dysfunction, it would be necessary to establish a diurnal pattern of either hormone to be tested. This would have required sampling at least twice in a 24-hour period, during light and dark phases respectively. Additionally, requiring sampling to commence pre-weaning in order to demonstrate rhythmicity disruption once kept under irregular L/D cycles. This was impractical as individuals were not housed individually pre-weaning. Further, during this time, both the mother and her pups are extremely sensitive to stress, and unless properly managed through ethical husbandry practices, may result in adverse outcomes such as pup abandonment or eating. As such, frequent sampling, in addition to the stressful nature of the sampling procedures, presented serious implications for the rats' welfare which would have confounded the results of both the circadian biomarkers and behavioural outcomes.

Further, inferring circadian dysfunction from metabolites of the hormones in either urine or saliva is less invasive, however still posed a number of logistical and ethical challenges. Firstly, the rats were housed in cages lined with aspen bedding to facilitate environmental enrichment. As such, urine collection would have proven to be difficult as it would have soaked the bedding prior to collection. Further, samples would have had to be collected within reasonable time intervals of one another in order to enable comparisons. However, as we could not predict when the rats would urinate, sampling was nearly impossible. Some researchers have suggested collection upon handling as many rat species involuntarily urinate when picked up (Touma *et al*., 2004). However, as there is no way to ensure urination each time, sampling would have been nearly impossible. Even if collection was successful, the use of urine samples has been unfavoured due to low sampling volumes (Touma *et al*., 2004) as well as the high incidence of contamination either from the contents of their cages or their fur. As such, increasing undue stress unto the rats from frequent handling for samples that would likely be unusable. Similar constraints are relevant to saliva sampling, specifically as it relates to handling, low sampling volumes and contamination.

2.4.6.3 Absence of objective sleep measures

In addition to circadian biomarkers, objective measures of sleep exist to describe and quantify sleep/wake cycles and the characteristics of their various stages (Hall & Hall, 2020). The most frequently used of these objective methods is polysomnography, which utilises electroencephalography (EEG) to distinguish various states of sleep (REM and non-REM) and wakefulness by identifying the unique physiological signatures exhibited during the various sleep states (Hunt *et al.,* 2022), measured as waves of electrical activity received telemetrically by electrodes which are implanted on an individual's scalp (Missig *et al.,* 2020). The electrophysiological data signals are then analysed and used to classify sleep states using scoring conventions (Zeng *et al*., 2012).

Due to its proven effectiveness in discriminating various sleep/wake states, EEG is considered the "gold standard" as a measure of sleep (Yaghouby *et al.,* 2016; Vanneau *et al.,* 2021) however, was not utilised in the present study for several reasons. Firstly, due to its invasive nature. Surgical implantation of the electrodes is a resource-intensive procedure (Goldman *et al.,* 2009; Yaghouby *et al.,* 2016) requiring a high level of skill and expertise for the surgery itself as well as the reading and analysis of resulting data (Bastianini *et al.,* 2017). Furthermore, animal welfare is often jeopardised by high stress levels resulting from surgery, reducing the survival chances of study subjects. This is especially the case in smaller mammals such as rats which were used in the present study (Bastianini *et al.,* 2017). For surviving individuals, post-operative recovery periods are often lengthy (Vanneau *et al.,* 2021) and require extensive care (Zeng *et al*., 2012), with no guarantee of a reduction in stress levels. These would have potentially altered behaviour (Zeng *et al*., 2012; Yaghouby *et al.,* 2016) and limit movement, thus affecting the reliability of the behaviours being observed (Yaghouby *et al.,* 2016).

These adverse effects on behaviour are worsened by the tethered restraints required for relaying recorded measurements. In a recent study, the effects of tethered restraints on rat activity and sleeping patterns were assessed by Aulehner and colleagues (2022). The authors used various parameters to assess the clinical state and sleeping behaviours of rats with tethered versus untethered EEG devices. Despite not revealing any statistically significant differences in nest building activity (a behavioural assessment often used to measure clinical state, most commonly affected by research-induced stress and pain during experimentation) and saccharin preference (the loss of which indicates anhedonia-associated behaviours in rats), their results

revealed an increase in individual variance of sweet-solution consumption, suggesting individual differences in the ability to cope to the experimental conditions and associated stress induced.

Thirdly and in addition to the impact of tethered devices, the interaction dynamics of pups with their mother and littermates were likely to be negatively impacted. The interaction dynamics between mothers and their pups, particularly during the lactation period have been shown to be necessary for the pups' environmental habituation.

Notably, the use of alternative techniques has been suggested to address the shortcomings in the use of EEGs in animal research (Zeng *et al.*, 2012). These techniques are based on the ability to discriminate sleep/wake states through body movements, muscle tone and physiological measures such as respiratory and heart rates (Zeng *et al.*, 2012; Hall & Hall, 2020) as well as blood pressure and blood oxygen levels (Šušmáková & Krakovská, 2008). The accuracy of which has been assessed in studies comparing the results of EEG measures conducted concurrently. These have yielded varying results, with actigraphy considered the most reliable.

Actigraphy is a sleep assessment method wherein an actigraph or wristwatch-like device is attached to an individuals' body to assess gross body activity to discriminate rest/activity cycles (Esaki *et al.*, 2021). Given that rats display distinct body movements (e.g. twitching etc.) during the various states of sleep and wake, the data obtained from actigraphy can be used as a proxy for sleep/wake states. This technique was also found to be unsuitable in the present study, largely due to the size of the pups at birth. Much like the EEGs, the actigraph would have needed to be attached to the pups shortly after birth to establish baseline readings and illustrate sleep disturbance following light manipulation. However, the attachment of the actigraph may have been perceived negatively by the mothers, potentially increasing the likelihood of abandonment and eating.

2.4.6.4 VPA rodent model

In addition to the challenges faced in conducting the experiments of the present study, further challenges were faced concerning the factors limiting the ability to make inferences of this study's findings based on comparisons to previous studies. These challenges are discussed here in terms of the variability in the use of the VPA model.

The VPA model is, perhaps, the most extensively studied environmental model of autism in rodents. However, variations in the methodologies used in its induction have been recognised as a factor limiting comparison across studies (Mabunga *et al*., 2015; Larner *et al*., 2021). Two sources of variation are considered here, the first relating to the gestational day of VPA administration. This is important as it informs the model's construct validity, relating to the similarities in the mechanisms underlying the autism-like phenotypes produced in animal models and those observed in diagnosed human individuals (Mabunga *et al*., 2015). Variations in the timing of administration affect the stage of neurodevelopment which is affected and potentially the mechanisms underlying autism-like dysfunction. For instance, VPA was administered on GD12 in the present study according to the GD12.5 commonly used in accordance with the original research establishing the model (Rodier *et al*., 1996; Schneider & Przewlocki, 2005). Administration at this time has been associated with abnormalities in neural tube closure, occurring around GD11-12 in rats (Schneider & Przewlocki, 2005). Further, Kataoka and colleagues (2013) show evidence for the importance of the timing of administration. In their study, the authors found that later exposure (GD14.5) decreased neuronal cell development in prefrontal and somatosensory cortices to a lesser extent than GD12.5. Further, showing that the most correlation to behavioural and neuronal phenotypes reminiscent of autism-like states was found when administered on GD12.5 instead of GD09 or GD14. Despite these findings, a number of studies have opted for earlier (Dufour-Rainfray *et al*., 2010; Kim *et al*., 2014b) or later (Gandal *et al*., 2010; Kang & Kim, 2015) with more mixed results.

The second source of variation lies in the varying dosages administered. There is currently no standardised dosage used across literature (Larner *et al*., 2021) with evidence for a wide range of dosages inducing varying levels of dysfunction in rats. For instance, a recent review found deficits in stereotypies and sociability typical of autism phenotypes could be observed from dosages ranging between 200 and 600 mg/kg, with some rats treated with dosages as low as 25-50 mg/kg showing increased self-grooming. However, consistent disruptive effects were observed more consistently at dosages between 400 and 600 mg/kg (Chaliha *et al*., 2020). What these findings indicate is two-fold: firstly, that the consistent presentation of autism-like behaviours is dosage dependent, with greater variability in outcomes observed at dosages less or greater than 400 and 600 mg/kg respectively. This finding is supported by earlier research into the VPA model showing compelling evidence for this dosage-dependent effect regarding various indicators of foetal development including, weight,

skeletal ossification and defects as well as cardiac anomalies (Binkerd *et al*., 1988). Furthermore, showing that comparisons across studies are strengthened when dosage is controlled for. Secondly, and perhaps more notably, is that the variability in the presentation of autism-like phenotypes is still observed even when dosage is standardised across studies (Chaliha *et al*., 2020), reflective of the spectral nature of the disorder. As such, enabling the disorder to be studied holistically, while still ensuring reliable comparisons across studies.

2.5 Conclusion

The aim of this study was to determine the effect of sleep disturbance on autism-like behaviours in both males and females. This relationship has been suggested to be bidirectional whereby existing autism pathology may cause sleep dysregulations and, in turn, a worsening of existing or appearance of additional deficits (Burman et al., 2023). However, this relationship has largely been assessed in males, with outcomes generalised to females. The present study utilised a three-way ANOVA to assess the impact of sex on the interaction between autism pathology and sleep condition. Despite failing to find significant differences in most of the results, the findings show patterns for the sex-specific effects of both autism pathology and sleep condition in the behavioural domains relating to autism such as RRBIs, sociability and cognitive flexibility. These findings support the unique female presentation of autism and highlight the need for their widened representation in autism research. Further, this shows the need for more inclusive methods of assessing behaviours in order to encompass diverse presentations.

Additionally, despite the wealth of literature existing on autism behaviours, the present study identified factors limiting comparisons amongst studies due to methodological heterogeneity, highlighting the need for standards in the assessment of autism-like behaviours in animal studies and in humans.

CHAPTER 3: Sleep effects on the lateral hypothalamic area

3.1 Literature Review

3.1.1 Sleep and the orexinergic system

Neurotransmitter systems have been implicated in sleep irregularities observed in autism (Burman *et al*., 2023). The basis for this is suggested to lie in common pathways underlying autism pathology and the regulation of sleep/wake cycles (Mazzone *et al*., 2018; Burman *et al*., 2023). The role of neurotransmitters in regulating sleep is well documented in literature, particularly as it relates to the serotonergic, cholinergic, and adrenergic systems (Saad *et al.,* 2022). However, less documented with regards to autism symptomatology is the orexinergic system.

Orexins (also known as hypocretins) are neuropeptides composed of two subtypes, namely Orexin A and B (alternatively Hypocretin 1 and 2) (Nambu *et al*., 1999). Further, orexins also bind two receptors, namely orexin 1 (OX1R) and orexin 2 (OX2R) receptors, which have varying affinities for binding each subtype (Vraka *et al*., 2023). Specifically, OX1R has a greater affinity for binding orexin A, while OX2R is comparatively non-selective (Sakurai *et al*., 1998; Zhang *et al*., 2013; Vraka *et al*., 2023). The cell bodies of orexin-producing neurons (orexinergic neurons) are localised within the lateral hypothalamic area (LHA) of the hypothalamus (Nambu *et al.,* 1999), while their receptors are expressed throughout the whole brain except for the cerebellum (Yamashita & Yamanaka, 2016). Despite the localisation of orexinergic neurons, their axonal projections are widespread across various regions of the brain and spinal cord (Nambu *et al.,* 1999; Peyron *et al.,* 1998; Sakurai *et al.,* 1998). The orexinergic system's widespread projection patterns thus explain the participation of orexinergic neurons in widespread functions across the brain (Saad *et al.,* 2022). These functions have been shown to include involvement in feeding, appetite regulation, arousal, reward seeking and the regulation of sleep/wake cycles (Chieffi *et al*., 2017). The present study focuses on the system's role in the regulation of sleep/wake cycles.

The role of orexins in the regulation of the sleep/wake cycle was first recognised in association with Type I narcolepsy, a neurological disorder characterised, amongst other symptoms, by extreme daytime sleepiness due to the selective loss of orexinergic neurons (Yamashita & Yamanaka, 2016; Chieffi *et al.,* 2017). Furthermore, an early study utilising knockout experiments showed that the absence of orexins alters sleep architecture, particularly

reducing the amount of REM sleep in the dark phase (Chemelli *et al*., 1999), findings which have also been supported by other studies (Hagan *et a*l., 1999; Bourgin *et al*., 2000).

Bourgin and colleagues (2000) have suggested that the potential mechanism by which orexins influence sleep involves the areas to which they project. Neuroanatomical studies of orexinergic fibre distribution have identified various brain regions receiving dense orexinergic innervation (Peyron *et al.,* 1998; Saper *et al.*, 2001) and containing high expressions of their receptors (Zhang *et al*., 2013). These include regions comprising monoamine systems (Zhang *et al*., 2013) such as the dopaminergic ventral tegmental area, noradrenergic locus coeruleus, serotonergic dorsal raphe and histaminergic tuberomammillary nucleus (Saper *et al.*, 2001; Zhang *et al*., 2013; Yamashita & Yamanaka, 2016). Orexins are, therefore, thought to regulate sleep/wake cycles through the activation of these monoaminergic neurons which promote arousal (Zhang *et al*., 2013; Yamashita & Yamanaka, 2016). Specifically, promoting arousal according to their firing patterns (Zhang *et al*., 2013). The activity of these neurons is increased during wake, decreased during non-REM sleep and inactive during REM sleep (Zhang *et al*., 2013). As such, orexins are neuroexcitatory in nature (de Lecea *et al*., 1998) and regulate sleep/wake cycles by promoting wakefulness and inhibiting REM sleep (Bourgin *et al*., 2000).

3.1.2 Orexinergic functioning in autism

Sleep disorders are the most frequently reported comorbidities in autistic individuals (Maurer *et al.,* 2023), the most common of these disorders related to sleep onset and maintenance insomnias (Cohen *et al.*, 2014; Kohyama, 2016). As such, it has been hypothesised that an alteration of the orexinergic system, which is responsible for maintaining consolidated periods of wakefulness (Al-Kuraishy *et al*., 2020), could be implicated in the pathogenesis of autistic symptoms (Piri *et al*., 2024). Several authors have suggested that this role of orexins in maintaining consolidated periods of wakefulness (Al-Kuraishy *et al*., 2020) is hyperactive in autistic individuals, thus excessively exciting sleep-inhibiting, and wake-promoting brain regions, thus inducing sleep disturbance (Prober *et al*., 2006; Kohyama, 2016; Tang *et al*., 2017).

It has been suggested that the hyperactive functioning of orexins in autistic individuals may be attributed to abnormal amygdala functioning (Kohyama, 2016; Marotta *et al*., 2020; Ji & Zhu 2023). Etkin and colleagues (2011) have suggested that the medial prefrontal cortex, which is implicated in autism dysfunction (Eilam-Stock *et al*., 2016; Long *et al*., 2016), is responsible for the downregulation of amygdala functioning. The interaction between the two

brain regions is known to play a crucial role in the regulation of emotions (Sakaki *et al*., 2016). The amygdala is one of the main sources of input to orexinergic neurons in the LHA (Sakurai *et al*., 2005), which additionally receives some innervation from neurons in the region (Schmitt *et al.*, 2012). As such, Kohyama (2016) suggests that the functioning of the orexinergic and reward systems is inherently interconnected. Similarly, Park and colleagues (2016) support the possibility that the amygdala is a key brain region responsible for the development of autistic traits. As such, the functioning of the amygdala may be a common mechanism underlying the core behavioural symptoms as well as the comorbid sleep disturbances in autism (Etkin *et al*., 2011; Mazzone *et al*., 2018; Burman *et al*., 2023).

Several studies have shown evidence for the role of the amygdala in inducing sleep disturbance through its connections with other brain regions. For instance, to confirm that increased amygdala activity promotes REM sleep (Ji & Zhu, 2023), Hasegawa and colleagues (2022) examined the extracellular dopamine levels of mice to determine the firing patterns of their neurons across the various sleep/wake transitions. The authors used a G protein–coupled receptor activation–based sensor which was expressed in multiple brain regions including the basolateral amygdala (Hasegawa *et al*., 2022). Following incremental increases in dopamine levels in the region, the mice exhibited faster transitions from non-REM to REM sleep. Furthermore, Wellman and colleagues (2022) illustrated how inhibited amygdala activation reduces REM sleep.

Additionally, orexinergic neurons project to brain regions releasing excitatory and inhibitory neurotransmitters, thus influencing their actions (Knez *et al.*, 2022). Particularly, gamma-aminobutyric acid (GABA) and glutamate are the brain's primary inhibitory and excitatory neurotransmitters respectively and have been implicated in dysfunction observed in multiple neurodevelopmental disorders including autism (Peerboom & Wieranga, 2021). Multiple authors have suggested that this dysfunction may be associated with defects in the so called "GABA shift" (Schulte et al., 2018; Peerboom & Wieranga, 2021) which describes the transition of GABAergic signalling from excitatory during embryological development to inhibitory postnatally (Herlenius & Lagercrantz, 2001). This transition has been shown to be delayed or even absent in some individuals with neurodevelopmental disorders including autism (Peerboom & Wieranga, 2021). Further, evidence for dysregulated excitatory/inhibitory signally in autism has been shown in an animal model of Fragile x syndrome (FXS) (He *et al*., 2019), which is the most prevalent form of syndromic autism (Napolitano *et al.*, 2022). The

restoration of GABAergic inhibitory signalling following restricted postnatal development was shown to improve brain functioning (He *et al*., 2019).

As such, sleep disturbance in autism related to the hyperactive functioning of orexinergic neurons (Kohyama, 2016) may be attributed to its widespread reciprocal connections throughout the brain (Bourgin *et al*., 2000) through which orexinergic neurons are able to mediate the excitatory and inhibitory balance (Knez et al., 2022).

3.1.3 Cellular phenotype of autism

Studies of the molecular and cellular basis of autism development have focussed primarily on the frontal cortex, amygdala, cerebellum, and hippocampus (Al Dera *et al*., 2022). Further, impairments in the development, and thus functional capacity, of these brain regions has most frequently been related to the reduction of neuron size, increased neuron numbers, abnormal orientation of neuronal population, reductions in white matter as well as dendritic abnormalities (de la Torre-Ubieta *et al*., 2016). However, as diverse neurophysiological processes are involved in the pathogenesis and development of ASD (Al Dera *et al*., 2022), it is necessary to expand the understanding of the neuroanatomy and functioning of all the brain regions implicated in the disorder's pathogenesis. To broaden this understanding, Málaga and colleagues (2019) have suggested that considerations of the neurogenesis, maturation, differentiation, and degradation of neuronal populations are required. However, these considerations are currently not well documented in literature, especially with regards to the co-occurrence of other disorders (Hyman *et al*., 2020) which are increasingly implicated in underlying behavioural symptoms (Richdale et al., 2009; Souders et al., 2009; Reynolds & Malow, 2011). Further, greater understanding of the cellular phenotype of autism alongside its co-morbid disorders has been suggested to be valuable for identifying treatment interventions (Hyman *et al*., 2020, Al Dera *et al*., 2022).

Evidently, cellular considerations may be useful in elucidating aspects of autism's underlying pathogenesis. Further, as sleep conditions are the most prevalent of these disorders co-occurring with autistic behaviours (Maurer *et al*., 2023), investigations of the sleep systems in the brain may prove valuable. The orexinergic system is one such system which is not as well represented as other sleep systems, therefore making investigations into its functioning in autism valuable (Saad *et al.,* 2022).

Orexinergic neurons are a distinct neuronal population within the LHA, and among the best characterised in the region at the molecular and cellular levels (deLecea *et al*., 1998; Sakurai *et al*., 1998). At the molecular level, identification of these neurons has been aided using molecular profiling techniques such as RNA-sequencing (Sokolowski *et al*., 2015; Yelin-Beckerman *et al*., 2015). Further, the cellular phenotype of the neurons has been identified by markers of their expressed neuropeptides and neurotransmitters by employing immunolabeling techniques (Bonnavion *et al*., 2016). Orexinergic neurons are known to overlap with other neuronal populations, likely even co-expressing other neuropeptides and transmitters (Bonnavion *et al*., 2016) such as the neuropeptide dynorphin (Muschamp *et al*., 2014). The full extent of the co-expression of orexinergic neurons within the LHA is not known (Bonnavion *et al*., 2016). This provides insights into the need for the use of a multitude of classification techniques in addition to the currently used labelling and profiling techniques. This is especially true given the varying specificity of these markers which may result in inconsistent labelling of entire cell populations (García-Cabezas *et al*., 2016), thus making it difficult to discriminate true differences among sample groups (Bonnavion *et al*., 2016).

As such, it has been suggested that Nissl staining be used alongside cellular and molecular identification techniques, especially in brains where pathology exists and may be unidentifiable compared to controls using these labelling techniques (García-Cabezas *et al*., 2016). This is because the Nissl technique stains all neuronal populations (García-Cabezas *et al*., 2016) and enables differences to be detected among various cell types of a specific brain region. The detection of these differences has been enabled by cytological descriptions of the various cell types in the brain (Ling *et al*., 1973; Gabbot & Stewart, 1987).

3.2 Materials and Methods

3.2.1 Tissue preparation

All dams (following weaning of their pups) and pups (after undergoing behavioural testing) were euthanised with 0.8ml of an intraperitoneal injection of sodium pentobarbital. At the discretion of the WRAF veterinary technician, an additional 0.2ml was provided for those rats who maintained consciousness for longer periods following the first injection. The brains were intracardially perfused with cold (4°C) isotonic (0.9%) saline until the runoff turned from red to clear. The brains were then extracted and sectioned along the midsagittal plane into left and right hemispheres. The left hemispheres were snap frozen in liquid nitrogen and stored at -80°C. The right hemispheres were postfixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB) at 4°C for one week. Following this, they were transferred to a 30% sucrose in 0.1M PB solution until equilibrated and then placed in an antifreeze solution at 4°C for two days. The brains were then stored in the antifreeze solution at -20°C for future use in several studies.

3.2.2 Immunohistochemistry and Nissl staining

For this study, the right brain hemispheres of four males (n=1 per sample group) were used for further analysis. The brains were removed from the antifreeze and transferred to a 30% sucrose in 0.1M PB solution for four days, mounted to an aluminium stage with a tissue freezing medium (Leica Biosystems, IL, USA) and sectioned at a thickness of 50μm using a cryostat (Thermo Scientific, MA, USA) into a 1-in-3 series.

The first sections in each series (from the whole brain) were used for Nissl staining to compare the relative volumes of the lateral hypothalamic area (LHA) amongst groups. Prior to staining, the selected sections were mounted on 0.5% gelatine coated slides and allowed to air dry for one week. Thereafter, the dry sections were placed in a solution of 50% alcohol and 50% chloroform overnight. They were then rehydrated in a decreasing alcohol series (2x 100% for five minutes, 1x 95% for two minutes, 70% for two minutes and 50% for two minutes) and stained with Cresyl Violet for one minute. Following this, the sections were immersed in distilled water for one minute and then dehydrated in an increasing graded ethanol series (50% for two minutes, 70% with a few drops of acetic acid under gentle agitation for variable time assessed by observation, 90% for variable time and 2x 100% for five minutes). The sections were then cleared twice in Xylene for two minutes, cover slipped with Depex and allowed a week to dry before visualisation.

The second sections in each series (within the series of sections within the region of interest) then underwent orexin-A (OX-A) immunohistochemistry (IHC) adapted from Olateju and colleagues (2017) as well as Swiegers and colleagues (2019) and performed as follows: The sections first underwent an endogenous peroxidase stage using a solution containing 50% methanol, 50% 0.1M phosphate buffer and 1.6% of 30%H2O2. The sections were incubated in this solution for 30 mins and followed by three ten-minute rinses in a 0.1M buffered phosphate solution. Thereafter, the sections were incubated in a blocking buffer solution containing 3% normal goat serum (NGS, BioWest), 2% bovine serum albumin (BSA, Sigma) and 0.25% Triton X-100 (Merck) in 0.1 phosphate buffer for two hours at room temperature. The sections were then incubated with the primary antibody to orexin-A (OxA, AB3704, Merck, raised in rabbit) at a dilution of 1:1000 for 48 hours at 4°C. This was followed by three ten-minute rinses in 0.1M PB, and a two-hour incubation with a biotinylated goat anti-rabbit IgG secondary antibody (BA-1000, Vector Labs) at 1:1000 dilution in a solution containing 3% NGS and 2% BSA in 0.1 M PB. This was followed by a further three ten-minute rinses in 0.1M PB and incubation with an avidin-biotin (AB) solution (Vector Labs) for one hour. The sections were then rinsed three times for ten minutes in a 0.1M phosphate buffer solution, and then incubated with a diaminobenzidine (DAB) solution containing 0.05% DAB in 0.1 M PB. For each 1ml of DAB solution, 3.3µl of 30% H2O2 was used according to the manufacturer's instructions (DAB Substrate Kit, 957D, Sigma). Sections were then incubated for five min or until sufficient colour changes were observed. The precipitation process was stopped by immersing the sections in 0.1M PB and then rinsing them twice more in 0.1 M PB for ten minutes each time. To check for non-specific staining, the incubation with the primary antibody was omitted for selected sections (negative controls). These were instead of the primary antibody, incubated with BB only. After staining, all sections were mounted onto 0.5% gel coated slides and dried for at least 48 hours, then dehydrated in increasing ethanol concentrations (70%/95%/100%/100%), cleared twice in xylene and cover slipped with Depex.

The remaining sections in the series were returned to an antifreeze solution and stored at -20℃ unstained and stored in an antifreeze solution at -20°C. These were kept for use as replacements in the event of damage or loss of any of the first two sections. This was done to ensure consistent distancing between sections throughout the brain. As such, enabling accurate comparisons within and amongst series.

3.2.3 Region of interest delineation

Orexinergic neurons are localised to the LHA (Nambu *et al.*, 1999; Nixon & Smale, 2007) and surrounding perifornical areas (Bonnavion *et al*., 2016) of the hypothalamus. As such, these regions were chosen as the regions of interest in the present study. The LHA was delineated according to descriptions of the rat hypothalamus provided by Bon and colleagues (2023). The authors describe a collection of nuclei subgroups that are arranged longitudinally into three main zones or columns along the rostro-caudal axis of the hypothalamus. Further, noting that the LHA lies in the middle or tuberal subregion, between the most rostral (supraoptic) and caudal (mammillary) subregions. Several atlases were used to identify these regions (Slotnick & Leonard, 1975; Paxinos & Watson, 1982) from the Nissl-stained series of sections. Further, the nomenclature described in the present study was adopted from Slotnick & Leonard (1975).

3.2.4 Cytological descriptions of LHA cell population

One male brain from each sample group was selected randomly and used to provide a preliminary account of the cellular composition of the LHA using the Nissl-stained sections. A MicroBrightfield (MBF) system (Vermont, USA), consisting of a Zeiss.Z2 Axio Imager Vario microscope and StereoInvestigator software (MBF, 64-bit), was used to visualise the hypothalamus of each individual using a 5x objective at Bregma -1.2. Part of the LHA seen at this level was then captured at a higher magnification using a 40x objective to visualise individual cells. These images were then used to categorise the cells according to descriptions of nuclear structures provided by Frost (1986). Figure 3.1 illustrates the structures used for these descriptions.

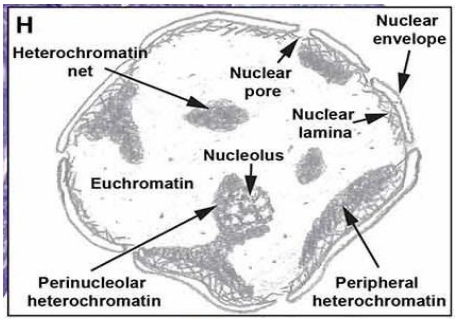


Figure 3.1: Illustration of cytological features used for characterising cell types from Nissl-stained sections (taken from García-Cabezas *et al*., 2016).

3.3 Results

3.3.1 Orexin-A immunohistochemistry

Several trials were conducted to optimise the IHC protocol used in the present study. Following a dilution series including 1:1000, 1:3000 and 1:7500, the 1:1000 was found to be the most suitable concentration for the primary antibody. However, following faint staining of only a few neurons using the DAB powder, an additional trial was conducted using a 1:1000 dilution of the primary and secondary antibodies to assess the most suitable DAB. A powder and kit were used on different sections of the hypothalami of a VPA-nSD male. The results of this trial revealed high levels of non-specific staining in both applications (Figure 3.3B - DAB powder; Figure 3.2B - DAB kit). Further, a high degree of background staining was observed from the use of the DAB kit (Figure 3.2). As such, no further analysis was conducted.

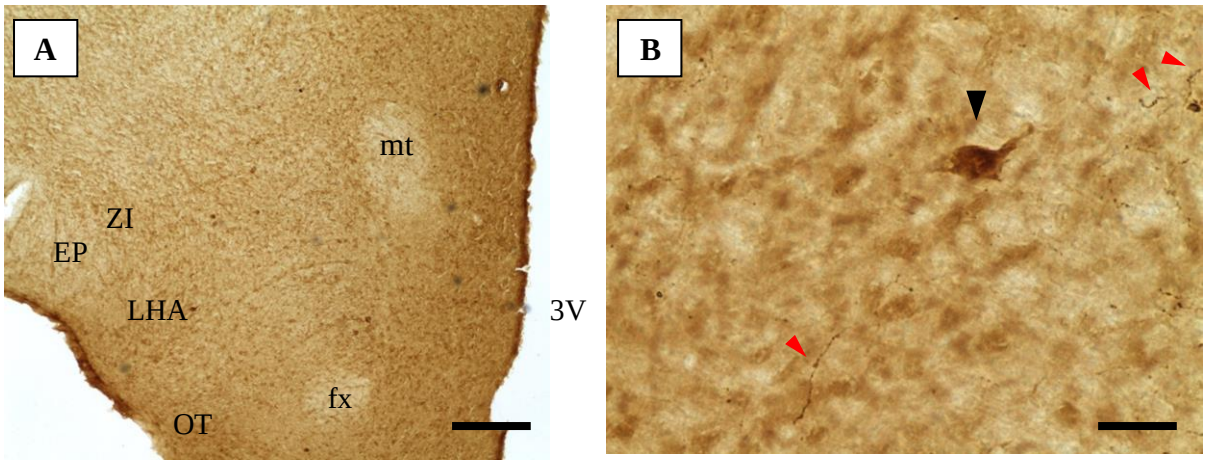


Figure 3.2: Photomicrographs of coronal sections through the hypothalamus of the *Rattus norvegicus* (Sprague Dawley rat) at bregma -1.2 (Slotnick & Leonard, 1975) immune-stained for the orexin-A antibody with peroxidase detection using a diaminobenzidine (DAB) kit. (A) shows the hypothalamus at 5x magnification; scale = 10µm. (B) shows the lateral hypothalamic area (LHA) at 40x magnification. Non-specific staining is indicated by the black arrowhead and nerve fibres with no cell body attachments indicated by the red arrowheads; scale = 100µm.

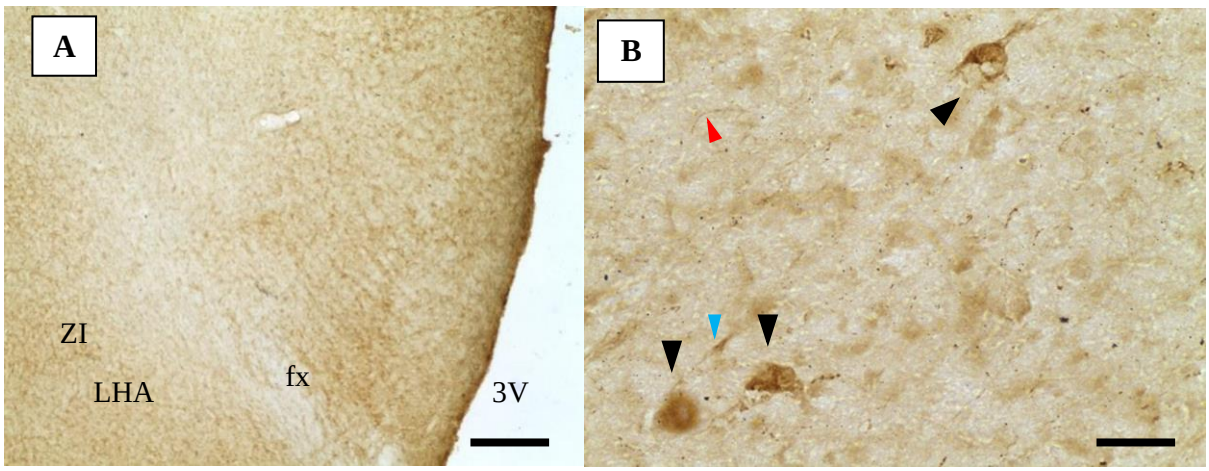


Figure 3.3: Photomicrographs of coronal sections through the hypothalamus of the *Rattus norvegicus* (Sprague Dawley rat) at bregma -1.2 (Slotnick & Leonard, 1975) immune-stained for the orexin-A antibody with peroxidase detection using a diaminobenzidine (DAB) powder. (A) shows the hypothalamus at 5x magnification; scale = 10µm. (B) shows the lateral hypothalamic area (LHA) at 40x magnification. Non-specific staining is indicated by the black arrowhead, nerve fibres with no cell body attachments indicated by the red arrowheads and a bouton-like structure indicated by the blue arrowhead; scale = 100µm.

3.3.2 Cytological descriptions of LHA cell population

The cellular arrangement of the hypothalamus at bregma -1.2 was relatively similar for the C-nSD, C-SD and VPA-SD groups. In the VPA-nSD rat, the zona incerta, internal capsule and ventro- and dorsomedial hypothalamic were not identified, with no distinct boundaries delineating their nuclei as opposed to the other three groups. However, as the fornix and mammillothalamic tracts could be delineated, the lateral hypothalamic and peri-fornical areas could be observed and the section included for comparison (Figure 3.4).

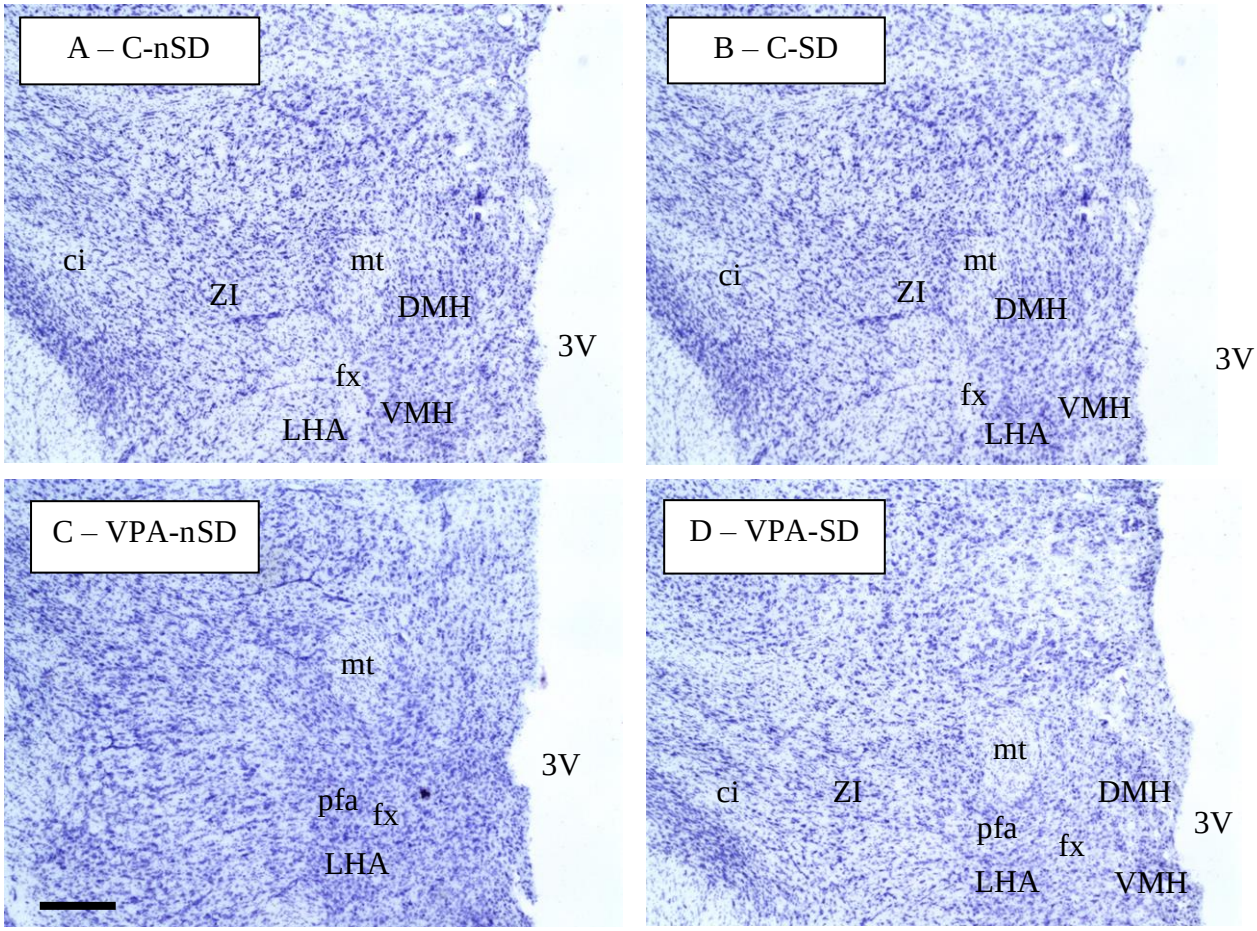


Figure 3.4: Photomicrographs of male *Rattus norvegicus* (Sprague Dawley rat) hypothalami at bregma -1.2 (Slotnick & Leonard, 1975). Abbreviations: 3V, third ventricle; ci, internal capsule; DMH, dorsomedial hypothalamus; EP, entopeduncular nucleus; fx, fornix; LHA, lateral hypothalamic area; mt, mammillothalamic tract; OT, optic tract; pfa, peri-fornical area; VMH, ventromedial hypothalamus; ZI, zona incerta.; scale = 10μm and applies to all.

Four cell types were distinguishable from the brains of the four rats, according to descriptions provided by Fawcett (1966) and Frost (1986). Firstly, large neurons (indicated by black arrowheads in Figure 3.5) were distinguished from other cell types by the visible ring of cytoplasm, typically lightly stained, surrounding their nuclei. The nuclei of these large neurons were round or ovoid in shape with dark-stained nucleoli, with minimal size variance despite the size of the neuron. This cell type was the most prevalent in the LHA of rats belonging to each group (Figure 3.5B, C and D) except for C-nSD (Figure 3.5A). Further, the nuclear envelope (or membrane surrounding their nuclei) of large neurons may also have indentations or folds, although this was not observed in any of the rats.

Secondly, small neurons (indicated by orange arrowheads in Figure 3.5) were distinguished, not necessarily by size but typically by the absence of the cytoplasm ring observed in the large neurons. This cell type was characterised by more visible accumulations of peripheral heterochromatin (observed as darkly stained dots within the cell) that were smaller than the nucleus. These small neurons were among the least prevalent cell types in the LHA of all the rats' brains. However, both subtypes of neurons were collectively the most numerous when compared to the other cell types.

Thirdly, astrocytes (indicated by green arrowheads in Figure 3.5) were distinguished from the small neurons by the presence of several heterochromatin granules attached to the peripheral heterochromatin as well as the heterochromatin net. Despite these cytological features not being clearly visible from the images used, these granules were seen as numerous and small darkly stained accumulations throughout the cell, giving them a dotted appearance. Thus, the astrocytes were morphologically distinct from the small neurons due to a variably visible nucleus and which when present, was indistinguishable in size from the granules. In the LHAs of the control rats, the astrocytes were similarly prevalent as the large neurons, irrespective of sleep condition (Figure 3.5A and B). Astrocytes were less prevalent than the large neurons in the VPA rats, however, the VPA-nSD notably had the least of all the sample groups (Figure 3.5C).

Finally, oligodendrocytes (indicated by red arrowheads in Figure 3.5) were identified by their small size and very darkly stained euchromatin, masking the appearance of other cytological features. Although, in some instances, dark round-shaped nuclei could be observed. This cell type was among the least prevalent in all the rats' groups except for C-nSD (Figure 3.5B) where the cell types were relatively the most balanced in number.

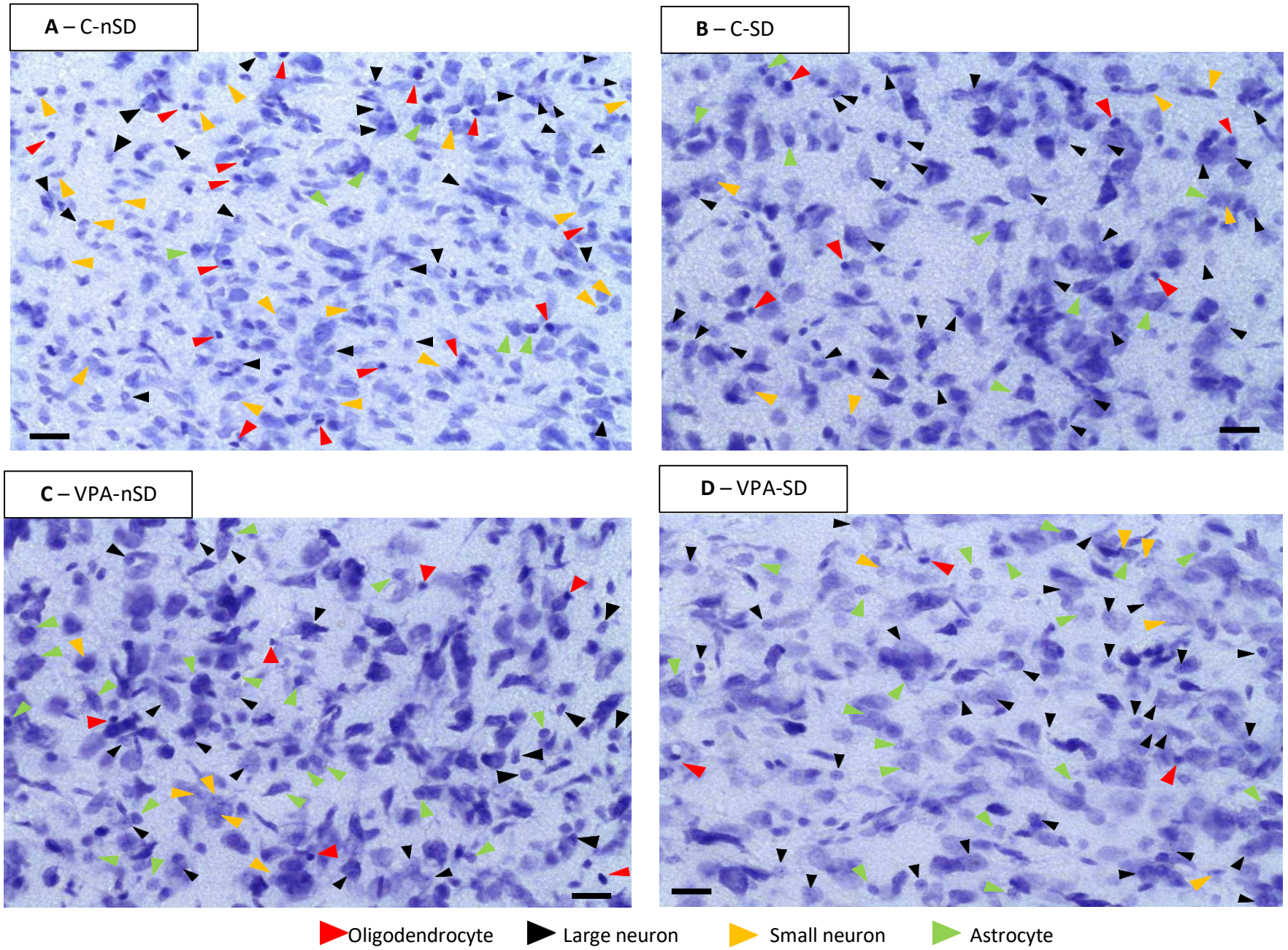

A – C-nSD
B – C-SD
C – VPA-nSD
D – VPA-SD
Oligodendrocyte
Large neuron
Small neuron
Astrocyte

Figure 3.5: Photomicrographs of the cell diversity from coronal sections through the lateral hypothalamic area at bregma -1.2 (Slotnick & Leonard, 1975) of male *Rattus norvegicus* (Sprague Dawley rat) under various treatment and sleep conditions, namely (A) C-nSD, (B) C-SD, (C) VPA-nSD and (D) VPA-SD. Large neurons (indicated by black arrowheads) and astrocytes (indicated by the green arrowheads) are equally prevalent. However, the total neuronal population including the small neurons (indicated by the orange arrowheads) is more numerous than any of the cell types observed; scale = 1000μm.

3.4 Discussion

3.4.1 Cellular diversity of the LHA

The functioning of the mammalian brain relies heavily on the specialisation and distribution of its diverse cell types (Langlieb *et al*., 2023). The exact number of distinct cell types as well as their distribution across brain regions is currently incompletely understood (Langlieb *et al*., 2023). Siletti and colleagues (2023) suggest that this is partly due to the vast research attention directed towards the cerebral cortex (García-Cabezas *et al*., 2016; Al Dera *et al*., 2022), thus resulting in gaps in the understanding of the cellular diversity of sub-cortical structures (Siletti *et al*., 2023). The understanding of this cellular diversity may be enabled through studies of morphology or their cellular and molecular phenotypes (Hyman *et al*., 2020, Al Dera *et al*., 2022), however much of the current understanding has been enabled by advancements in molecular techniques such as single-cell ribonucleic acid (RNA) sequencing (Langlieb *et al*., 2023; Siletti *et al*., 2023) and spatial transcriptomics (Moffitt *et al*., 2018; Ortiz *et al*., 2020; Chen *et al*., 2021). Given that the specialisation and distribution of cell types are dependent on patterned developmental trajectories (Siletti *et al*., 2023), the importance of their understanding has been identified with respect to neurodevelopmental disorders such as autism (Hyman *et al*., 2020, Al Dera *et al*., 2022). However, the knowledge gaps with regards to autism parallels the global lack of understanding in the neurotypical brain (Hyman *et al*., 2020). This is exacerbated by instances where existing autism pathology is comorbid by other disorders (Hyman *et al*., 2020) such as sleep dysregulations which are most frequently reported alongside the disorders core behavioural features (Lorsung *et al*., 2021). As such, the present study aimed to aid the understanding of the cellular diversity of the LHA which houses orexinergic neurons responsible for the regulation of sleep/wake cycles (Zhang *et al*., 2013; Yamashita & Yamanaka, 2016).

The LHA is one of the brain regions with the highest cellular diversity (Welch *et al*., 2019; Langlieb *et al*. 2023). Likewise, four cell types, namely small and large neurons as well as astrocytes and oligodendrocytes were found in varying proportions in all four sample groups in the present study (Figure 3.5). Notably, the cellular diversity in the brain is much greater (García-Cabezas *et al*., 2016), however, these four types were the only ones that could be clearly distinguished from the Nissl- stained sections of the LHA.

As all four cell types could be identified in all four sample groups, the proportion of those cells which were present allowed inferences regarding the role of autism pathology and sleep condition on the cellular diversity and subsequent functioning of the LHA. For each sample group, neurons (small and large) constituted most of the cell types observed, in accordance with the dense neuronal populations said to be present in the region (Cai *et al*., 2022). Further, the C-nSD rat had the most of this cell type compared to other groups (Figure 3.5A), followed the VPA groups where similar numbers were observed (Figure 3.5C and D). The C-SD rat had the least neurons of all the groups (Figure 3.5B). Interestingly, the proportion of astrocytes to the neurons differed among the groups according to the presence of sleep disturbance. In both control and VPA groups, the presence of sleep disturbance increased the proportion of astrocytes observed, constituting more than half compared to the neuronal population (Figure 3.5B and D). To contrast, in the absence of sleep disturbance, and regardless of autism pathology, the proportion of astrocytes was markedly decreased (Figure 3.5A and C).

Astrocytes play a role in modulating the activity of nearby neurons (Araque *et al*., 2014). In the LHA, this role is said to relate to the maintenance of the depolarisation of orexinergic neurons (Eggerman *et al*., 2003). It is worth noting that without any form of identification of orexinergic neurons, the morphological characteristics used to identify neurons in the present study cannot be assumed to be orexinergic. This is especially because the LHA houses additional neuronal populations that are not orexinergic (Burt *et al*., 2011). Further, as the entire LHA was not sampled, it is possible that the observed cell proportions are not representative of the organisation of the whole region. As such, the role of astrocytes as a potential mechanism underlying the cellular differences cannot be accurately inferred from the findings obtained in the present study. Still, these results provide an opportunity for further and more holistic investigation into the cellular organisation of the LHA in attempts to understand the mechanisms underlying sleep disturbance in autism.

The role of astrocytes has been the focus of research related to the regulation of sleep/wake cycles (Cai *et al*., 2022), largely due to the fluctuations of their intracellular calcium levels throughout the various stages of sleep and wake (Blum *et al*., 2021). Furthermore, disruptions of these typical fluctuations have been evidenced to disrupt sleep (Cai et al., 2022). For instance, Bojarskaite and colleagues (2020) showed that the genetic suppression of an important calcium signalling pathway in astrocytes resulted in microarousals and disrupted slow wave sleep in mice, evidenced by abnormal EEG rhythmicity.

Orexinergic neurons are maintained in a depolarised or active state, presumably to prolong their neuroexcitatory role (de Lecea *et al*., 1998), to sufficiently maintain wakefulness and execute their other functions through positive feedback mechanisms (Cai *et al*., 2022). This active state is enabled by several local mechanisms (Cai *et al*., 2022), among them being their ion calcium channels (Cvetkovic-Lopes *et al*., 2010; Burt *et al*., 2011) and excitatory action of the neurotransmitters released by the neurons themselves (Burt *et al*., 2011; Cai *et al*., 2022). Astrocytes can indirectly influence orexinergic functioning through the amplification of these excitatory inputs (Burt *et al*., 2011). For instance, as a primary excitatory neurotransmitter of the hypothalamus, glutamate is also the major excitatory input of orexinergic neurons (Eyigor *et al*., 2012). Glutamate also stimulates lactate production in astrocytes which locally lowers extracellular pH through the action of its transport mechanisms (Halestrap & Price, 1999), as such, depolarising orexinergic neurons (Williams *et al*., 2007). Additionally, astrocyte activation can also stimulate the release of ATP, which may act as a transport molecule (Druzin *et al*., 2002) that depolarises orexinergic neurons via receptors that are expressed on their plasma membrane (Burt *et al*., 2011).

Kim and colleagues (2014a) illustrated the effects of sleep disturbance (particularly chronic sleep deprivation) in facilitating increased astrocytic activation that potentially underlies dysfunction in a range of neurological disorders. The authors conducted an analysis of the proteins identified to be differentially expressed in the astrocytes of the rat hypothalamus. Of the 139 proteins that were identified, 86 of them presented with increased astrocytic activity. Furthermore, of those 86, the majority were associated with the generation of pre-curser metabolites and the regulation of neurotransmitter levels (Kim *et al*., 2014a).

As such, it is possible that the increased astrocyte populations relative to neuronal population as observed in the C-SD and VPA-SD groups may cause further excitation of already neuroexcitatory orexinergic neurons (de Lecea *et al*., 1998), resulting in imbalances in the negative feedback mechanisms mediating the actions of the orexinergic neurons (Burt *et al*., 2011). Thus, the astrocyte-induced overexcitation of orexinergic neurons may maintain these neurons in the hyperactive state said to underlie sleep disturbance in autism (Prober *et al*., 2006; Kohyama, 2016; Tang *et al*., 2017).

3.4.2 Limitations

In the present study, high levels of non-specific binding were observed in the orexin-A immune-stained sections (Figures 3.3 & 3.4). Thus, no further analysis could be conducted due to the inability to discriminate individual neurons. Further, sections were incubated with the primary antibody to orexin-A (OxA, AB3704, Merck, raised in rabbit) which was polyclonal. Polyclonal antibodies are typically favoured for IHC applications due to their ability to recognise multiple epitopes on a target protein (Paavilainen *et al.*, 2008). This characteristic of polyclonal antibodies is necessary because post-fixation methods (such as the use of PFA in the present study) are known to denature protein structure, thus increasing difficulty in antibody binding (Paavilainen *et al.*, 2008). As such, the use of monoclonal antibodies (which bind to a single epitope) may produce weakly-stained sections from failing to recognise truncated or masked epitopes (Paavilainen *et al.*, 2008). However, polyclonal antibodies may have high levels of reactivity with non-target proteins (Paavilainen *et al.*, 2008), resulting in non-specific binding as was observed in the present study. In future, the use of different antibodies and further optimisation will be required to successfully identify orexinergic neurons for further analysis. Unfortunately, in the present study, attempts to optimise the IHC protocols adapted from several studies (Olateju *et al.*, 2017; Swiegers *et al.*, 2019) were ceased due to costs and time constraints.

Following the unsuccessful use of orexin-A immunohistochemistry, Nissl-stained sections were used to compare the cytology of the LHA among the four sample groups. Notably, inferences regarding the orexinergic system specifically were limited due to the absence of markers identifying specific orexinergic neurons. Further, due to time constraints, only a preliminary account was provided in the present study. Still, Nissl-stained sections may be used alongside IHC to obtain holistic accounts of the effect of sleep disturbance on autism-like outcomes. For instance, autistic children have been found to possess abnormal neuronal growth across widespread brain regions, indicative of global encephalopathy within the disorder (Wegiel *et al.*, 2014). As such, Nissl-stained sections may be assessed with Stereological estimation tools such as the Cavalieri probe and may be used to quantify total and relative brain volumes.

3.5 Conclusion

The aim of the present study was to determine the effect of sleep disturbance on autism-like behaviours. Further, orexinergic functioning has been implicated as a potential mechanism underlying dysregulated sleep in autism (Kohyama, 2016). As such, the present study examined the neuroanatomy of the LHA, in which orexinergic neurons are localised (Nambu *et al*., 1999), in sleep-disturbed VPA model of the disorder. After failing to label orexinergic neurons using immunohistochemistry, a preliminary description of the diversity of cell types in the LHA was provided. The results showed the presence of two types of neurons (small and large) as well as glial cells (astrocytes and oligodendrocytes) in all sample groups. However, the proportions of those cell types which were observed differed among groups according to sleep condition. Particularly, the ratio of neuronal and astrocyte populations was increased following sleep disturbance, irrespective of autism pathology. Given the neuromodulatory role of astrocytes (Araque *et al*., 2014), these results support the potential role for astrocytes in underlying orexinergic dysfunction previously suggested (Bojarskaite *et al*., 2020; Cai *et al*., 2022). Further, supporting the hyperexcitation of orexinergic neurons of autism (Kohyama, 2016) as astrocytes are known to maintain orexinergic depolarisation through various mechanisms (Williams *et al*., 2007; Burt *et al*., 2011). Notably, these results provided only a preliminary account due to the sampling of only a single frame within the LHA. As such, requiring further investigation with more representative samples in order to increase the reliability of the results obtained.

CHAPTER 4: General Discussion

The present study provided an account of the role of sleep disturbance in the autism-like behaviours observed in a VPA rat model of the disorder. This account was based, firstly, on the interaction between sleep condition, autism pathology and sex and secondly, on the cellular composition of the LHA where orexinergic neurons are localised (Nambu *et al.*, 1999).

The results of these two accounts failed to provide consistent evidence for the worsening of existing autism symptoms due to sleep disturbance as previously reported (Richdale *et al.*, 2009; Souders *et al.*, 2009; Reynolds & Malow, 2011). The cytological descriptions of the LHA revealed differences in the proportion of cell types based on sleep condition. Particularly in that the proportion of astrocytes relative to the neuronal population was increased in the presence of sleep disturbance, regardless of autism pathology. Notably, this result may possibly be attributed to the exclusion of females in this preliminary investigation. Similar differences based on sleep condition could not be detected from the results of the behavioural tests which revealed more mixed results, likely due to the inclusion of both males and females. Particularly, the worsening of autistic-like behaviours in the presence of sleep disturbance could not be consistently observed in both males and females for any of the behaviours assessed. Thus, suggesting a role for sex in mediating the relationship between sleep condition and autism pathology. Further, the manner in which sex mediated these behaviours was dependent on the behaviour. For instance, stereotyped or repetitive motor movements assessed from the number of marbles buried were observed to be increased in VPA males compared to controls, whereas the opposite was observed in the females (Figure 2.13). According to this single measure, the absence of autism-like symptomology has been generalised across all domains of repetitive behaviours, neglecting the possibility of female dysfunction across different measures. As such, this instance is reflective of the present study showing support for sex-specific presentations of autism (Hull *et al.*, 2020) which are frequently overlooked due to male-biased criteria (Loomes *et al.*, 2017; Napolitano *et al.*, 2022). In addition to showing how the limitations of these male-centric criteria may be disadvantageous for identifying autism-symptomology in females, the present study also identified how limiting the behavioural outcomes assessed may hinder the ability to infer the role of comorbidities in the observed behaviours.

Further, autism is a spectral disorder with high degrees of heterogeneity in its presentation (Lorsung *et al.*, 2021). However, it was unclear whether this inherent variability was the reason for some of the inconsistencies observed when compared to previous studies.

This highlights the importance of improvements in the methodological approaches to autism research, particularly related to the standardisation of behavioural assessment protocols in order to increase reproducibility and inferences across studies.

References

Aathira, R., Gulati, S., Tripathi, M., *et al*. (2017). Prevalence of sleep abnormalities in Indian children with autism spectrum disorder: a cross-sectional study. *Pediatric Neurology*. 74: 62-67.

Al Backer, N.B., Alzawad, M., Habibullah, H., *et al*. (2018). The relationship between sleep and cognitive performance in Autism Spectrum Disorder (ASD): a pilot study. *Children*. 5(11):153.

Al-Kuraishy, H.M., Abdulhadi, M.H., Hussien, N.R., *et al*. (2020). Involvement of orexinergic system in psychiatric and neurodegenerative disorders: A scoping review. *Brain Circulation*. 6(2):70-80.

Allada, R, Siegel, JM. (2008). Unearthing the phylogenetic roots of sleep. *Curr Biol*. 18: R670–R679.

Amaral, D.G., Bauman, M.D. and Mills Schumann, C. (2003). The amygdala and autism: implications from non-human primate studies. *Genes, Brain and Behavior*. 2(5): 295-302.

American Psychiatric Association. (2013). Diagnostic and statistical manual of mental disorders (DSM-5®).

Antezana, L., Factor, R.S., Condy, E.E., *et al*. (2019). Gender differences in restricted and repetitive behaviors and interests in youth with autism. *Autism Research*. 12(2):274-283.

Araque, A., Carmignoto, G., Haydon, P.G., *et al*. (2014). Gliotransmitters travel in time and space. *Neuron*. 81(4):728-739.

Aulehner, K., Bray, J., Koska, I., *et al.* (2022). The impact of tethered recording techniques on activity and sleep patterns in rats. *Scientific Reports*, *12*(1):1-16.

Baker, K.B., Wray, S.P., Ritter, R., *et al*. (2010). Male and female Fmr1 knockout mice on C57 albino background exhibit spatial learning and memory impairments. *Genes, Brain and Behavior*. 9(6):562-574.

Barone, I., Hawks-Mayer, H. and Lipton, J.O. (2019). Mechanisms of sleep and circadian ontogeny through the lens of neurodevelopmental disorders. *Neurobiology of learning and memory*. *160*:160-172.

Baronio, D., Castro, K., Gonchoroski, T., *et al.* (2015). Effects of an H3R antagonist on the animal model of autism induced by prenatal exposure to valproic acid. *PloS one*. 10(1): e0116363.

Baron-Cohen, S. (2002). The extreme male brain theory of autism. *Trends in cognitive sciences*. 6(6):248-254.

Baron-Cohen, S. (2004). *The essential difference*. Penguin UK.

Baron-Cohen, S. (2005). Autism «Autos»: Literally. *The Lost Self: Pathologies of the Brain and Identity: Pathologies of the Brain and Identity*. pp.166-180.

Bastianini, S., Alvente, S., Berteotti, C., *et al.* (2017). Accurate discrimination of the wake-sleep states of mice using non-invasive whole-body plethysmography. *Scientific reports*. *7*(1):1-10.

Ben-Sasson, A., Hen, L., Fluss, R., *et al.* (2009). A meta-analysis of sensory modulation symptoms in individuals with autism spectrum disorders. *Journal of autism and developmental disorders*. 39:1-11.

Bernier, R., Dawson, G., Panagiotides, H., *et al*. (2005). Individuals with autism spectrum disorder show normal responses to a fear potential startle paradigm. *Journal of Autism and Developmental Disorders*. 35:575-583.

Binkerd, P.E., Rowland, J.M., Nau, H., *et al.* (1988). Evaluation of valproic acid (VPA) developmental toxicity and pharmacokinetics in Sprague-Dawley rats. *Fundamental and Applied Toxicology*. 11(3):485-493.

Blum, I.D., Keleş, M.F., Baz, E.S., *et al.* (2021). Astroglial calcium signaling encodes sleep need in Drosophila. *Current Biology*. 31(1):150-162.

Bodfish, J.W., Symons, F.J., Parker, D.E., *et al*. (1999). Repetitive behavior scale–revised. *Journal of Autism and Developmental Disorders*.

Bojarskaite, L., Bjørnstad, D.M., Pettersen, K.H., *et al*. (2020). Astrocytic Ca2+ signaling is reduced during sleep and is involved in the regulation of slow wave sleep. *Nature communications*. 11(1):3240.

Bon, E.I., Maksimovich, N.Y., Zimatkin, S.M., *et al*. (2023). Morphofunctional organisation of the rat hypothalamus. *Clinical Genetic Research*. 2(1).

Bonnavion, P., Mickelsen, L.E., Fujita, A., *et al*. (2016). Hubs and spokes of the lateral hypothalamus: cell types, circuits and behaviour. *The Journal of physiology*. 594(22):6443-6462.

Bonnin, A., Goeden, N., Chen, K., *et al*. (2011). A transient placental source of serotonin for the fetal forebrain. *Nature*. 472(7343): 347-350.

Boucher, J., Mayes, A. and Bigham, S. (2012). Memory in autistic spectrum disorder. *Psychological bulletin*. 138(3):458.

Bourgin, P., Huitron-Resendiz, S., Spier, A.D., *et al*. (2000). Hypocretin-1 modulates rapid eye movement sleep through activation of locus coeruleus neurons. *Journal of neuroscience*. 20(20):7760-7765.

Brand, S., Jossen, S., Holsboer-Trachsler, E., *et al*. (2015). Impact of aerobic exercise on sleep and motor skills in children with autism spectrum disorders–a pilot study. *Neuropsychiatric Disease and Treatment*. pp.1911-1920.

Büchel, C. and Dolan., R.J. (2000) Classical fear conditioning in functional neuroimaging. *Current opinion in neurobiology*. 10(2): 219-223.

Burman, D., Ramanujam, K., Manzar, D., et al. (2023). Sleep and Autism Spectrum Disorder: A Comprehensive Review of Diagnosis, Markers, Interventions, and Treatments. *Sleep and Vigilance*. pp.1-14.

Cai, P., Huang, S.N., Lin, Z.H., *et al*. (2022). Regulation of wakefulness by astrocytes in the lateral hypothalamus. *Neuropharmacology*. 221:109275.

Campolongo, M., Kazlauskas, N., Falasco, G., *et al*. (2018). Sociability deficits after prenatal exposure to valproic acid are rescued by early social enrichment. *Molecular autism*. 9(1):1-17.

Cardillo, R., Vio, C. and Mammarella, I.C. (2020). A comparison of local-global visuospatial processing in autism spectrum disorder, nonverbal learning disability, ADHD and typical development. *Research in developmental disabilities*. 103:103682.

Cash-Padgett, T., Sawa, A. and Jaaro-Peled, H. (2016). Increased stereotypy in conditional Cxcr4 knockout mice. *Neuroscience research*. 105:75-79.

Castro, K., Baronio, D., Perry, I.S., *et al*. (2017). The effect of ketogenic diet in an animal model of autism induced by prenatal exposure to valproic acid. *Nutritional neuroscience*. 20(6):343-350.

Cezar, L.C., Kirsten, T.B., da Fonseca, C.C.N., *et al*. (2018). Zinc as a therapy in a rat model of autism prenatally induced by valproic acid. *Progress in neuro-psychopharmacology and biological psychiatry*. 84:173-180.

Chaliha, D., Albrecht, M., Vaccarezza, M., *et al*. (2020). A systematic review of the valproic-acid-induced rodent model of autism. *Developmental neuroscience*. 42(1):12-48.

Chemelli, R.M., Willie, J.T., Sinton, C.M., *et al*. (1999). Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell*. 98(4):437-451.

Chen, R., Blosser, T.R., Djekidel, M.N., *et al*. (2021). Decoding molecular and cellular heterogeneity of mouse nucleus accumbens. *Nature neuroscience*. 24(12):1757-1771.

Chieffi, S., Carotenuto, M., Monda, V., *et al*. (2017). Orexin System: The Key for a Healthy Life. *Front. Physiol.* 8:357.

Cho, H., Kim, C.H., Knight, E.Q., *et al*. (2017). Changes in brain metabolic connectivity underlie autistic-like social deficits in a rat model of autism spectrum disorder. *Scientific reports*. *7*(1):13213.

Chugani, D.C., Muzik, O., Rothermel, R., *et al*. (1997). Altered serotonin synthesis in the dentatothalamocortical pathway in autistic boys. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society*. 42(4):666-669.

Cleal, M., Fontana, B.D., Ranson, D.C., *et al*. (2021). The Free-movement pattern Y-maze: A cross-species measure of working memory and executive function. *Behavior research methods*. 53(2):536-557.

Cohen, S., Conduit, R., Lockley SW. (2014). The relationship between sleep and behaviour in autism spectrum disorder (ASD): a review. *J Neurodev Disord*. 6:44.

Courchesne, E., Townsend, J. and Saitoh, O. (1994). The brain in infantile autism: posterior fossa structures are abnormal. *Neurology*. 44(2):214-214.

Crespi, B., Read, S., Ly, A., *et al*. (2019). AMBRA1, autophagy, and the extreme male brain theory of autism. *Autism research and treatment*. 2019(1):1-6.

Cusmano, D.M., Mong, J.A. (2014). In utero exposure to valproic acid changes sleep in juvenile rats: a model for sleep disturbances in autism. *Sleep*. 37:1489–99.

Couturier, J.L., Speechley, K.N., Steele, M., *et al*. (2005). Parental perception of sleep problems in children of normal intelligence with pervasive developmental disorders: prevalence, severity and pattern. *J Am Acad Child Adolesc Psychiatry*. 44:815-822.

Cvetkovic-Lopes, V., Eggermann, E., Uschakov, A., *et al*. (2010). Rat hypocretin/orexin neurons are maintained in a depolarized state by TRPC channels. *PloS one*. 5(12): e15673.

Dai, X., Yin, Y. and Qin, L. (2017). Valproic acid exposure decreases the mRNA stability of Bcl-2 via up-regulating miR-34a in the cerebellum of rat. *Neuroscience Letters*. 657:159-165.

Dajani, D.R. and Uddin, L.Q. (2015). Demystifying cognitive flexibility: Implications for clinical and developmental neuroscience. *Trends in neurosciences*. 38(9): 571-578.

Davis, M. and Whalen, P.J. (2001). The amygdala: vigilance and emotion. *Molecular psychiatry*. 6(1):13-34.

Doldur de la Torre-Ubieta, L., Won, H., Stein, J.L., *et al*. (2016). Advancing the understanding of autism disease mechanisms through genetics. *Nature medicine*. 22(4):345-361.

de Lecea, L., Kilduff, T.S., Peyron, C., *et al*. (1998). The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proceedings of the National Academy of Sciences*. 95(1):322-327.

De Theije, C.G., Koelink, P.J., Korte-Bouws, G.A., *et al*. (2014). Intestinal inflammation in a murine model of autism spectrum disorders. *Brain, behavior, and immunity*. 37:240-247.

Desaunay, P., Briant, A.R., Bowler, D.M., *et al*. (2020). Memory in autism spectrum disorder: A meta-analysis of experimental studies. *Psychological Bulletin*. 146(5):377.

Deliens, G. and Peigneux, P. (2019). Sleep–behaviour relationship in children with autism spectrum disorder: Methodological pitfalls and insights from cognition and sensory processing. *Developmental Medicine & Child Neurology*. 61(12):1368-1376.

Devnani, P.A., Hedge, A.U. (2015). Autism and sleep disorders. *J Pedia Neurosci*. 10:304-307.

Diaz-Roman, A., Zhang, J., Delorme, R., *et al.* (2018). Sleep in youth with autism spectrum disorders; systematic review and meta-analysis of subjective and objective studies. *Evid Based Ment Health*. 21:146-154.

Ding, Q., Sethna, F. and Wang, H. (2014). Behavioral analysis of male and female Fmr1 knockout mice on C57BL/6 background. *Behavioural brain research*. 271:72-78.

Drummond, S.P., Brown, G.G., Gillin, J.C., *et al*. (2000). Altered brain response to verbal learning following sleep deprivation. *Nature*. 403(6770):655-657.

Drummond, S.P., Gillin, J.C. and Brown, G.G. (2001). Increased cerebral response during a divided attention task following sleep deprivation. *Journal of sleep research*. 10(2):85-92.

Druzin, M., Haage, D., Malinina, E., *et al*. (2002). Dual and opposing roles of presynaptic Ca2+ influx for spontaneous GABA release from rat medial preoptic nerve terminals. *The Journal of physiology*. 542(1):131-146.

Dufour-Rainfray, D., Vourc'h, P., Le Guisquet, A.M., *et al*. (2010). Behavior and serotonergic disorders in rats exposed prenatally to valproate: a model for autism. *Neuroscience letters*. 470(1):55-59.

Edalatmanesh, M.A., Nikfarjam, H., Vafaee, F., *et al*. (2013). Increased hippocampal cell density and enhanced spatial memory in the valproic acid rat model of autism. *Brain research*. 1526:15-25.

Edwards, H., Wright, S., Sargeant, C., *et al*. (2024). Research Review: A systematic review and meta-analysis of sex differences in narrow constructs of restricted and repetitive

behaviours and interests in autistic children, adolescents, and adults. *Journal of Child Psychology and Psychiatry*. 65(1):4-17.

Eggermann, E., Bayer, L., Serafin, M., *et al*. (2003). The wake-promoting hypocretin–orexin neurons are in an intrinsic state of membrane depolarization. *Journal of Neuroscience*. 23(5):1557-1562.

Eilam-Stock, T., Wu, T., Spagna, A., *et al.* (2016). Neuroanatomical alterations in high-functioning adults with autism spectrum disorder. *Frontiers in neuroscience*. 10:198812.

Eissa, N., Jayaprakash, P., Azimullah, S., *et al*. (2018). The histamine H3R antagonist DL77 attenuates autistic behaviors in a prenatal valproic acid-induced mouse model of autism. *Scientific reports*. 8(1):13077.

Elrod, M.D. and Hood, B.S. (2015). Sleep differences among children with autism spectrum disorders and typically developing peers: a meta-analysis. *J Dev Beh Pedia*. 3:1-10.

Esaki, S., Nakayama, M., Arima, S., *et al*. (2021). Use of Actigraphy for a Rat Behavioural Sleep Study. *Clocks & Sleep*. 3(3):409-414.

Esposito, G., Venuti, P., Maestro, S., *et al*. (2009). An exploration of symmetry in early autism spectrum disorders: analysis of lying. *Brain and Development*. 31(2):131-138.

Etkin, A., Egner, T. and Kalisch, R. (2011). Emotional processing in anterior cingulate and medial prefrontal cortex. *Trends in cognitive sciences*. 15(2):85-93.

Eyigor, O., Minbay, Z., and Kafa, I.M. (2012). Glutamate and orexinergic neurons. *Vitam. Horm.* 89:209-222.

Fang, K., Liu, D., Pathak, S.S., *et al*. (2021). Disruption of circadian rhythms by ambient light during neurodevelopment leads to autistic-like molecular and behavioral alterations in adult mice. *Cells*. 10(12):3314.

Fatemi, S.H., Aldinger, K.A., Ashwood, P., *et al*. (2012). Consensus paper: pathological role of the cerebellum in autism. *The Cerebellum*. 11:777-807.

Fischer, S., Hallschmid, M., Elsner, A.L., *et al*. (2002). Sleep forms memory for finger skills. *Proceedings of the National Academy of Sciences*. 99(18):11987-11991.

Fournier, K.A., Hass, C.J., Naik, S.K., *et al*. (2010). Motor coordination in autism spectrum disorders: a synthesis and meta-analysis. *Journal of autism and developmental disorders*. 40:1227-1240.

Franken, P., Tobler, I. and Borbély, A.A. (1995). Varying photoperiod in the laboratory rat: profound effect on 24-h sleep pattern but no effect on sleep homeostasis. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*. 269(3): R691-R701.

Gabbot, P.L. and Stewart, M.G. (1987). Distribution of neurons and glia in the visual cortex (area 17) of the adult albino rat: a quantitative description. *Neuroscience*. 21:833-845.

Gallahue, D.L. and Ozmun, J.C. (2006). Understanding motor development: Infants, children, adolescents, adults. *No Title*.

Galli, J., Loi, E., Visconti, L.M., *et al*. (2022). Sleep disturbances in children affected by autism spectrum disorder. *Frontiers in psychiatry*. 13:736696.

Gandal, M.J., Edgar, J.C., Ehrlichman, R.S., *et al*. (2010). Validating γ oscillations and delayed auditory responses as translational biomarkers of autism. *Biological psychiatry*. 68(12):1100-1106.

Gandotra, A., Kotyuk, E., Szekely, A., *et al*. (2020). Fundamental movement skills in children with autism spectrum disorder: A systematic review. *Research in Autism Spectrum Disorders*. 78:101632.

García-Cabezas, M.Á., John, Y.J., Barbas, H., *et al*. (2016). Distinction of Neurons, Glia and Endothelial Cells in the Cerebral Cortex: An Algorithm Based on Cytological Features. *Frontiers in Neuroanatomy*. 10:107.

Geoffray, M.M., Nicolas, A., Speranza, M., *et al*. (2016). Are circadian rhythms new pathways to understand Autism Spectrum Disorder?. *Journal of Physiology-Paris*. 110(4):434-438.

Ghahremani, R., Mohammadkhani, R., Salehi, I., *et al*. (2022). Sex differences in spatial learning and memory in valproic acid rat model of autism: possible beneficial role of exercise interventions. *Frontiers in Behavioral Neuroscience*. 16:869792.

Goldman, S.E., Surdyka, K., Cuevas, R., *et al.* (2009). Defining the sleep phenotype in children with autism. *Developmental neuropsychology*. 34(5):560-573.

Goldstein, G., Johnson, C.R. and Minshew, N.J. (2001). Attentional processes in autism. *Journal of autism and developmental disorders*. 31:433-440.

Hagan, J.J., Leslie, R.A., Patel, S., *et al*. (1999). Orexin A activates locus coeruleus cell firing and increases arousal in the rat. *Proceedings of the National Academy of Sciences*. 96(19):10911-10916.

Halestrap, A.P. and Price, N.T. (1999). The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochemical Journal*. 343(2):281-299.

Halberg, F. (1960, January). Temporal coordination of physiologic function. In *Cold Spring Harbor symposia on quantitative biology*. 25:289-310.

Hall, J.E. and Hall, M.E. (2020). *Guyton and Hall textbook of medical physiology e-Book*. Elsevier Health Sciences.

Hara, Y., Maeda, Y., Kataoka, S., *et al*. (2012). Effect of prenatal valproic acid exposure on cortical morphology in female mice. *Journal of pharmacological sciences*. 118(4):543-546.

Haratizadeh, S., Parvan, M., Mohammadi, S., *et al.* (2021). An overview of modeling and behavioral assessment of autism in the rodent. *International Journal of Developmental Neuroscience*. *81*(3):221-228.

Hardy, L.L., King, L., Farrell, L., *et al*. (2010). Fundamental movement skills among Australian preschool children. *Journal of science and medicine in sport*. 13(5):503-508.

Harrop, C., Gulsrud, A. and Kasari, C. (2015). Does gender moderate core deficits in ASD? An investigation into restricted and repetitive behaviors in girls and boys with ASD. *Journal of autism and developmental disorders*. 45:3644-3655.

Hasegawa, E., Miyasaka, A., Sakurai, K., *et al*. (2022). Rapid eye movement sleep is initiated by basolateral amygdala dopamine signaling in mice. *Science (New York, NY)*. 375:994–1000.

He, Q., Arroyo, E.D., Smukowski, S.N., *et al.* (2019). Critical period inhibition of NKCC1 rectifies synapse plasticity in the somatosensory cortex and restores adult tactile response maps in fragile X mice. *Molecular psychiatry*. 24(11):1732-1747.

Heredia-López, F.J., Álvarez-Cervera, F.J., Collí-Alfaro, J.G., *et al.* (2016). An automated Y-maze based on a reduced instruction set computer (RISC) microcontroller for the assessment of continuous spontaneous alternation in rats. *Behavior Research Methods*. 48:1631-1643.

Herlenius, E. and Lagercrantz, H. (2001). Neurotransmitters and neuromodulators during early human development. *Early human development*. 65(1):21-37.

Hiller, R.M., Young, R.L. and Weber, N. (2014). Sex differences in autism spectrum disorder based on DSM-5 criteria: evidence from clinician and teacher reporting. *Journal of abnormal child psychology*. 42:1381-1393.

Hodge, D., Carollo, T.M., Lewin, M., *et al.* (2014). Sleep patterns in children with and without autism spectrum disorders: developmental comparisons. *Res Dev Disabil.* 35:1631–1638.

Hou, Q., Wang, Y., Li, Y., *et al.* (2018). A developmental study of abnormal behaviors and altered GABAergic signaling in the VPA-treated rat model of autism. *Frontiers in behavioral neuroscience*. 12:182.

Hughes, M.D. (2005). Multiplicity in clinical trials. *Encyclopedia of Biostatistics*. *5*.

Hyman, S.L., Levy, S.E., and Myers, S.M. (2020). Council on Children with Disabilities, Section on Developmental and Behavioral Pediatrics. Identification, evaluation, and management of children with autism spectrum disorder. *Pediatrics*. 145(1): e20193447.

Hull, L., Petrides, K.V. and Mandy, W. (2020). The female autism phenotype and camouflaging: A narrative review. *Review Journal of Autism and Developmental Disorders*. 7:306-317.

Hunt, J., Coulson, E.J., Rajnarayanan, R., *et al.* (2022). Sleep and circadian rhythms in Parkinson's disease and preclinical models. *Molecular neurodegeneration*. 17(1):1-21.

Jabarin, R., Netser, S. and Wagner, S. (2022). Beyond the three-chamber test: toward a multimodal and objective assessment of social behavior in rodents. *Molecular Autism*. 13(1):41.

Janíková, M. (2023). Cognitive flexibility in selected animal models of psychiatric disorders. (Doctoral thesis). Retrieved from Charles University Digital Repository. (183435)

Janušonis, S. (2014). Serotonin dynamics in and around the central nervous system: is autism solvable without fundamental insights?. *International Journal of Developmental Neuroscience*. 39:9-15.

Jeon, S.J., Gonzales, E.L., Mabunga, D.F.N., *et al.* (2018). Sex-specific behavioral features of rodent models of autism spectrum disorder. *Experimental neurobiology*. 27(5):321.

Ji, Q. and Zhu, Z.R. (2023). Genetic and neural mechanisms of sleep disorders in children with autism spectrum disorder: a review. *Frontiers in Psychiatry*. 14:1079683.

Kahne, D., Tudorica, A., Borella, A., *et al*. (2002). Behavioral and magnetic resonance spectroscopic studies in the rat hyperserotonemic model of autism. *Physiology & behavior*. 75(3):403-410.

Kamara, D. and Beauchaine, T.P. (2020). A review of sleep disturbances among infants and children with neurodevelopmental disorders. *Review journal of autism and developmental disorders*. 7:278-294.

Kang, J. and Kim, E. (2015). Suppression of NMDA receptor function in mice prenatally exposed to valproic acid improves social deficits and repetitive behaviors. *Frontiers in molecular neuroscience*. 8:17.

Kanner, L. (1943). Autistic disturbances of affective contact. *Nervous child*. 2(3):217-250.

Karmel, B.Z., Gardner, J.M., Meade, L.S., *et al*. (2010). Early medical and behavioral characteristics of NICU infants later classified with ASD. *Pediatrics*. 126(3):457-467.

Kataoka, S., Takuma, K., Hara, Y., *et al*. (2013). Autism-like behaviours with transient histone hyperacetylation in mice treated prenatally with valproic acid. *International journal of Neuropsychopharmacology*. 16(1):91-103.

Katz-Zetler, N., Gal, E. and Engel Yeger, B. (2023). Predictors of Daily Activity Performance of Children with Autism and its Association to Autism Characteristics. *The Open Journal of Occupational Therapy*. 11(1):1-9.

Kim, H., Kim, J.H., Kim, J., *et al*. (2023). Subjective and objective sleep alterations in medication-naïve children and adolescents with autism spectrum disorder: a systematic review and meta-analysis. *Epidemiology and Psychiatric Sciences*. 32: e48.

Kim, K.C., Kim, P., Go, H.S., *et al*. (2013a). Male-specific alteration in excitatory post-synaptic development and social interaction in pre-natal valproic acid exposure model of autism spectrum disorder. *Journal of neurochemistry*. 124(6):832-843.

Kim, P., Park, J.H., Kwon, K.J., *et al*. (2013b). Effects of Korean red ginseng extracts on neural tube defects and impairment of social interaction induced by prenatal exposure to valproic acid. *Food and chemical toxicology*. 51:288-296.

Kim, J.H., Kim, J.H., Cho, Y.E., *et al*. (2014a). Chronic sleep deprivation-induced proteome changes in astrocytes of the rat hypothalamus. *Journal of Proteome Research*. 13(9):4047-4061.

Kim, J.W., Seung, H., Kwon, K.J., *et al*. (2014b). Subchronic treatment of donepezil rescues impaired social, hyperactive, and stereotypic behavior in valproic acid-induced animal model of autism. *PloS one*. 9(8): e104927.

Kemper, T.L. and Bauman, M. (1998). Neuropathology of infantile autism. *Journal of neuropathology and experimental neurology*. 57(7):645-652.

Kempler, L. and Richmond, J.L. (2012). Effect of sleep on gross motor memory. *Memory*. 20(8):907-914.

Kling, A., Lancaster, J. and Benitone, J. (1970). Amygdalectomy in the free-ranging vervet (Cercopithecus aethiops). *Journal of Psychiatric Research*.

Kling, A.S. and Brothers, L.A. (1992). The amygdala and social behavior. *(No Title)*

Knez, R., Stevanovic, D., Fernell, E., *et al*. (2022). Orexin/Hypocretin system dysfunction in ESSENCE (early symptomatic syndromes eliciting neurodevelopmental clinical examinations). *Neuropsychiatric Disease and Treatment*. pp. 2683-2702.

Kohyama, J. (2016). Possible neuronal mechanisms of sleep disturbances in patients with autism

spectrum disorders and attention-deficit/hyperactivity disorder. *Medical Hypotheses*. 97:131-133.

Komariah, K., Kiranadi, B., Winarto, A., *et al.* (2018). Valproic Acid Administration in Pregnant Rats Affects Litter Size, Birth Weight, and Postnatal Growth of the Offspring. *Toxicological research*. 34(2):173-182.

Kootz, J.P., Marinelli, B. and Cohen, D.J. (1982). Modulation of response to environmental stimulation in autistic children. *Journal of autism and developmental disorders*. 12(2):185-193.

Kouklari, E.C., Tagkouli, E., Tsermentseli, S., *et al.* (2023). Cross-sectional Developmental Trajectories of Executive Function and Relations to Theory of Mind in Autism Spectrum Disorder. *Journal of Pediatric Neuropsychology*. 9(3):157-174.

Krakowiak P., Goodlin-Jones B., Hertz-Picciotto I., *et al.* (2008). Sleep problems in children with autism spectrum disorders, developmental delays, and typical development: a population-based study. *J Sleep Res*. 17:197-208

Kumar, D., Dedic, N., Flachskamm, C., *et al*. (2015). Cacna1c (Cav1. 2) modulates electroencephalographic rhythm and rapid eye movement sleep recovery. *Sleep*. *38*(9):1371-1380.

Kumar, N.S., Malhi, P., Bharti, B., *et al.* (2022). Restricted and Repetitive Behaviors and Interests in Young Children with Autism: A Comparative Study. *Indian Journal of Pediatrics*. 89(12):1216-1221.

Kumar, H. and Sharma, B. (2016a). Memantine ameliorates autistic behavior, biochemistry & blood brain barrier impairments in rats. *Brain research bulletin*. 124:27-39.

Kumar, H. and Sharma, B. (2016b). Minocycline ameliorates prenatal valproic acid induced autistic behaviour, biochemistry and blood brain barrier impairments in rats. *Brain research*. 1630:83-97.

Kuriyama, K., Stickgold, R. and Walker, M.P. (2004). Sleep-dependent learning and motor-skill complexity. *Learning & memory*. 11(6):705-713.

Lage, C., Smith, E.S. and Lawson, R.P. (2023). A Meta-Analysis of Cognitive Flexibility in Autism Spectrum Disorder. *Neuroscience & Biobehavioral Reviews*. p.105511.

Langlieb, J., Sachdev, N.S., Balderrama, K.S., *et al.* (2023). The cell type composition of the adult mouse brain revealed by single cell and spatial genomics. *bioRxiv*.

Lambert, A., Tessier, S., Rochette, A.C., *et al*. (2016). Poor sleep affects daytime functioning in typically developing and autistic children not complaining of sleep problems: A questionnaire-based and polysomnographic study. *Research in autism spectrum disorders*. 23:94-106.

Landa, R.J., Gross, A.L., Stuart, E.A., *et al*. (2013). Developmental trajectories in children with and without autism spectrum disorders: the first 3 years. *Child development*. 84(2):429-442.

Larner, O., Roberts, J., Twiss, J., *et al*. (2021). A need for consistency in behavioral phenotyping for ASD: analysis of the valproic acid model. *Autism Research and Treatment*. 2021: 8863256.

LeBarton, E.S. and Landa, R.J. (2019). Infant motor skill predicts later expressive language and autism spectrum disorder diagnosis. *Infant Behavior and Development*. 54:37-47.

LeDoux, J.E. (2000). Emotion circuits in the brain. *Annual review of neuroscience*. 23(1):155-184.

LeDoux, J. (2003). The emotional brain, fear, and the amygdala. *Cellular and molecular neurobiology*. 23:727-738.

Levin, J., Khokhlovich, E. and Vyshedskiy, A. (2022). Sleep problems effect on developmental trajectories in children with autism. *MedRxiv*. pp.2022-03.

Levy, D., Ronemus, M., Yamrom, B., *et al*. (2011). Rare de novo and transmitted copy-number variation in autistic spectrum disorders. *Neuron*. 70(5):886-897.

Ling, E.A., Paterson, J.A., Privat, A., *et al*. (1973). Investigation of glial cells in semithin sections. I. Identification of glial cells in the brains of young rats. *J. Comp. Neurol.* 149:43-71.

Loesch, D.Z., Huggins, R.M. and Hagerman, R.J. (2004). Phenotypic variation and FMRP levels in fragile X. *Mental retardation and developmental disabilities research reviews*. 10(1):31-41.

Long, Z., Duan, X., Mantini, D., *et al.* (2016). Alteration of functional connectivity in autism spectrum disorder: effect of age and anatomical distance. *Scientific reports*. 6(1):26527.

Loomes, R., Hull, L. and Mandy, W.P.L. (2017). What is the male-to-female ratio in autism spectrum disorder? A systematic review and meta-analysis. *Journal of the American Academy of Child & Adolescent Psychiatry*. 56(6):466-474.

Lorsung, E., Karthikeyan, R. and Cao, R. (2021). Biological timing and neurodevelopmental disorders: a role for circadian dysfunction in autism spectrum disorders. *Frontiers in Neuroscience*. 15(12): 642745.

Lueptow, L.M. (2017). Novel object recognition test for the investigation of learning and memory in mice. *Journal of Visualized Experiments.* (126): e55718.

MacDuffle, K.E., Shen, M.D., Dager, S.R., *et al*. (2020). Sleep onset problems and subcortical development in infants later diagnosed with autism spectrum disorder. *American Journal of Psychiatry*. 177(6): 518-525.

Mahmood, U., Ahn, S., Yang, E.J., *et al*. (2018). Dendritic spine anomalies and PTEN alterations in a mouse model of VPA-induced autism spectrum disorder. *Pharmacological research*. 128:110-121.

Mabunga, D.F.N., Gonzales, E.L.T., Kim, J.W., *et al*. (2015). Exploring the validity of valproic acid animal model of autism. *Experimental neurobiology*. 24(4):285.

Main, S.L. and Kulesza, R.J. (2017). Repeated prenatal exposure to valproic acid results in cerebellar hypoplasia and ataxia. *Neuroscience*. 340:34-47.

Malow, B.A., Marzec, M.L., McGrew, S.G., *et al*. (2006). Characterizing sleep in children with autism spectrum disorders: a multidimensional approach. *Sleep*. 29(12):1563-1571.

Malow, B., Adkins, K.W., McGrew, S.G., *et al*. (2012). Melatonin for sleep in children with autism: a controlled trial examining dose, tolerability, and outcomes. *Journal of autism and developmental disorders*. 42:1729-1737.

Málaga, I., Blanco Lago, R., Hedrera-Fernández, A., *et al*. (2019). Prevalence of autism spectrum disorders in children in the United States, Europe and Spain: coincidences and discrepancies. *Medicine (Buenos Aires)*. 79(1):4-9.

Markram, K., Rinaldi, T., Mendola, D.L., *et al*. (2008). Abnormal fear conditioning and amygdala processing in an animal model of autism. *Neuropsychopharmacology*. 33(4):901-912.

Marotta, R., Risoleo, M.C., Messina, G., *et al*. (2020). The neurochemistry of autism. *Brain Sciences*. 10:163.

Maski, K., Holbrook, H., Manoach, D., *et al*. (2015). Sleep dependent memory consolidation in children with autism spectrum disorder. *Sleep*. 38(12):1955-1963.

Mathiasen, J.R. and DiCamillo, A. (2010). Novel object recognition in the rat: a facile assay for cognitive function. *Current protocols in pharmacology*. *49*(1):5-59.

Maurer, J.J., Choi, A., An, I., *et al*. (2023). Sleep disturbances in autism spectrum disorder: Animal models, neural mechanisms, and therapeutics. *Neurobiology of Sleep and Circadian Rhythms*. p.100095.

Mazzone, L., Postorino, V., Siracusano, M., *et al*. (2018). The relationship between sleep problems, neurobiological alterations, core symptoms of autism spectrum disorder, and psychiatric comorbidities. *J Clin Med*. 7:102.

McArthur, G.E., Lee, E. and Laycock, R. (2023). Autism traits and cognitive performance: Mediating roles of sleep disturbance, anxiety and depression. *Journal of autism and developmental disorders*. 53(12):4560-4576.

McCann, M., Bayliss, D.M., Pestell, C., *et al*. (2018). The relationship between sleep and working memory in children with neurological conditions. *Child Neuropsychology*. 24(3):304-321.

Melancia, F., Schiavi, S., Servadio, M., *et al.* (2018). Sex-specific autistic endophenotypes induced by prenatal exposure to valproic acid involve anandamide signalling. *British journal of pharmacology*. 175(18):3699-3712.

Melke, J., Goubran Botros, H., Chaste, P., *et al*. (2008). Abnormal melatonin synthesis in autism spectrum disorders. *Molecular psychiatry*.13(1):90-98.

Miedel, C.J., Patton, J.M., Miedel, A.N., *et al*. (2017). Assessment of spontaneous alternation, novel object recognition and limb clasping in transgenic mouse models of amyloid-β and tau neuropathology. *Journal of Visualized Experiments.* (123): e55523.

Mirmiran, M., Maas, Y.G., and Ariagno, R.L. (2003). Development of fetal and neonatal sleep and circadian rhythms. *Sleep Medicine Reviews*. 7(4): 321-334.

Missig, G., McDougle, C.J. and Carlezon, W.A. (2020). Sleep as a translationally-relevant endpoint in studies of autism spectrum disorder (ASD). *Neuropsychopharmacology*. 45(1):90-103.

Miyazaki, K., Narita, N. and Narita, M. (2005). Maternal administration of thalidomide or valproic acid causes abnormal serotonergic neurons in the offspring: implication for pathogenesis of autism. *International Journal of Developmental Neuroscience*. 23(2-3):287-297.

Moffitt, J.R., Bambah-Mukku, D., Eichhorn, S.W., *et al*. (2018). Molecular, spatial, and functional single-cell profiling of the hypothalamic preoptic region. *Science*. 362(6416): eaau5324.

Mohd Nordin, A., Ismail, J. and Kamal Nor, N. (2021). Motor development in children with autism spectrum disorder. *Frontiers in pediatrics*. 9:598276.

Murta, V., Seiffe, A. and Depino, A.M. (2023). Sex Differences in Mouse Models of Autism Spectrum Disorders: Their Potential to Uncover the Impact of Brain Sexual Differentiation on Gender Bias. *Sexes*. 4(3):358-391.

Muschamp, J.W., Dominguez, J.M., Sato, S.M., *et al*. (2007). A role for hypocretin (orexin) in male sexual behaviour. *J. Neurosci*. 27:2837–2845.

Mychasiuk, R., Richards, S., Nakahashi, A., *et al*. (2012). Effects of rat prenatal exposure to valproic acid on behaviour and neuro-anatomy. *Developmental neuroscience*. 34(2-3):268-276.

Nambu, T., Sakurai, T., Mizukami, K., *et al*. (1999). Distribution of orexin neurons in the adult rat brain. *Brain research*. 827(1-2):243-260.

Napolitano, A., Schiavi, S., La Rosa, P., *et al*. (2022). Sex differences in autism Spectrum disorder: diagnostic, neurobiological, and behavioral features. *Frontiers in Psychiatry*. 13:889636.

Netser, S., Haskal, S., Magalnik, H., *et al*. (2017). A novel system for tracking social preference dynamics in mice reveals sex-and strain-specific characteristics. *Molecular autism*. 8:1-14.

Neul, J.L. (2012). The relationship of Rett syndrome and MECP2 disorders to autism. *Dialogues in clinical neuroscience*. 14(3):253-262.

Nicholas, J.S., Charles, J.M., Carpenter, L.A., *et al*. (2008). Prevalence and characteristics of children with autism-spectrum disorders. *Annals of Epidemiology*. 18:130–136.

Nixon, J.P. and Smale, L. (2007). A comparative analysis of the distribution of immunoreactive orexin A and B in the brains of nocturnal and diurnal rodents. *Behavioral and Brain Functions*. 3:1-27.

Nolan, S.O., Reynolds, C.D., Smith, G.D., *et al*. (2017). Deletion of Fmr1 results in sex-specific changes in behavior. *Brain and behavior*. 7(10): e00800.

Ortiz, C., Navarro, J.F., Jurek, A., *et al*. (2020). Molecular atlas of the adult mouse brain. *Science advances*. 6(26): eabb3446.

Owens, J.A., Spirito, A., McGuinn, M. (2000). The Children's Sleep Habits Questionnaire (CSHQ): psychometric properties of a survey instrument for school-aged children. *Sleep*. 23:1043-1051.

Paavilainen, L., Wernérus, H., Nilsson, P., *et al*. (2008). Evaluation of monospecific antibodies: a comparison study with commercial analogs using immunohistochemistry on tissue microarrays. *Applied Immunohistochemistry & Molecular Morphology*. 16(5):493-502.

Pandi-Perumal, S.R., Cardinali, D.P., Zaki, N.F., *et al*. (2022). Timing is everything: circadian rhythms and their role in the control of sleep. *Frontiers in neuroendocrinology*. p.100978.

Park, H.R., Lee, J.M., Moon, H.E., *et al*. (2016). A short review on the current understanding of autism spectrum disorders. *Experimental neurobiology*. 25(1):1.

Paxinos, G. and Watson, C. (2006). The rat brain in stereotaxic coordinates: hard cover edition. Amsterdam. Elsevier.

Peerboom, C. and Wierenga, C.J. (2021). The postnatal GABA shift: a developmental perspective. *Neuroscience & Biobehavioral Reviews*. 124:179-192.

Peyron, C., Tighe, D.K., Van Den Pol, A.N., *et al*. (1998). Neurons containing hypocretin (orexin) project to multiple neuronal systems. *Journal of Neuroscience*. 18(23):9996-10015.

Pierce, K. and Courchesne, E. (2001). Evidence for a cerebellar role in reduced exploration and stereotyped behavior in autism. *Biological psychiatry*. 49(8):655-664.

Piri, F., Salmani, M.E. and Sepehri, H. (2024). Improvement of autistic-like behaviors in adult rats prenatally exposed to valproic acid through early suppression of orexin receptor. *Annals of Medicine and Surgery*. 86(1):166-171.

Pizza, F., Barateau, L., Dauvilliers, Y., *et al*. (2022). The orexin story, sleep and sleep disturbances. *Journal of Sleep Research*. p.e13665.

Prior, M. and Macmillan, M.B. (1973). Maintenance of sameness in children with Kanner's syndrome. *Journal of Autism and Childhood Schizophrenia*. 3(2):154-167.

Prober, D.A., Rihel, J., Onah, A.A., *et al*. (2006). Hypocretin/Orexin Overexpression Induces An Insomnia-Like Phenotype in Zebrafish. *J. Neurosci.* 26:13400–13410.

Proschan, M.A. and Waclawiw, M.A. (2000). Practical guidelines for multiplicity adjustment in clinical trials. *Controlled clinical trials*. 21(6):527-539.

Reynolds, A.M. and Malow, B.A. (2011). Sleep and autism spectrum disorders. *Pediatric Clinics*. 58(3):685-698.

Richards, L. (2022). Analyzing the genetic mechanisms for the sex-biased penetrance of autism spectrum disorder. (Senior thesis). Retrieved from Claremont Theses. (2806)

Richdale, A.L. and Schreck, K.A. (2009). Sleep problems in autism spectrum disorders: prevalence, nature, & possible biopsychosocial aetiologies. *Sleep medicine reviews*. 13(6):403-411.

Rivet, T.T. and Matson, J.L. (2011). Review of gender differences in core symptomatology in autism spectrum disorders. *Research in Autism Spectrum Disorders*. 5(3):957-976.

Robinson, E.B., Lichtenstein, P., Anckarsäter, H., *et al*. (2013). Examining and interpreting the female protective effect against autistic behavior. *Proceedings of the National Academy of Sciences*. 110(13):5258-5262.

Rodier, P.M., Ingram, J.L., Tisdale, B., *et al*. (1996). Embryological origin for autism: developmental anomalies of the cranial nerve motor nuclei. *Journal of Comparative Neurology*. 370(2):247-261.

Ross, J. (2015). *Crash Course Nervous System Updated Edition-E-Book*. Elsevier Health Sciences.

Rudy, J.W., Huff, N.C. and Matus-Amat, P. (2004). Understanding contextual fear conditioning: insights from a two-process model. *Neuroscience & Biobehavioral Reviews*. 28(7):675-685.

Safari, S., Ahmadi, N., Mohammadkhani, R., *et al*. (2021). Sex differences in spatial learning and memory and hippocampal long-term potentiation at perforant pathway-dentate gyrus (PP-DG) synapses in Wistar rats. *Behavioral and Brain Functions*. 17:1-11.

Sakaki, M., Yoo, H.J., Nga, L., *et al*. (2016). Heart rate variability is associated with amygdala functional connectivity with MPFC across younger and older adults. *Neuroimage*. 139:44-52.

Sakurai, T. (2007). The neural circuit of orexin (hypocretin): maintaining sleep and wakefulness. *Nature Reviews Neuroscience*. 8(3):171-181.

Sakurai, T., Nagata, R., Yamanaka, A., *et al*. (2005). Input of orexin/hypocretin neurons revealed by a genetically encoded tracer in mice. *Neuron*. 46(2):297-308.

Saper, C. B., Chou, T. C., and Scammell, T. E. (2001). The sleep switch: hypothalamic control of sleep and wakefulness. *Trends Neurosci.* 24:726–731.

Saper, C.B., Fuller, P.M., Pedersen, N.P., *et al*. (2010). Sleep state switching. *Neuron*. 68:1023-1042.

Scalise, C.B. (2023). *Effects of Sleep Deprivation on Memory in Mouse Model of Autism: Awake but at What Cost?*. (Doctoral dissertation). Retrieved from Southeastern Louisiana University ProQuest Dissertations Publishing. (30813327)

Schain, R.J. and Freedman, D.X. (1961). Studies on 5-hydroxyindole metabolism in autistic and other mentally retarded children. *The Journal of pediatrics*. 58(3):315-320.

Schiavi, S., Iezzi, D., Manduca, A., *et al*. (2019). Reward-related behavioral, neurochemical and electrophysiological changes in a rat model of autism based on prenatal exposure to valproic acid. *Frontiers in cellular neuroscience*. 13:479.

Schmitt, O., Usunoff, K.G., Lazarov, N.E., *et al*. (2012). Orexinergic innervation of the extended amygdala and basal ganglia in the rat. *Brain Structure and Function*. 217:233-256.

Schneider, T. and Przewłocki, R. (2005). Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. *Neuropsychopharmacology*. 30(1):80-89.

Schreck, K.A., Mulick, J.A., Smith, A.F. (2004). Sleep problems as possible predictors of intensified symptoms of autism. *Res Dev Disabil*. 25:57-66.

Schulte, J.T., Wierenga, C.J. and Bruining, H. (2018). Chloride transporters and GABA polarity in developmental, neurological and psychiatric conditions. *Neuroscience & Biobehavioral Reviews*. 90:260-271.

Schumann, C.M., Hamstra, J., Goodlin-Jones, B.L., *et al*. (2004). The amygdala is enlarged in children but not adolescents with autism; the hippocampus is enlarged at all ages. *Journal of neuroscience*. 24(28):6392-6401.

Schumann, C.M. and Amaral, D.G. (2006). Stereological analysis of amygdala neuron number in autism. *Journal of Neuroscience*. 26(29):7674-7679.

Siletti, K., Hodge, R., Mossi, A.A., *et al*. (2023). Transcriptomic diversity of cell types across the adult human brain. *Science*. 382(6667): eadd7046.

Sipes, M., Matson, J.L., Worley, J.A., *et al*. (2011). Gender differences in symptoms of autism spectrum disorders in toddlers. *Research in Autism Spectrum Disorders*. 5(4):1465-1470.

Skefos, J., Cummings, C., Enzer, K., *et al*. (2014). Regional alterations in purkinje cell density in patients with autism. *PloS one*. 9(2): e81255.

Slotnick, B.M. and Leonard, C.M. (1975). A stereotaxic atlas of the albino mouse forebrain (Vol. 75). Washington, DC. US Department of Health, Education, and Welfare, Public Health Service, Alcohol, Drug Abuse, and Mental Health Administration.

Smith, E.E., Jonides, J. and Koeppe, R.A. (1996). Dissociating verbal and spatial working memory using PET. *Cerebral cortex*. 6(1):11-20.

Sokolowski, K., Esumi, S., Hirata, T., *et al*. (2015). Specification of select hypothalamic circuits and innate behaviours by theembryonicpatterninggenedbx1. *Neuron*. 86:403–416.

Soorya, L., Kiarashi, J. and Hollander, E. (2008). Psychopharmacologic interventions for repetitive behaviors in autism spectrum disorders. *Child and Adolescent Psychiatric Clinics of North America*. 17(4):753-771.

Souders, M.C., Mason, T.B., Valladares, O., *et al*. (2009). Sleep behaviors and sleep quality in children with autism spectrum disorders. *Sleep*. 32(12):1566-1578.

Sparks, B.F., Friedman, S.D., Shaw, D.W., *et al*. (2002). Brain structural abnormalities in young children with autism spectrum disorder. *Neurology*. 59(2):184-192.

Staples, K.L. and Reid, G. (2010). Fundamental movement skills and autism spectrum disorders. *Journal of autism and developmental disorders*. 40:209-217.

Šušmáková, K. and Krakovská, A. (2008). Discrimination ability of individual measures used in sleep stages classification. *Artificial intelligence in medicine*. 44(3):261-277.

Takumi, T., Tamada, K., Hatanaka, F., *et al*. (2020). Behavioral neuroscience of autism. *Neuroscience & Biobehavioral Reviews*. 110:60-76.

Tam, S.K., Bannerman, D.M. and Peirson, S.N. (2020). Mechanisms mediating the effects of light on sleep and alertness: current challenges. *Current Opinion in Physiology*. 15:152-158.

Tang, S., Huang, W., Lu, S., *et al*. (2017). Increased Plasma Orexin-A Levels in Patients with Insomnia Disorder Are Not Associated with Prepro-Orexin or Orexin Receptor Gene Polymorphisms. *Peptides*. 88:55–61.

Tordjman, S., Anderson, G.M., Cohen, D., *et al*. (2013). Presence of autism, hyperserotonemia, and severe expressive language impairment in Williams-Beuren syndrome. *Molecular Autism*. *4*:1-10.

Touma, C., Palme, R. and Sachser, N. (2004). Analyzing corticosterone metabolites in fecal samples of mice: a noninvasive technique to monitor stress hormones. *Hormones and behavior*. 45(1):10-22.

Tsujino, N., Nakatani, Y., Seki, Y., *et al.* (2007). Abnormality of circadian rhythm accompanied by an increase in frontal cortex serotonin in animal model of autism. *Neuroscience research*. 57(2):289-295.

Vanneau, T., Quiquempoix, M., Trignol, A., *et al.* (2021). Determination of the sleep–wake pattern and feasibility of NREM/REM discrimination using the non-invasive piezoelectric system in rats. *Journal of Sleep Research*. 30(6): e13373.

Verhoeff, M.E., Blanken, M.L.E., Kocevska, D., *et al.* (2018). The bidirectional association between sleep problems and autism spectrum disorder: a population-based cohort study. *Molecular Autism*. 9:8.

Vitaterna, M.H., Takahashi, J.S. and Turek, F.W. (2001). Overview of circadian rhythms. *Alcohol research & health*. 25(2):85.

Walker, M.P., Brakefield, T., Morgan, A., *et al.* (2002). Practice with sleep makes perfect: sleep-dependent motor skill learning. *Neuron*. 35(1):205-211.

Walker, M.P. (2005). A refined model of sleep and the time course of memory formation. *Behavioral and brain sciences*. 28(1):51-64.

Wang, Y., Zhang, Y.B., Liu, L.L., *et al.* (2017). A meta-analysis of working memory impairments in autism spectrum disorders. *Neuropsychology review*. 27:46-61.

Wang, J., Feng, S., Li, M., *et al*. (2019). Increased expression of Kv10. 2 in the hippocampus attenuates valproic acid-induced autism-like behaviors in rats. *Neurochemical Research*. 44:2796-2808.

Wegiel, J., Flory, M., Kuchna, I., *et al.* (2014). Brain-region–specific alterations of the trajectories of neuronal volume growth throughout the lifespan in autism. *Acta neuropathologica communications*. 2:1-18.

Welch, J.D., Kozareva, V., Ferreira, A., *et al.* (2019). Single-cell multi-omic integration compares and contrasts features of brain cell identity. *Cell*. 177(7):1873-1887.

Wellman, L.L., Lonart, G., Adkins, A.M., *et al*. (2022). Regulation of dark period sleep by the amygdala: a microinjection and optogenetics study. *Brain Res*. 1781:147816.

Wiggs, L. and Stores, G. (2004). Sleep patterns and sleep disorders in children with autistic spectrum disorders: insights using parent report and actigraphy. *Developmental medicine and child neurology*. 46(6):372-380.

Williams, D.L., Goldstein, G., Carpenter, P.A., *et al.* (2005). Verbal and spatial working memory in autism. *Journal of autism and developmental disorders*. 35:747-756.

Wilson, C.E., Murphy, C.M., McAlonan, G., *et al.* (2016). Does sex influence the diagnostic evaluation of autism spectrum disorder in adults?. *Autism*. 20(7):808-819.

Yaghouby, F., Donohue, K.D., O'Hara, B.F., *et al.* (2016). Noninvasive dissection of mouse sleep using a piezoelectric motion sensor. *Journal of neuroscience methods*. 259:90-100.

Yelin-Bekerman, L., Elbaz, I., Diber, A., *et al.* (2015). Hypocretin neuron-specific transcriptome profiling identifies the sleep modulator Kcnh4a. *eLife*. 4: e08638

Zeif, D. and Yechiam, E. (2020). Autism is not associated with poor or enhanced performance on the Iowa Gambling Task: A Meta-Analysis. *Neuroscience & Biobehavioral Reviews*. 113:440-447.

Zeng, T., Mott, C., Mollicone, D., *et al*. (2012). Automated determination of wakefulness and sleep in rats based on non-invasively acquired measures of movement and respiratory activity. *Journal of neuroscience methods*. 204(2):276-287.

Zhang, X.Y., Yu, L., Zhuang, Q.X., *et al*. (2013). Central functions of the orexinergic system. *Neuroscience bulletin*. 29:355-365.

Zhang, R., Zhou, J., Ren, J., *et al.* (2018). Transcriptional and splicing dysregulation in the prefrontal cortex in valproic acid rat model of autism. *Reproductive Toxicology*. *77*:53-61.

APPENDIX A: Animal Research Ethics

Clearance certificate No. 2022/10/03/B

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: 2022/10/03/B

APPLICANT: Ms M Gaffane
School: School of Anatomical Sciences; Department: ; Location: WRAF

PROJECT TITLE: Does sleep disturbance affect the neuroanatomical basis of sleep systems and thereby exacerbate symptoms in Autism? A detailed account on the Orexinergic system in a valproic acid *Rattus norvegicus* (Sprague Dawley) rat model of autism. Category: B; Species and Numbers involved: 84 total (12 dams + 72 pups), Dams at 10-12 weeks old nulliparous, pups from birth to 50 days, male & 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 25 Oct 2022. This approval remains valid until 13 Nov 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: ____14/11/2022____

(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

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)

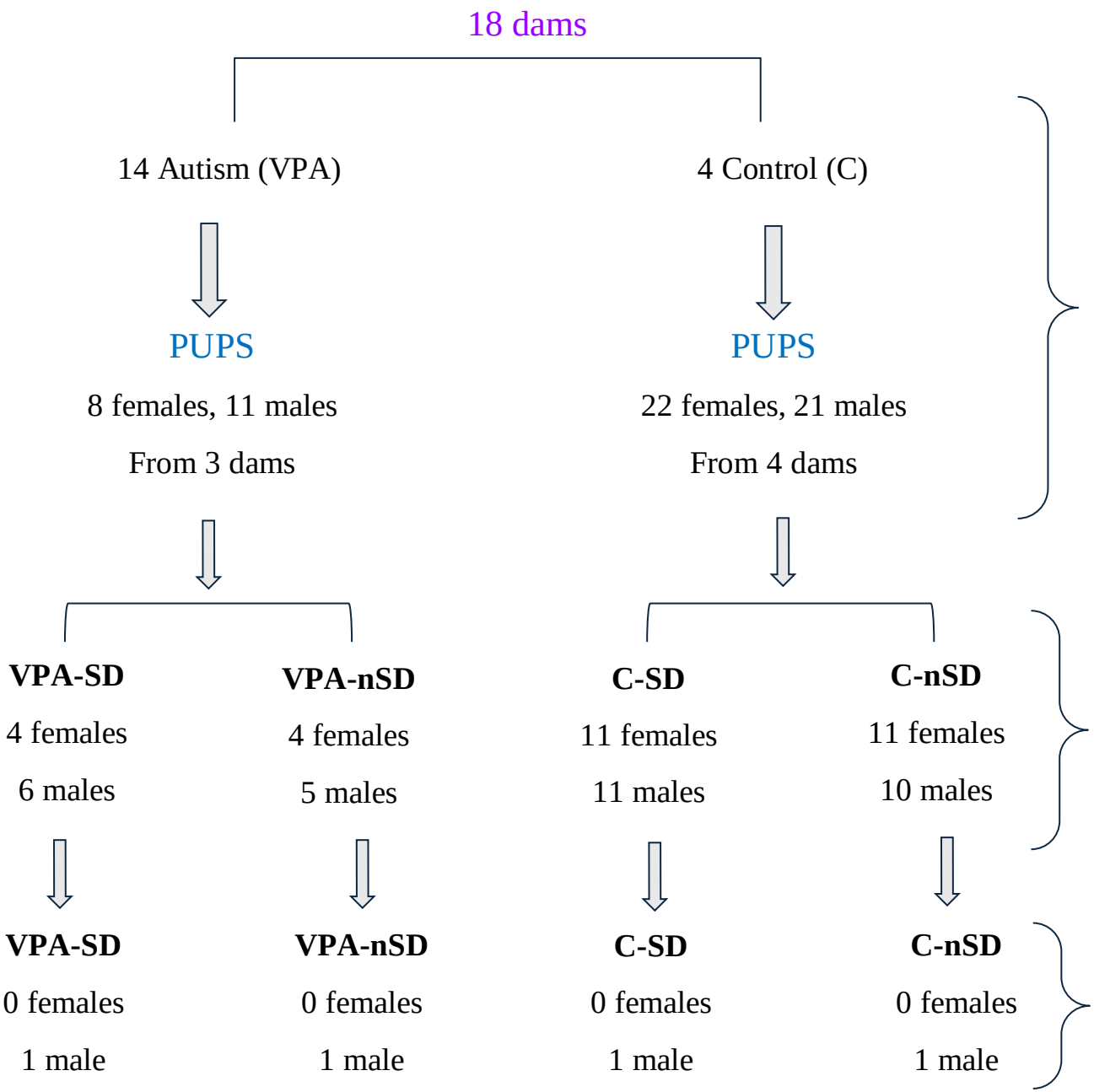
UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Signed: __  _____ Date: __14/11/2022_____

(Registered Veterinarian)

APPENDIX B: Sprague Dawley Sampling

Sprague Dawley (SD) Sampling



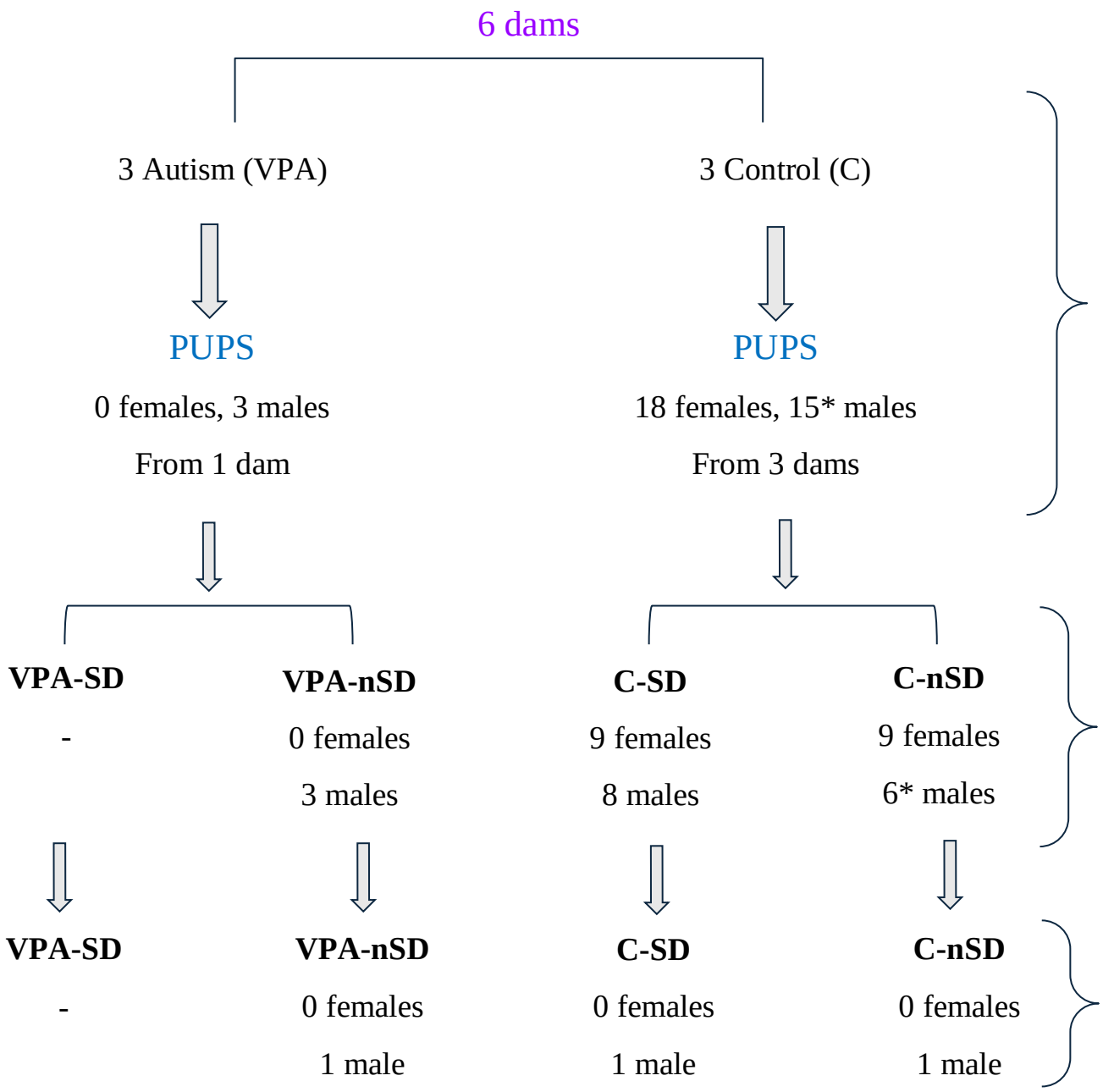
18 dams were assigned to either the VPA or C sample groups according to relative pup litter sizes across the 3 rounds.

At weaning, males and females from each dam's litter were separated.

The males and females were then randomly separated into 2 further sample groups for the sleep disturbance protocol and behavioural testing.

For neuroanatomical analysis, one male from each group were selected to comprise each sample group for the preliminary orexinergic analysis.

SD Sampling – First Round



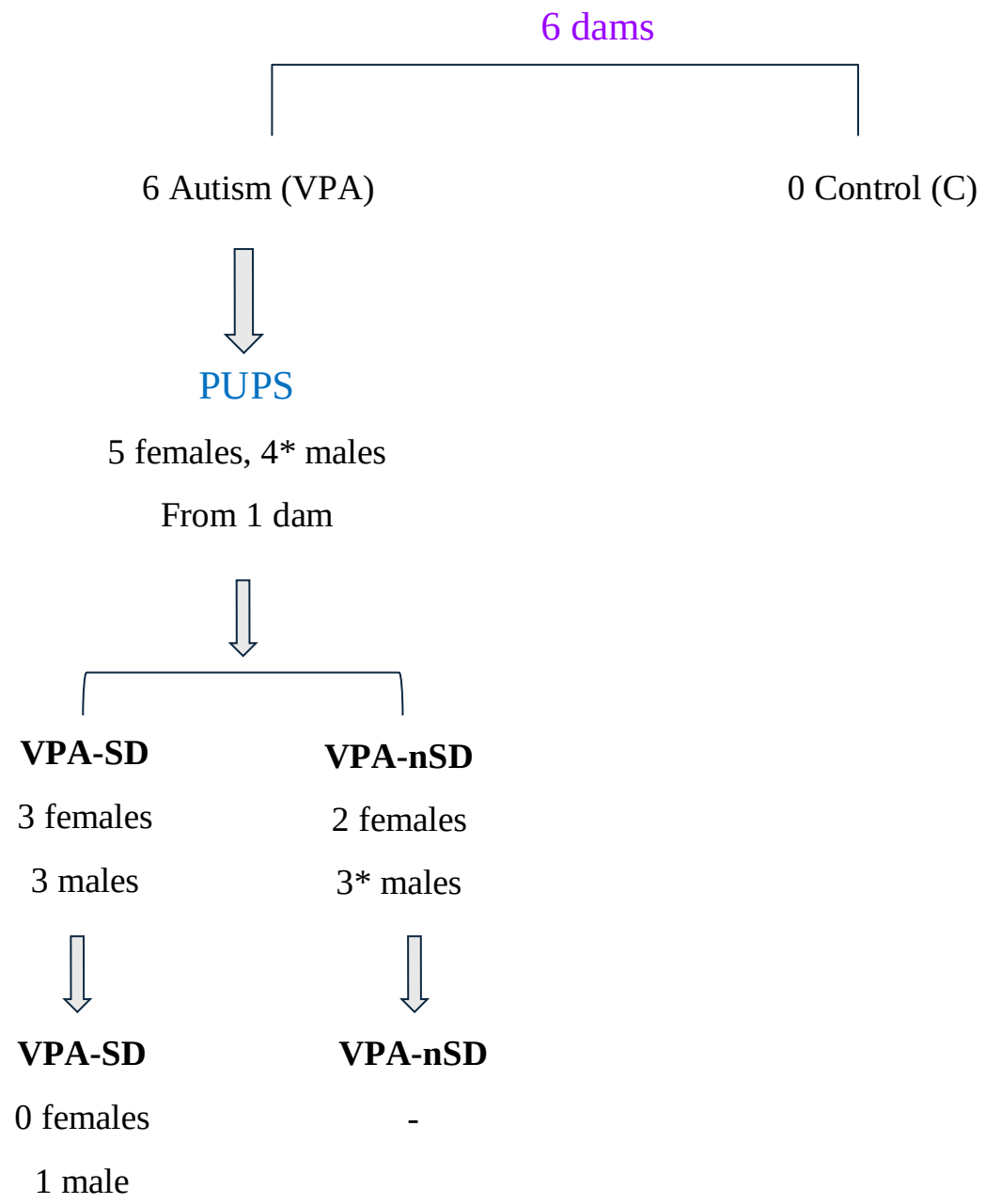
6 dams were randomly assigned to either the VPA or C sample groups.

At weaning, males and females from each dam's litter were separated.

The males and females were then randomly separated into 2 further sample groups for the sleep disturbance protocol and behavioural testing.

**Only 14 C males surviving at start of testing – 1 male from C-nSD found dead in cage resulting in 6 remaining.*

SD Sampling – Second Round



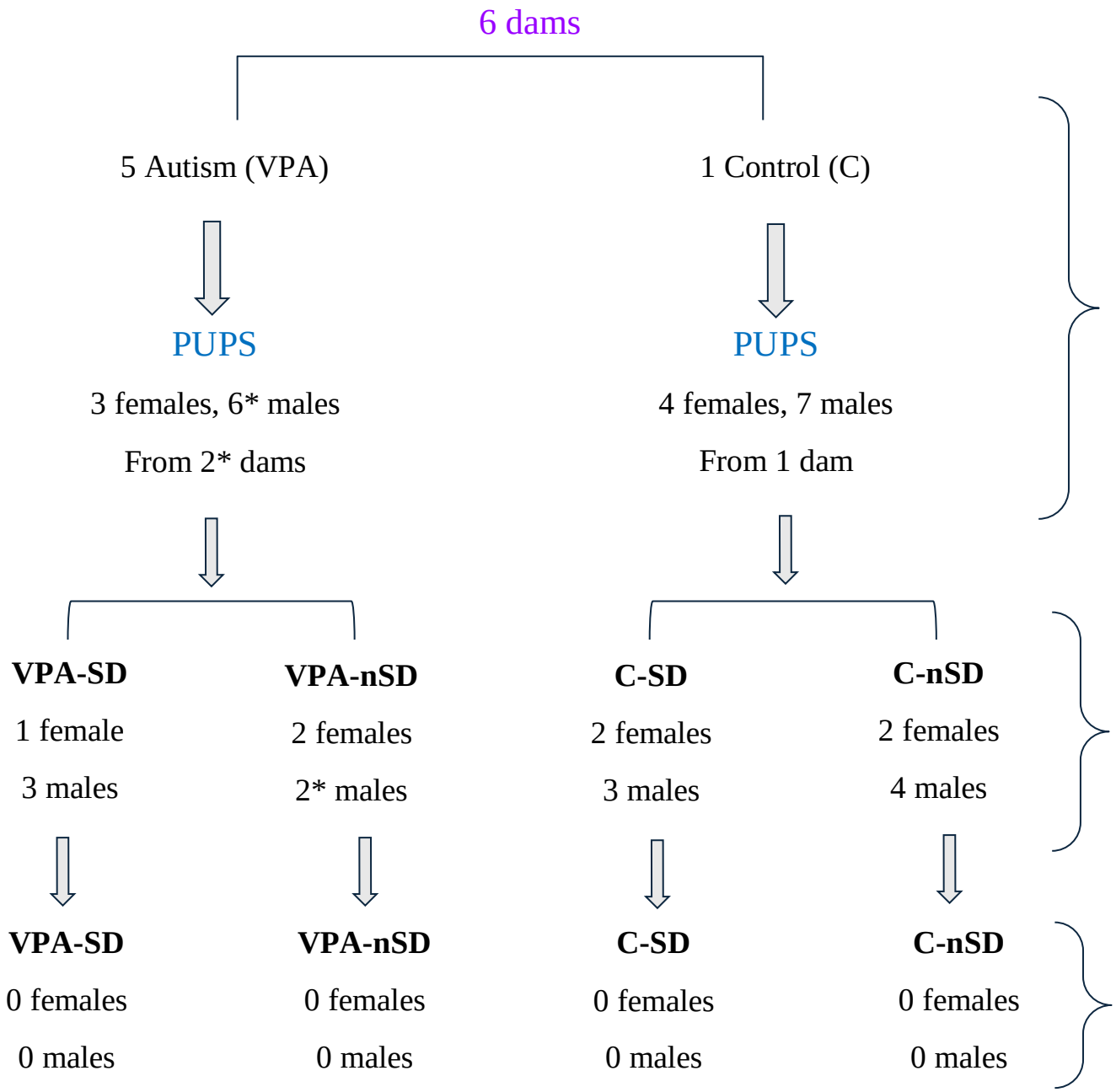
Following poor outcomes from the VPA dams in the first round (only 3 males from 1/3 dams), all 6 dams in this round were assigned to the VPA sample group.

At weaning, males and females from each dam's litter were separated.

The males and females were then randomly separated into 2 further sample groups for the sleep disturbance protocol and behavioural testing.

** Only 3 surviving males at the start of testing – only male from VPA-nSD euthanized to minimize suffering from emaciation and ruptured eyeball.*

SD Sampling – Third Round



6 dams were unevenly assigned at random to either the VPA or C sample groups.

At weaning, males and females from each dam's litter were separated.

The males and females were then randomly separated into 2 further sample groups for the sleep disturbance protocol and behavioural testing.

Only 5 males surviving at start of testing – 1 male from VPA-nSD (from separate litter) found dead in cage resulting in 5 remaining pups from a single dam.

APPENDIX C: Wits Research Animal Facility Welfare Scoring Sheet

PI NAME:							AREC#:			
PROCEDURE/ INTERVENTION							DATE:			
	Day:	ID#:	Day:	ID#:	Day:	ID#:	Day:	ID#:	Day:	ID#:
UNDISTURBED OBSERVATION	comment									
Eating and drinking: *Yes, No*										
Posture*: Normal, Huddled*										
Mood: *Calm, Restless, Lethargic, Depressed*										
Movement: *Normal, Hunched*										
Fur*: Groomed, Ruffled*										
Breathing: *Normal, Laboured, gasping*										
Face: See *Grimace scale*										
Vocalisation: *None, whimper*										
CAGE OPEN/ HANDLING	comment									
Alert/ Inquisitive, Avoidance										
Approachable, Aggressive, Fear										
Diarrhoea, Blood on bedding										
CLINICAL SIGNS	comment									
Eyes: *Bright, Red rimmed, Dim*										
Nose: *Clean, Discharge*										
Skin: *Firm elastic, Tented dry*										
Intervention site: *Clean, Inflamed, oozing, blood*										
Self- Mutilation: *Chew, bite, scratch self*										
Body mass: *Same, Gain, Loss*										
OTHER OBSERVATION/ COMMENT										

Observations ONCE per day: Record animal ID in line when recording of abnormality/ concern

Score: 0 Normal; 1 Mild 2 Moderate; 3 Severe
Actions to take: 0- 1 Record
2 Record, REPORT and observe more often
3 Record, REPORT and evaluate for humane termination

Developed by: M Costello Checked by: K Jardine 2018/12/18**Version #:** 001AREC Approval:Click or tap to enter a date.

APPENDIX D: Multiple Linear Regression Assumptions

Multiple linear regressions (MLR) were run for each behavioural test to determine the extent to which the three independent variables, namely, (1) sex, (2) sleep disturbance and (3) autism pathology (injection) could be used to predict the outcome of the tests' respective outcome variables. As such, the data of each behavioural test were tested to ensure that the eight necessary assumptions were satisfied to ensure valid interpretation of the output of the statistical analysis.

The first assumption required that two or more of the independent variables be measured at either the continuous or categorical scale. This assumption was satisfied as all three independent variables were dichotomous nominal (categorical) variables.

Secondly, the MLR required that the outcome variable be measured on the continuous scale. The number of marbles buried obtained from the marble burying test were counts (i.e. discrete) data and, thus, transformed in a manner that enabled the other assumptions to be met. This was found to be rank transformation. The number of hind-paw foot slips obtained from the balance beam test were not discrete, however, had to be transformed in order to satisfy the other assumptions. The square root transformation was found to be most appropriate. The outcome variables of all the other tests (YM – SAS (%), NOR – proportion of total test time spent investigating novel object (s), 3C – time spent investigating social stimulus (s)) were continuous and were analysed without transformation.

The third assumption required the independence of the residuals (no autocorrelation). This was assessed using the Durbin Watson (DW) Statistic generated by the MLR test itself. This statistic measures the extent to which variables are correlated to one another across the data set and assumes any value between zero and four. According to the classification of values across this scale (Table 1), the data of all of the tests satisfied this assumption (Table 2).

Table 1: Degree of correlation obtained from the Durbin Watson test statistic.

d	Autocorrelation
$d < 1.50$	Negative
$1.50 < d < 2.50$	None
$d > 2.50$	Positive

KEY: "d" represents the value of the test statistic.

Table 2: Durbin Watson test statistics.

Behavioural test	DW statistic
MB	1.496
BB	1.580
YM	1.554
NO	2.267
3C	1.925

The fourth assumption required the homoscedasticity of the residuals, otherwise described as the equality of the variance in the error of the residuals. This was assessed by observation of a scatterplot showing the relationship between standard predicted values and their residuals. For the assumption to be satisfied, the Loess line of best fit should appear near parallel to the x-axis. Three of the behavioural tests (BB, NOR and 3C) (Figure 1 B, D and E respectively) satisfied this assumption however, MB and YM appeared to exhibit slight deviations (Figure 1, A and C respectively).

The fifth assumption of the MLR required the normality of the residuals which tests the normality of the theoretical and not observed (standardised) residuals. These theoretical values were not available and, thus, could not be tested. Instead, the normality of the standardised residuals was assessed through observation of observed and expected cumulative probability values on a Normal Probability-Probability (P-P) plot. This assumption was satisfied for those values observed to adhere closely to the slope line.

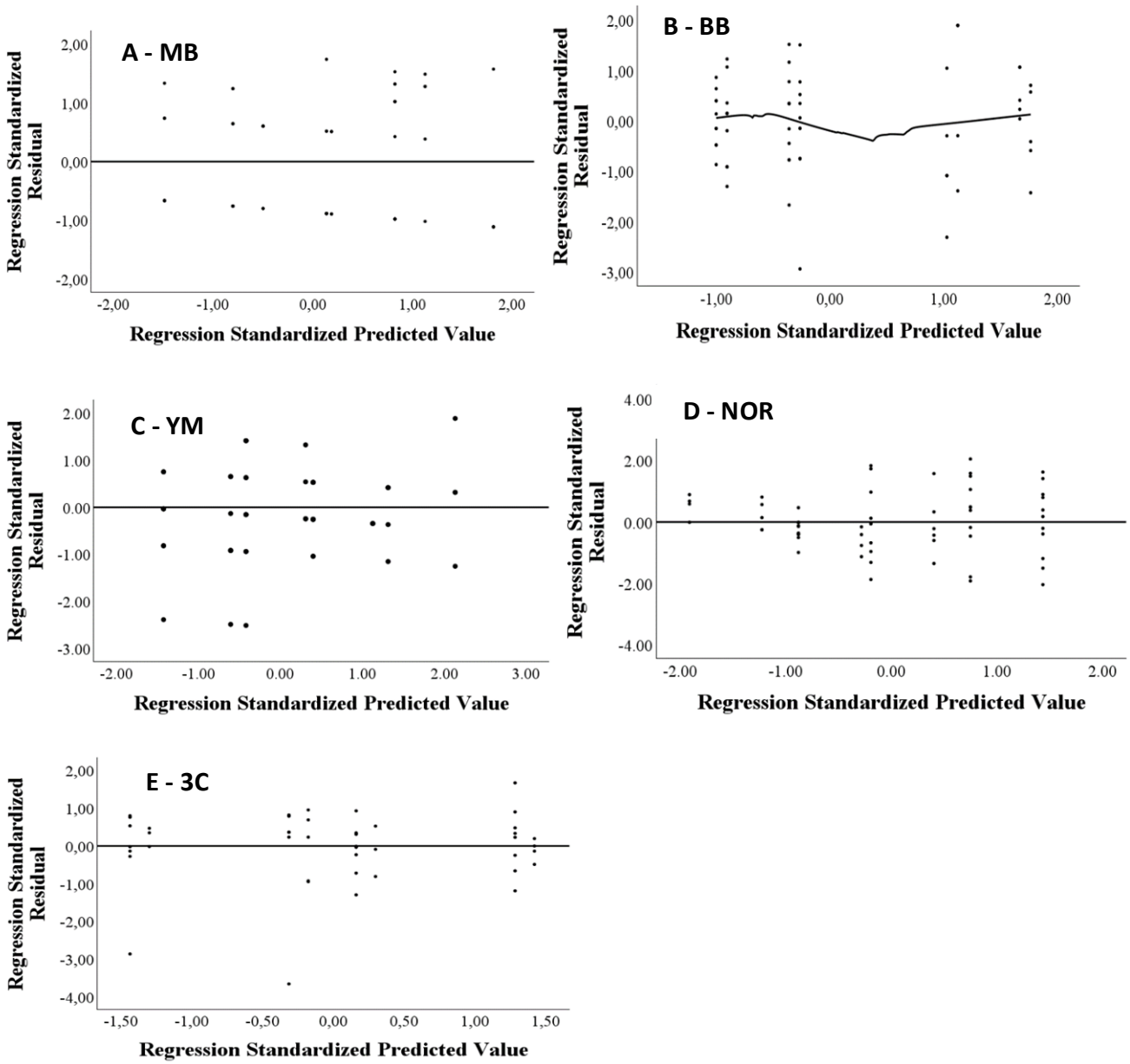


Figure 1: Scatterplots for each behavioural test [A – Marble Burying (MB), B – Balance Beam (BB), C – Y-Maze (YM), D – Novel Object Recognition (NOR), E – Three Chamber (3C)] showing the relationship between the regression standard predicted values on the x-axis and regression standard residuals on the y-axis. Near parallel Loess lines of best fit show acceptable degree of homoscedasticity.

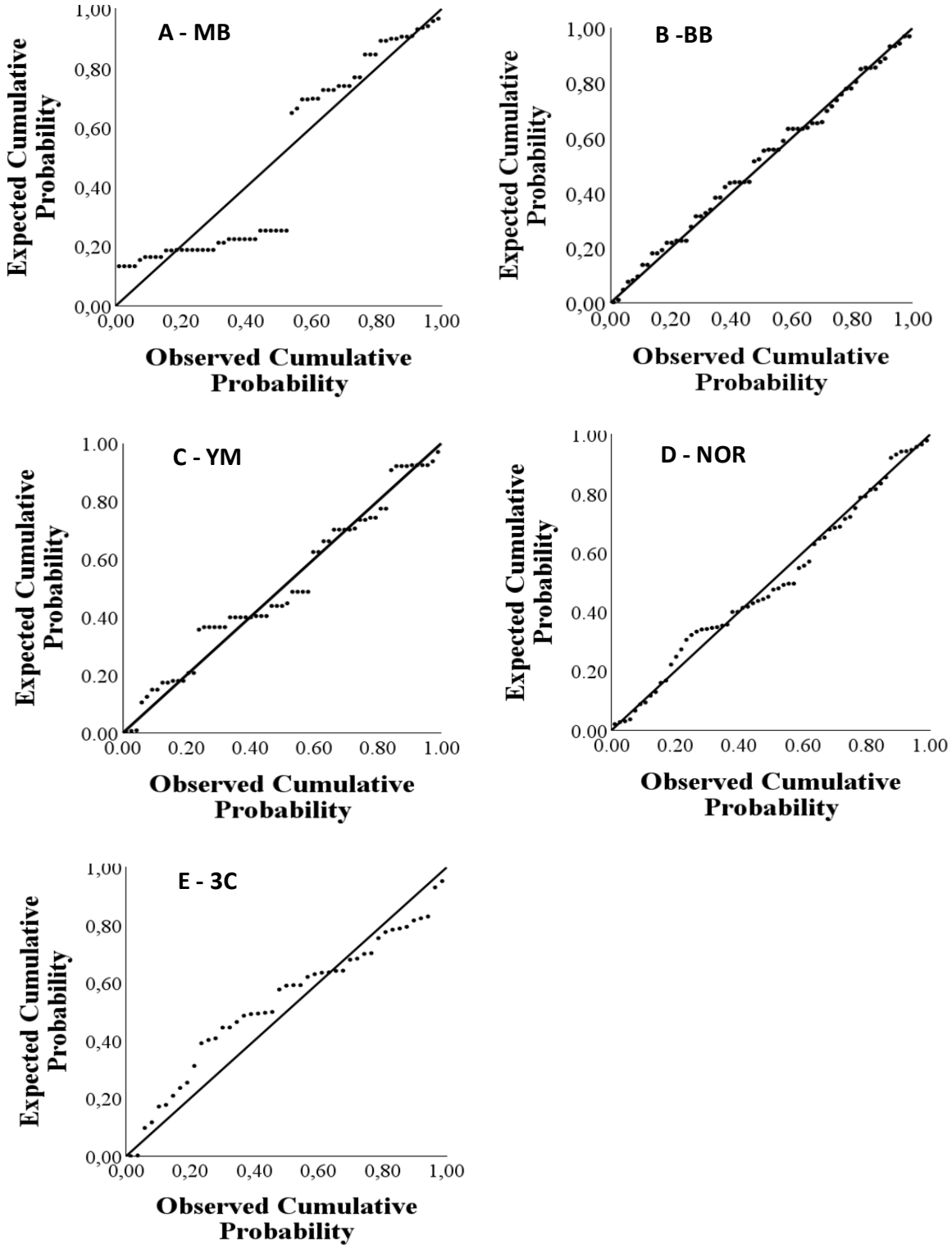


Figure 2: Normal Probability-Probability (P-P) plots for each behavioural test [A – Marble Burying (MB), B – Balance Beam (BB), C – Y-Maze (YM), D – Novel Object Recognition (NOR), E – Three Chamber (3C)] showing the relationship between observed and expected cumulative probabilities generated by the multiple linear regression statistical test.

The sixth assumption of the MLR required the presence of a linear relationship between each test's outcome variable and each of the three independent variables. This is satisfied by correlation co-efficient values greater than 0.03. Three of the tests, namely, MB, BB and YM failed to satisfy this assumption for Sex and NOR and 3C for Autism Pathology (Table 3).

Table 3: Correlations between outcome variables of each behavioural test and the three independent variables.

Behavioural test	Correlation Co-efficient		
	Autism Pathology	Sleep Condition	Sex
MB	0.063	0.113	**-0.045**
BB	0.404	0.144	**-0.007**
YM	0.088	0.046	**-0.052**
NOR	**-0.235**	0.442	0.178
3C	**0.018**	0.169	0.123

KEY: Bolded entries indicate assumption violations.

The seventh assumption of the MLR required the absence of outliers assessed using Mahalanobi's distance. Using the degrees of freedom of each test, the minimum and maximum values were determined, and outliers identified accordingly. One outlier was identified in the 3C test (Table 4) however, was not excluded from the analysis. This is because extreme outliers are considered for values outside of three standard deviations. The minimum and maximum values identified by Mahalanobi's distance fall within these standard deviations and were therefore, non-extreme outliers.

Table 4: MLR outliers in the outcome variables assessed from Mahalanobi's distance.

Behavioural test	Mahalanobi's distance		Outliers	
	Minimum value	Maximum value	value	Sample group (sex)
MB	2.313	4.411	-	-
BB	2.313	4.411	-	-
YM	2.313	4.411	-	-
NOR	2.345	4.577	-	-
3C	2.263	4.582	2.41504	C-nSD (F)

The eighth assumption of the MLR is the absence of multicollinearity of the independent variables, satisfied by Pearson's correlation values less than 0.07. This assumption was satisfied for all the IV interactions for all the behavioural tests except for autism pathology and sleep condition in the NOR and 3C test (Table 5).

Table 5: MLR Pearson's correlations amongst independent variables.

Behavioural test	Pearson's Correlation		
	Autism Pathology & Sleep Condition	Sleep Condition & Sex	Autism Pathology & Sex
MB	0.014	0.000	0.035
BB	0.014	0.000	0.035
YM	0.014	0.000	0.035
NOR	0.014	0.031	**0.084**
3C	-0.063	0.022	**0.126**

KEY: Bolded table entries indicate assumption violations.

APPENDIX E: Three-way (2x2x2) ANOVA Assumptions

Six assumptions need to be considered when performing a three-way analysis of variance (ANOVA). The first three relate to the study design. Firstly, a single dependent variable measured at the continuous (interval or ratio) level is required. This assumption was satisfied with the raw data of each behavioural test except for the MB test which was transformed in order to satisfy this assumption as its raw data were counts of the number of marbles buried (i.e. discrete). As such, the raw data of the MB test are not presented. Further, despite the dependent variables of the other behavioural tests satisfying this assumption, the data of BB, YM, NO and 3C tests were also transformed in order to satisfy the other assumptions.

The second assumption requires the use of three independent variables measured at the categorical (nominal or ordinal) scale. The present study utilised a 2x2x2 between subjects factorial study design. This is because the three independent variables, namely, autism pathology (C or VPA), condition (nSD or SD) and sex (M or F) were dichotomous nominal variables, thus, this assumption was satisfied.

The third assumption relating to the study design requires the independence of observations in each of the groups. In other words, there is no relationship between the outcomes of any of the cases assessed across all sample groups. This was satisfied in this study as none of the outcomes assessed were repeated measures.

The following three assumptions relate to the characteristics of the data being assessed. As such, the fourth assumption requires the absence of outliers from the dependent variable data. Cases or observations outside of three standard deviations were considered extreme outliers and excluded. However, as the presence of outliers is related to the normality of the data distribution, exclusions were only conducted following data transformations to correct for non-normality in the datasets as well as consideration of normality characteristics. These characteristics are skewness and kurtosis which assess the symmetry and peak of distributions respectively (Table 1).

The fifth assumption requires that the standard residuals of the dependent variable be approximately normally distributed. This was assessed using the Shapiro Wilk's Test with p-values greater than 0.05 indicating that this assumption was satisfied. The transformation used for each test was based on the agreement of the final assumption with the fourth and fifth

assumptions (Table 3). This sixth assumption requires the homogeneity of the variances of the standard residuals of the dependent variable.

Table 1: Skewness and kurtosis scales.

	Negative	Normal	Positive
Skewness	-1.0 to -0.5	-0.5 to 0.5	0.5 to 1.0
Kurtosis	<-2	-2 to 2	>2

Table 2: Normality characteristics of the standard residuals of dependent variables under various transformations.

	Behavioural test according to transformation of dependent variable											
	MB		BB			YM		NOR			3C	
	Raw	Log_{10}	Raw	Rank	Square root	Raw	Reflected	Raw	Rank	Square root	Raw	Rank
Skewness	*	**0.804**	0.267	0.004	0.019	-0.495	0.365	**0.643**	0.000	0.074	**-1.832**	0.000
Kurtosis	*	-0.624	-0.100	-1.199	0.248	**0.739**	0.567	-0.622	-1.200	-0.417	**5.515**	-1.200

KEY: Bolded table entries indicate non-normal values; * = the raw data of the MB test are not included as these were discrete.

Table 3: Normality and homogeneity characteristics of the residuals according to transformation of dependent variable.

	Behavioural test											
	MB		BB			YM		NOR			3C	
	Raw	Log_{10}	Raw	Rank	Square root	Raw	Reflected	Raw	Rank	Square root	Raw	Rank
W (p-value)	*	**(<0.001)**	0.997 (0.282)	0.952 **(0.016)**	0.985 (0.650)	0.913 **(<0.001)**	0.892 (0.100)	0.930 **(0.002)**	0.955 **(0.022)**	0.970 (0.139)	0.846 **(<0.001)**	0.956 (0.085)
F (p-value)	*	0.808 (0.585)	4.044 **(0.001)**	1.203 (0.317)	1.475 (0.196)	2.533 **(0.025)**	1.989 (0.080)	2.736 **(0.017)**	1.317 (0.260)	2.116 (0.057)	1.149 (0.355)	1.229 (0.312)
Presence of extreme outliers – sample group (sex)	*	-	-	-	-	**C-nSD (F); C-nSD (M)**	-	-	-	-	**C-nSD (F); C-nSD (M)**	-

KEY: Bolded table entries indicate assumption violations; "-" indicates the absence of outliers; "W" = Shapiro Wilk's test statistic; "F" = Levene's test statistic.

APPENDIX F: Plagiarism Declaration

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I, Mokgatjane Gaffane (Student number: 1840911), am a student registered for the degree of Master of Science (Medicine) in the academic year 2024.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature:  Date: 21 October 2024

APPENDIX G: Turn-It-In Similarity Report

M.Gaffane_MSc Dissertation_28-03-2024_Tunitin.docx

ORIGINALITY REPORT

14%	10%	11%	2%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS