THE DEVELOPMENT OF ANIMAL MODELS FOR AUTISM:

A GENE-ENVIRONMENT APPROACH

BY

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"When nothing seems to help, I go and look at a stonecutter hammering away at his rock perhaps a hundred times without as much as a crack showing in it. Yet at the hundred and first blow it will split in two, and I know it was not that blow that did it, but all that had gone before." – Jacob August Riis.

Contents

List of Abbreviations

5-HT	Serotonin
5-HTT	Serotonin transporter (also SERT)
5-HTTLPR	Serotonin transporter linked polymorphic region
ADHD	Attention deficit hyperactivity disorder
ANOVA	Analysis of variance
AP2	Activating protein 2
ASD	Autism spectrum disorder
BDNF	Brain-derived neurotrophic factor
CDC	Centres for disease control and prevention
$CHCl_3$	Chloroform
CNS	Central nervous system
CPP	Conditioned place preference
Ct	Cycle threshold
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DSM	Diagnostic and stastitical manual of mental disorders
EDTA	Ethylenediaminetetraacetic acid
ENU	N-ethyl-N-nitrosourea
FDA	Food and Drug Administration
GD	Gestational day
GM-CFS	Granulocyte-macrophage colony-stimulating factor
GSH	Glutathione
GSSG	Glutathione disulfide (oxidised form of glutathione)
H_20	Water
HDAC	Histone deacetylase
HDAC-I	Histone deacetylase inhibition
HIV	Human immunodeficiency virus
HR	Homologous recombination
i.p	Intraperitoneal
IL	Interleukin
LPO	Lipoxygenase
LPS	Lipopolysaccharide

MCP	Macrophage chemoattractant protein
$MgCl_2$	Magnesium chloride
MIA	Maternal immune activation
mRNA	Messenger ribonucleic acid
mTOR	Mechanistic target of rapamycin
NMDA	N-methyl-D-aspartate
PHS	Prostaglandin H synthase
PND	Postnatal day
POLY (I:C)	Polyriboinosinic-polyribocytidilic acid
RCF	Relative centrifugal force
RNA	Ribonucleic acid
RNase	Ribonuclease
RPM	Revolutions per minute
RT-PCR	Real time polymerase chain reaction
s.c	Subcutaneous
SERT	Serotonin transporter (also 5-HTT)
SLC6A4	Serotonin transporter gene
SOD	Superoxide dismutase
SSRI	Selective serotonin reuptake inhibitor
TNF	Tumor necrosis factor
VPA	Valproic acid, or valproate

Abstract

Autism Spectrum Disorder (ASD) is a pervasive neurodevelopmental disorder characterised by social, communicative, and behavioural deficits. Despite decades of research in this field, effective pharmacological treatments for ASD are still lacking and better animal models for this disorder are urgently needed. Although it is now well understood that both genetic and environmental influences play a role in the aetiology of ASD, most existing animal models for this disorder only take into account one of these aetiological contributors and have largely ignored investigating an interaction. The main aim of this thesis was to develop a novel animal model for ASD that demonstrated higher construct validity than traditional models by using a gene-environment approach. To this aim, two previously established environmental risk factor-based models for ASD were each combined with a genetic rat model that mimicked a genotype associated with ASD. Specifically, a maternal immune activation model (modelled via prenatal administration of lipopolysaccharide) and a prenatal exposure to valproate model (modelled via prenatal administration of valproate) were both combined with a serotonin transporter (SERT) knockout rat model. Next, experimental rats were investigated in a variety of paradigms designed to detect behavioural, biochemical, and immunological outcomes related to ASD. This thesis tested the hypothesis that rats with a genetically compromised SERT function would be more vulnerable to the impacts of the two environmental risk factors. Collectively, the data from this thesis show that rats with a genetically compromised SERT function are *not* more vulnerable to the impacts of a maternal immune activation or prenatal exposure to VPA. In fact, at least with regards to prenatal exposure to valproate, rats with a compromised SERT function actually appeared more resilient to ASD-like outcomes.

CHAPTER 1: GENERAL INTRODUCTION

Autism

Autism (or Autism Spectrum Disorder, ASD) is a pervasive neurodevelopmental disorder characterised by a triad of behavioural symptoms: impaired social behaviour, impaired communication, and repetitive behaviour (Belzung, Leman, Vourc'h, & Andres, 2005). Clinical symptoms are expected by age 3 but signs of ASD have been observed as early as 6-12 months of age (Levy, Mandell, & Schultz, 2009). These deficits usually persist over the individual's lifetime and only a small fraction of those with ASD function independently in adulthood (Kinney, Munir, Crowley, & Miller, 2008). Although this triad of symptoms forms the core of ASD, it is important to realise that many ASD patients also show a plethora of other symptoms. These auxiliary symptoms can be psychiatric (including depression and anxiety), behavioural (including aggression), intellectual (lower IQ in many cases), sensory (tactile hyperresponsiveness), and neurological (including pre-pulse inhibition deficits and epilepsy). Importantly, ASD is a spectrum disorder, showing a large heterogeneity with regards to its symptoms and severity (Hartley, Sikora, & McCoy, 2008; Levy et al., 2009; Perry, Minassian, Lopez, Maron, & Lincoln, 2007; Rogers, Hepburn, & Wehner, 2003; Simonoff et al., 2008; Tuchman & Rapin, 2002).

Although estimates vary according to region, race, and sex, the most recent data on the prevalence of ASD suggests it affects 1 in 68 children (1.47%); a number that appears to have increased dramatically over the last 50 years (Wingate et al., 2014). The increasing prevalence is apparent even over the last few years: The Centres for Disease Control and Prevention (CDC) have been tracking prevalence data for ASD in the USA since the year 2000. The figures show a steady increase in prevalence over time; rising from 0.67% in the year 2000 to 1.47% in 2010. It is not yet clear whether the increasing prevalence reflects a true increase in the disorder or simply an increased awareness or broadening diagnostic criteria (Weintraub, 2011). Interestingly, a study investigating the ASD prevalence in England found that the prevalence in adults (0.98%) was comparable

with that in children *at the time* – a finding that would not be expected if there was a true increase in prevalence (Brugha et al., 2011). However, note that this 0.98% figure is still lower than the more recent estimates of prevalence provided by the CDC. Monitoring the prevalence of ASD is difficult for many reasons, including: the complexity of the disease, the lack of established biomarkers, and the ever-changing diagnostic criteria (Wingate et al., 2014). Interestingly, males are 4-5 times more likely to have ASD than females (Wingate et al., 2014).

The impact of ASD is not only felt by its sufferers, but also by their families and society as a whole (Buescher, Cidav, Knapp, & Mandell, 2014; Cadman et al., 2012; Kinney et al., 2008). Families of individuals with ASD have to deal with significant emotional and financial burden (Barker et al., 2011; Cidav, Marcus, & Mandell, 2012; Stuart & McGrew, 2009). On average, families of ASD patients work less and earn less than families of healthy children (Cidav et al., 2012). These figures are, in part, presumably due to the time demands required in caring for a child with ASD. In fact, one Swedish study concluded that parents of individuals with ASD spend approximately 1000 hours annually caring for their affected child (Järbrink, 2007).

ASD is an extremely costly problem for society. The most recent research investigating the economic impact of ASD in the United Kingdom and the United States reported several key findings: first, the *lifetime* societal cost for *one* individual with ASD was approximately US$1.4 million in both countries. Second, this figure was significantly larger if the patient also had an intellectual disability. Third, when comparing the relative costs of children with ASD and adults with ASD, they found the aggregate societal cost for all children with ASD was US$4.5 billion annually in the United Kingdom and US$61 billion annually in the USA; and the aggregate societal cost for all adults with ASD was US$43 billion annually in the United Kingdom, and US$175 billion annually in the USA. The major contributors to cost were specialised education and accommodation, and productivity loss (Buescher et al., 2014). These figures echo earlier findings from a Swedish study that estimated the societal cost of ASD in Sweden was approximately €50,000 annually per child; with the major contributors to cost stemming from community support and specialised schooling (Järbrink, 2007).

Effective treatments for ASD are very limited – no pharmacological treatment currently exists that can cure ASD or ameliorate all of the core symptoms (Levy et al., 2009; Vorstman et al., 2014). However, there is effective medication available to target many of the co-morbid problems associated with ASD such as irritability, aggression,

anxiety, and behavioural problems (Hsia et al., 2014; Murray et al., 2014; Politte & McDougle, 2014; Steckler, Spooren, & Murphy, 2014; Vorstman et al., 2014). In fact, it was reported that children and adolescents with ASD are 7 and 12 times more likely, respectively, to be prescribed psychostimulant medication, than healthy controls (Murray et al., 2014). Despite there being considerable world-wide variation in the way medical practitioners prescribe medication for those with ASD, anti-psychotics are the most commonly prescribed class of drug, with risperidone being the most commonly prescribed drug within this class. In fact, there are only two Food and Drug Administration (FDA)-approved drugs for ASD, both second generation antipsychotics: risperidone and aripiprazole (Hsia et al., 2014). The primary reason for the lack of effective medication for ASD is that the neurobiological underpinnings of this disorder are poorly understood, thus drug developers have few molecular targets to investigate (Vorstman et al., 2014).

To reiterate: the exact causes of ASD are not well understood. However, results from twin and family studies suggest two important points: first, ASD is highly genetic with heritability estimates ranging from 50-95% (Colvert et al., 2015; Sandin et al., 2014). Second, as monozygotic twins do not have 100% concordance rates, environmental factors must also play a role in the aetiology of ASD (Bailey et al., 1995; Freitag, 2006; O'Roak & State, 2008; Ronald & Hoekstra, 2011; Steffenburg et al., 1989; Sykes & Lamb, 2007). The question of whether genetic or environmental influences play a bigger role in the aetiology of ASD is disputed; it has been claimed that environmental influences play a larger role than genetic factors, and that early estimates of the genetic liability to ASD were greatly overestimated (Hallmayer et al., 2011). However, the most recent evidence suggests genetic factors play a larger role than the environment in the development of ASD (Colvert et al., 2015). These conflicting results indicate that ASD may not just have heterogeneous symptomatology but also a heterogeneous aetiology (Tordjman et al., 2014). In other words, ASD patients with differing phenotypes may have developed ASD in differing ways. This hypothesis will be discussed in more detail in a later section. Together, the data suggest both genetic and environmental influences play a role in the aetiology of ASD, but the exact nature of this relationship remains unclear.

Thus, there are four clear points that make ASD an important focus of research: first, the disastrous consequences for the individuals, their families, and society; second, the prevalence of this disorder appears to have increased over the last 50 years; third, the lack

of effective treatments; and finally, the complex and poorly understood nature of its aetiology.

Two kinds of research are essential for the development of an effective treatment for ASD: first, clinical research aimed at identifying specific genetic and environmental factors that contribute to this disorder (Kinney et al., 2008; Vorstman et al., 2014); and second, preclinical research aimed at modelling these identified genetic and environmental risk factors in animals (Patterson, 2011). The current project is focused on the second kind of research. The objective is to develop a novel animal model for ASD by investigating the interaction between specific genetic and environmental risk factors that have been repeatedly linked to ASD.

Unravelling the relationship between genetic and environmental risk factors can provide a deeper understanding of the aetiology of complex disorders (Caspi & Moffitt, 2006; Di Forti, 2008; Rodier & Hyman, 1998). Research of this nature is achieved by examining the effect of an exposure to a particular environmental variable and observing how this same variable influences different genotypes (Caspi & Moffitt, 2006). Indeed, certain genotypes can make one more vulnerable or more resilient to the same environmental variable. Research investigating gene-environment interactions has shed light on disorders such as schizophrenia and depression, helping to answer questions like why only some people who experience negative life events become depressed (Caspi et al., 2005; Caspi et al., 2003). One of the objectives of the current project is to help improve the understanding of the aetiology of ASD through a gene-environment approach.

Due to the complexity of ASD and the heterogeneity of its symptoms, multiple candidates have been identified as genetic and environmental risk factors (Betancur, 2011; Kinney et al., 2008; Levy et al., 2009; Newschaffer et al., 2007; Sykes & Lamb, 2007). The current research will focus on one genetic candidate and two environmental candidates. The genetic risk factor under investigation will be a genetic polymorphism in the promoter region of the serotonin transporter: the so-called serotonin transporter linked polymorphic region (5-HTTLPR). The environmental risk factors under investigation will be a maternal infection and prenatal exposure to valproate (VPA). The following sections of this paper will describe each risk factor in more detail, and outline why they are the focus of the current research. First the genetic risk factor will be discussed, followed by both the environmental risk factors.

Genetics and the 5-HTTLPR

Serotonin

Numerous studies suggest serotonin (5-HT) is involved in the pathophysiology of ASD (Cook Jr & Leventhal, 1996; Muller, Anacker, & Veenstra-VanderWeele, 2015). In fact, a seminal review of the neurochemical literature concluded that 5-HT was the neurochemical with the most empirical evidence for a role in ASD (Lam, Aman, & Arnold, 2006). Pioneering research on mental retardation and autistic disorder in children observed an elevated level of whole blood 5-HT (hyperserotonemia) in individuals with ASD (Schain & Freedman, 1961). Since then, hyperserotonemia has become the neurochemical finding most consistently replicated in patients with ASD (Devlin et al., 2005). Interestingly, a recent study found that taking selective serotonin reuptake inhibitors (SSRIs: a class of drugs primarily used as antidepressants that produce a state of increased extracellular 5-HT) during pregnancy significantly increased the risk of ASD in the resulting children (Boukhris, Sheehy, Mottron, & Bérard, 2015). In addition, immunocytochemical analysis of post-mortem brain tissue revealed a dramatic increase in brain 5-HT axons in individuals with ASD relative to controls (Azmitia, Singh, & Whitaker-Azmitia, 2011). On the other hand, short-term dietary depletion of tryptophan, the precursor to 5-HT, was seen to exacerbate behavioural symptoms of autistic disorder in some adult patients (McDougle, Naylor, Cohen, Aghajanian, et al., 1996). Moreover, tryptophan hydroxylase 2 knock-out mice, lacking any brain 5-HT, exhibit the three major behavioural hallmarks of ASD and have been suggested as a promising model for this disorder (Kane et al., 2012; Mosienko, Beis, Alenina, & Wöhr, 2015). Combined, these findings suggest a *dysregulation* of 5-HT may be an important part of the aetiology of ASD. However, the question of *how* 5-HT levels play a role in autism remains unclear (Devlin et al., 2005; Lam et al., 2006).

Dysregulation, as opposed to either *too much* or *too little* is a common pattern within the ASD literature. In fact, numerous individual genes, synaptic functioning, NMDA receptors, and mTOR pathway expression are just a few of the biological factors that have been found to lead to ASD-type deficits when normal functioning is altered in either direction (Lee, Choi, & Kim, 2015; Nicolini, Ahn, Michalski, Rho, & Fahnestock, 2015; Zoghbi & Bear, 2012).

Serotonin Transporter

ASD research has focused primarily on the serotonin transporter gene (SLC6A4, located on chromosome 17q) for several reasons: first, it is the prime regulator of extra cellular 5-HT levels and thus may play a role in the elevated levels of whole-blood 5-HT seen in autistic patients (Muller et al., 2015; Schain & Freedman, 1961). Second, there is some evidence that SSRIs have clinical benefits in autistic patients (McDougle, Naylor, Cohen, Volkmar, et al., 1996). In addition, more recent evidence implicated the serotonin transporter (SERT) in autism; specifically, autistic children were found to have decreased SERT binding capacity in the medial frontal cortex (Makkonen, Riikonen, Kokki, Airaksinen, & Kuikka, 2008).

The SERT regulates the level of 5-HT in the synaptic cleft by transporting it back into the presynaptic neurons. This regulation leaves the SERT an integral part of serotonergic neurotransmission. In fact, this is the case from a particularly early stage of embryogenesis; SERT mRNA (messenger RNA) and protein begin to be expressed in the rat brain the same day as 5-HT itself, in the rostral raphe nuclei on embryonic day 12 (Hansson, Mezey, & Hoffman, 1998; Zhou, Sari, & Zhang, 2000).

The 5-HTTLPR

Importantly, transcription of the SERT gene is modulated by, among others, a polymorphism in the promoter region of this gene: the 5-HTTLPR (Lesch et al., 1996). The allelic structure of the 5-HTTLPR is complex. Although technically up to 14 allelic variants have been identified (Nakamura, Ueno, Sano, & Tanabe, 2000), and convincing evidence has demonstrated it to be functionally triallelic in structure, much of the research investigating the 5-HTTLPR has done so treating it as though it is biallelic, considering only the two major alleles: the short (S) and the long (L) allele (Hu et al., 2006). In fact, in a recent meta-analysis, of the thirty different reports compiled that investigated the role of the 5-HTTLPR on developmental outcomes, only three used triallelic genotyping (van Ijzendoorn, Belsky, & Bakermans-Kranenburg, 2012). Thus, the bulk of research tends to consider only the following three genotypes as relevant: S/S (those homozygous for the S allele), S/L (those heterozygous for the S allele), and L/L (those homozygous for the L allele). Moreover, when examining the association between ASD and 5-HTTLPR genotype, there is a tendency for researchers to group the S/L genotypes with the S/S genotypes, thus creating a binary distinction between those that express the S allele and

those that do not. The reason for grouping these two genotypes is that the S allele seems to work in a dominant manner (Hu et al., 2006).

The functional consequences of the allelic variation are what make the 5-HTTLPR an interesting focus of research. Relative to the L variant, the S variant reduces the transcriptional efficiency of the SERT gene and, theoretically, is associated with increased extracellular 5-HT levels (Kiser, SteemerS, Branchi, & Homberg, 2012; Lesch et al., 1996). Thus, an individual's 5-HTTLPR genotype can reveal important information about their serotonergic transmission. Given that serotonin is implicated in ASD, the 5-HTTLPR appears to be an excellent candidate for genetic research on this disorder.

The 5-HTTLPR and ASD

Human research investigating the genetic underpinnings of ASD has suggested a significant association between 5-HTTLPR genotype and ASD (Conroy et al., 2004; Cook et al., 1997; Devlin et al., 2005; Klauck, Poustka, Benner, Lesch, & Poustka, 1997; McCauley et al., 2004; Yirmiya et al., 2001). However, other studies have failed to replicate this significant association (Betancur et al., 2002; Huang & Santangelo, 2008; Maestrini et al., 1999; Persico et al., 2000; Ramoz et al., 2006; Zhong et al., 1999). The most notable aspect of the research investigating the association between 5-HTTLPR genotype and ASD is the large heterogeneity in the findings. Not only are studies divided on whether the 5-HTTLPR plays a role at all, but of the studies that do conclude the 5-HTTLPR plays a role in ASD, there remains a controversy as to which allelic variant confers the risk for ASD, or if the allelic variants actually play a different role in ASD altogether. The number of studies indicating the S variant to be overtransmitted in ASD is almost equal to the number of studies indicating the L variant to be overtransmitted in ASD (Devlin et al., 2005). Furthermore, the degree of heterogeneity is even reflected in the results of different meta-analyses. When grouping the available findings, one research team found an overall preferential transmission of the S allele from parents to their affected children (Guhathakurta et al., 2006); whereas two years later another meta-analysis failed to find an overall association between ASD and either of the allelic variants of the 5-HTTLPR (Huang & Santangelo, 2008). However, this latter study did observe an effect of ethnicity, with US mixed population samples showing a significant preferential transmission of the S variant while no transmission bias was seen in Asian or European samples (Huang & Santangelo, 2008).

The 5-HTTLPR is a good theoretical candidate for research. Although this notion is supported by numerous research groups, it is evident some research does not support this claim. However, failed replications of significant genetic associations with psychopathological conditions are by no means specific to ASD or the 5-HTTLPR (Burmeister, McInnis, & Zöllner, 2008; Trikalinos, Ntzani, Contopoulos-Ioannidis, & Ioannidis, 2004; Zöllner & Pritchard, 2007). The following sections of this paper will explain why such large heterogeneity in this field is possible, and why there is still reason to be optimistic that the 5-HTTLPR plays a role in ASD. There appear to be at least four major factors that may explain some of the large heterogeneity amongst research in this field. Each factor will be discussed in turn.

Explanations for Heterogeneity

The first possible explanation for these diverse findings is the hypothesis that perhaps the heterogeneity of 5-HTTLPR genotype research maps on to the clinical heterogeneity of ASD (Brune et al., 2006; Devlin et al., 2005). In other words, perhaps the S variant is associated with particular symptoms of ASD and the L variant associated with other symptoms of ASD. In fact, research investigating this idea provides support for such a hypothesis. In a sample of autistic children from the University of Chicago Developmental Disorders Clinic, the S variant was associated with more severe nonverbal communication deficits, whilst the L variant was associated with more severe stereotyped and repetitive behaviours (Brune et al., 2006). In addition, two recent studies found that within groups of ASD patients, the L variant was associated with increased tactile hyperresponsiveness (Schauder, Muller, Veenstra-VanderWeele, & Cascio, 2015), and self-injury (Kolevzon et al., 2014). It is possible these findings explain some of the reason why several research groups failed to find an association between 5-HTTLPR genotype and ASD. The clinical heterogeneity of ASD has also been used to explain the contradictory results found in the neuropathological underpinnings of ASD (Ciaranello & Ciaranello, 1995).

The second possible explanation for the large heterogeneity in this field stems from the ethnic and racial diversity of the populations being investigated. It appears ethnicity/race may play a vital role in allelic frequency and which polymorphic variant confers a higher risk of ASD, if at all (Arieff, Kaur, Gameeldien, Van der Merwe, & Bajic, 2010; Huang & Santangelo, 2008). Following the omnipresent theme of research

on ASD, research comparing the S and L allelic frequencies and transmission rates of different autistic populations around the world has produced varying results.

With regard to simple overall allelic frequency, there is a large difference between ethnic groups throughout the entire population (Hu et al., 2006). For example, people with northern European ancestry have a higher frequency of the L allele, whereas individuals of Asian and Indian ancestry have a higher frequency of the S allele (Cho, Yoo, Park, Lee, & Kim, 2007; Guhathakurta et al., 2006; Koishi et al., 2006; Tordjman et al., 2001).

Research investigating the preferential transmission of 5-HTTLPR allelic variants in ASD among different ethnic groups is also heterogeneous. A study of French autistic patients found that when examining the patients as one group, there appeared to be a small overtransmission of the L allele, but not enough to confidently conclude that it conveyed risk. However, when examining autistic patients in sub-groups, grouped by severity of phenotype, it was found that severely affected individuals were more likely to express the S variant, whereas moderately affected individuals were more likely to express the L variant. It was concluded that 5-HTTLPR genotype did not convey risk of ASD but rather it influenced the phenotypic expression of ASD (Tordjman et al., 2001).

In addition, although research conducted on a Korean population found that the L allele is preferentially transmitted in ASD, research on a predominantly Caucasian American population found that the S allele is preferentially transmitted (Cho et al., 2007; Cook et al., 1997). Despite this contrast, both samples support an association between 5-HTTLPR genotype and ASD. However, research on Indian and Japanese populations found no preferential transmission of either allelic variant and thus does not support an association between 5-HTTLPR genotype and ASD (Guhathakurta et al., 2006; Koishi et al., 2006).

One extreme example of the role that ethnicity/race plays in the relationship between 5-HTTLPR and ASD comes from a study done in South Africa. Using a sample consisting of three groups: African, mixed race, and Caucasian, it was seen that relative to the L/L genotype, having the S/S genotype increased the odds of ASD by more than 10,000 fold for Africans; 10.15 fold for those of mixed race and 2.74 fold for Caucasians (Arieff et al., 2010).

The third possible explanation for the heterogeneity of research investigating the association between the 5-HTTLPR and ASD is the way in which the allelic structure of the 5-HTTLPR is characterised. As previously mentioned, most linkage studies have

treated the 5-HTTLPR as functionally biallelic, acknowledging only the S and L alleles. However, more recently the 5-HTTLPR has been described as functionally triallelic (Hu et al., 2006). Specifically, the L allele can actually be divided into two different alleles: the L(a), corresponding to what is commonly referred to as the normal L allele, and the new L(g) allele. This third allele, the L(g), results from a single base substitution (adenine → guanine) in the normal L allele. The L(g) allele creates a binding site for the transcription factor AP2 (activating protein 2), resulting in a reduced expression rate and thus making it functionally similar to the S allele (Hu et al., 2006). Although the L(g) allele is less common, it is by no means rare (present in 10-15% of Caucasians, and approximately 24% of African Americans) and it could possibly explain the moderate effect size of the 5-HTTLPR in some papers (Kiser et al., 2012). The reason for this is that there is now an additional variable that may mask the true effect of the 5-HTTLPR: previously unrecognised allelic variation of the 5-HTTLPR. As the L(g) allele functions almost the same as the S allele, studies that have included L(g) allele carriers within the S/L and L/L genotypes have possibly underestimated the true effect of the 5-HTTLPR (Hu et al., 2006). In fact, computer simulations based on actual allelic frequencies in different ethnic groups demonstrated that knowledge of the L(g) allele vastly improved prediction of SERT expression (Hu et al., 2006). In summary, the inconsistent manner in which the alleles of the 5-HTTLPR are studied results in a potentially meaningful source of variability between studies supposedly studying the same polymorphism.

The fourth possible explanation for the heterogeneity in this field is the failure of the majority of linkage studies to take into account environmental influences. If research only examines the overall effect of genotype and a meaningful environmental influence is ignored then it is possible a significant gene-environment interaction can be missed and therefore the importance of the gene can be understated. One study investigating the 5-HTTLPR and its association with ASD symptoms in children with attention deficit hyperactivity disorder (ADHD) demonstrated this well (Nijmeijer et al., 2010). Whilst no main effect of 5-HTTLPR genotype was found, further analysis revealed that there was significant interaction between the S allele of the 5-HTTLPR and maternal smoking during pregnancy and low birth weight. Specifically, carriers of the S allele had increased social and behavioural problems, but only when encountering these negative environmental influences (Nijmeijer et al., 2010). If this paper had not investigated these environmental influences the role of the 5-HTTLPR would have been underestimated or, at worst, dismissed.

In conclusion, human research investigating the 5-HTTLPR in ASD suggests this particular polymorphism may play a role in ASD, but exactly *how* remains unclear. There appear to be four major reasons that may explain some of the large heterogeneity within the research in this field: the clinical heterogeneity of ASD, the ethnic and racial diversity of the populations being investigated, the way the allelic structure of the 5-HTTLPR is characterised, and finally the influence the environment may play in such research.

Animal Research and SERT

Animal research investigating genetic variation in the SERT also suggests the SERT may play an important role in components of ASD. Studies from mice, rats, and non-human primates have all found compromised SERT function associated with deficits in social behaviour – one of the core symptoms of ASD. When compared with wild-type mice, SERT knockout mice performed differently on a social interaction test, including significant reductions in 'initiating sniffing' and 'follows' (Kalueff, Fox, Gallagher, & Murphy, 2007). In addition, when compared with wild-type rats, SERT knockout rats demonstrated a significant reduction in social play behaviour; specifically, a reduction in pinning, pouncing, and boxing/wrestling (Homberg, Schiepers, Schoffelmeer, Cuppen, & Vanderschuren, 2007). This last finding involving rats is of particular importance as, compared with mice, rats are more social and have a richer repertoire of social behaviour, making them a better model for human social behaviour (Wöhr & Scattoni, 2013).

A study involving non-human primates also found that 5-HTTLPR genotype significantly influenced social behaviour and cognition. Specifically, rhesus macaques with the S/L genotype significantly differed from L/L genotyped macaques on several important dimensions: they spent less time staring at faces and eyes of conspecifics; they were less likely to take a risky gamble after being primed with a picture of a high-status conspecific, and were less likely to give up juice to see an image of a high-status male conspecific (Watson, Ghodasra, & Platt, 2009). The data from this rhesus macaque study support the idea that 5-HTTLPR genotype is associated with disorders of social behaviour and anxiety. Moreover, the authors concluded that 5-HTTLPR variation may be one of many genetic factors that contribute to a complex disorder like ASD (Watson et al., 2009).

In conclusion, animal research suggests that compromised SERT function may play a role in ASD. Thus, evidence from rodents, non-human primates, and humans suggest the 5-HTTLPR/SERT is an ideal candidate for genetic research on ASD. Whereas human

research indicates both alleles may play a role, the animal studies described here would appear to indicate that it is the S allele which may confer risk for ASD.

Other Genetic Candidates

The genetic underpinnings of ASD are extremely complex, with a combination of genetic factors likely contributing to an individual's susceptibility for developing ASD (Sykes & Lamb, 2007; Vorstman et al., 2014). It is important to note then, that there are many other plausible candidates for genetic research on ASD. In fact, over 100 genetic loci associated with ASD have been described and recent estimates of the total number of risk genes are even higher (Betancur, 2011; O'Roak et al., 2012). For instance: research has shown two single-nucleotide-polymorphisms in the SLC25A12 gene (located at chromosome 2q31.1) and a trinucleotide repeat polymorphism in the RELN gene (located at chromosome 7q22) to be associated with ASD (Ramoz et al., 2004; Segurado et al., 2005). However, as is the case with the 5-HTTLPR, other studies have failed to replicate these findings (Blasi et al., 2005; Bonora et al., 2003; Devlin et al., 2004; Li et al., 2004; Rabionet et al., 2006). In addition, genetic alterations in the Shank gene family, and alterations in genes encoding for neuroligins and neurexins (all related to synaptic functioning) have been associated with ASD (Jamain et al., 2003; Jiang & Ehlers, 2013; Kim et al., 2008; Leblond et al., 2014; Südhof, 2008; Yan et al., 2008). Although other genetic candidates exist, studying multiple candidates is beyond the scope of the current thesis. For reasons outlined above, the current project will focus only on the 5-HTTLPR/SERT. Moreover, many of these other genetic mutations (such as in the Shank genes, and in the genes encoding neuroligins and neurexins) are extremely rare; therefore, from a population point of view, the 5-HTTLPR may be much more relevant.

The preponderance of evidence suggests that ASD has a heterogeneous aetiology (Ronald, Happe, Bolton, et al., 2006; Tordjman et al., 2014), which may go a long way towards explaining its heterogeneous symptomatology. Thus, it is no wonder the genetic architecture of ASD is so complex, as individuals with ASD may have entirely different genetic (or environmental) factors contributing to their disorder. When these individuals are clustered in one large study to search for unified genetic risks, inconsistencies, contradictory findings, and failed replications should be expected. Furthermore, the concept that ASD has several causal pathways may explain the continued pattern of *dysregulation* found in the literature. When ASD is investigated for an association with numerous biological factors such as synaptic functioning, levels of specific

neurochemicals, neurotrophins, or proteins, evidence in both directions is apparent. In fact, it is difficult to find consistently replicated associations with ASD. However, if the mechanisms underlying these patients' pathologies are different, then these patterns are unsurprising. The issue of ASD's heterogeneous aetiology and what it means for ASD research as whole will be discussed in more depth in the final chapter of this thesis.

An important question to consider is whether the genetic complexity of ASD provides insurmountable problems for drug development and future treatment. According to Vorstman et al. (2014), although there may be hundreds of potential gene associations with ASD, many different genes often converge in networks and contribute to the same biological pathway or function. Thus, there are far fewer biological functions involved in ASD than there are candidate genes. As pharmacological therapies target the normalisation of these defective biological functions/pathways, rather than the risk genes themselves, it means the possibility of developing an effective pharmacological compound may be more promising than the genetic complexity would suggest (Vorstman et al., 2014).

The Environment and ASD

Although environmental influences are known to play a role in the aetiology of ASD, when and how they act is unclear. A wealth of evidence points to the prenatal period as a particularly sensitive period in which environmental agents may exert their effects on postnatal behaviour – a logical interpretation for a disorder that can show symptoms as early as 6 months of age. Therefore, both the environmental risk factors investigated in this thesis pertain to the prenatal developmental period.

Maternal Infection and the Immune System

The first environmental risk factor investigated in this thesis is a maternal infection and the corresponding maternal immune activation. In order to gain an understanding of the role of maternal infection in ASD, one must first understand the role of the immune system. Immune system dysregulation has been repeatedly associated with ASD (Ferretti & Hollander, 2015; Patterson, 2009; Sperner-Unterweger, 2005). The immune system, by which an organism protects against disease, has two main components: first, to recognise foreign material or pathogens, and second, to eliminate these from the host organism (Beck & Habicht, 1996). These two components are collectively known as an immune response (or immune activation) and can be achieved in multiple ways (Sperner-

Unterweger, 2005). An important part of the immune response system is the group of proteins called cytokines, which are proteins that regulate a host's reaction to immune responses, infection, and inflammation. Broadly speaking, cytokines can be divided into two types: pro-inflammatory cytokines that worsen disease, and anti-inflammatory cytokines that work to heal disease. There are several groups of cytokines, including tumor necrosis factor (TNF), macrophage chemoattractant protein (MCP) and interleukins (IL) (Dinarello, 2000). The human immune system is both sophisticated and remarkably complex, thus only a brief description is appropriate here; for more information see Beck and Habicht (1996).

Numerous findings have indicated a possible role of immune dysregulation in the pathogenesis of ASD. Specifically, research has observed an increase of the pro-inflammatory cytokine TNF-α in the cerebrospinal fluid of autistic children (Chez, Dowling, Patel, Khanna, & Kominsky, 2007); an increase of pro-inflammatory cytokines TNF-α, IL-6, and Granulocyte-macrophage colony-stimulating factor (GM-CFS) in the brains of ASD patients (Li et al., 2009); elevated levels of TNF-α, IL-6, and IL-1β in peripheral blood mononuclear cells of ASD patients (Jyonouchi, Sun, & Le, 2001); and increased activation of microglia and astroglia, along with a significant increase in MCP-1 in autistic patients (Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005).

Maternal Infection and Human Research

One environmental factor that can influence the foetal immune system is a maternal infection. (Libbey, Sweeten, McMahon, & Fujinami, 2005). In fact, maternal infection has been proposed as a leading candidate for initiating the immune changes seen in ASD, possibly via the production of cytokines during the maternal immune response (Libbey et al., 2005; Patterson, 2002, 2009; Smith, Li, Garbett, Mirnics, & Patterson, 2007). There are two major reasons for this hypothesis: first, the immune dysregulation in ASD is found in the adult and child autistic brain, indicating it is both a permanent state and that it begins at an early stage (Patterson, 2009). Second, there is strong evidence, from both human and animal research, for an association between maternal infection and ASD. In fact, some authors have even concluded that prenatal infections are the primary non-genetic cause of ASD (Ciaranello & Ciaranello, 1995).

Although few epidemiological studies have investigated the involvement of maternal infection with ASD, a recent landmark paper has provided exactly this type of research. Atladóttir et al. (2010) conducted a large-scale study in Denmark, investigating the link

between maternal infection and ASD diagnosis in over 1.6 million children. Using hospital records, a significant association was found between maternal infection in the first and second trimesters of pregnancy and ASD diagnoses in the resulting children. Specifically, pregnant mothers having a viral infection in their first trimester or a bacterial infection in their second trimester increased the odds of ASD in their children by 2.98 and 1.42 fold, respectively (Atladóttir et al., 2010). These findings suggest several important points: first, they support the hypothesis that maternal infections are a risk factor for developing ASD. Second, the findings suggest the timing of exposure to such infections is a crucial factor in their risk association. Third, the fact that not all infected mothers had children with ASD suggests other factors such as genotype influence this environmental variable (Patterson, 2009).

Maternal infection being associated with psychopathological outcomes is not specific to ASD. Numerous epidemiological studies have found a positive association between maternal infection and schizophrenia as well (Patterson, 2009). In fact, it was estimated that up to 14% of cases of schizophrenia would have been prevented if maternal infection had not occurred (Penner & Brown, 2007). Considering schizophrenia costs the USA alone approximately US$62.7 billion per annum (Wu et al., 2005), preventing 14% of the cases of schizophrenia would represent a remarkably significant sum every year (US$8.78 billion).

Interestingly, schizophrenia is a disorder where marked immune dysregulation has also been observed (Sperner-Unterweger, 2005). The numerous parallels between schizophrenia and ASD have led recent speculation that these disorders may actually be closer than traditionally believed. It has been proposed that a maternal infection leading to an acute inflammatory response can account for both the shared and differing symptomatology of ASD and schizophrenia. Specifically, the acute inflammation itself may account for the shared symptoms, whereas subsequent chronic or latent inflammation may lead to the unique phenotypes for ASD, and schizophrenia, respectively (Meyer, Feldon, & Dammann, 2011). More recently it has even been proposed that ASD and schizophrenia may indeed be one and the same disease but simply manifest at different developmental stages according to an individual's interaction with the environment (Wang, 2015).

Maternal Infection and Animal Research

In line with the human epidemiological data, animal research has suggested that maternal infection may play a role in ASD. Mice whose mothers were injected on gestational day (GD) 9.5 with a mouse-adapted human influenza virus, displayed significant behavioural changes and cerebellar abnormalities relating to ASD, including deficits in social interaction and pre-pulse inhibition, and a reduction in Purkinje cells (Shi, Fatemi, Sidwell, & Patterson, 2003; Shi et al., 2009). In addition, mice exposed to a prenatal influenza virus on GD 18 showed a reduction in 5-HT levels in adolescence (Fatemi et al., 2008). Finally, research with non-human primates found that rhesus macaques exposed prenatally to the influenza virus exhibited a reduction in overall brain volume that resembled the brain abnormalities found in schizophrenia, a disorder with numerous parallels to ASD (Short et al., 2010). The data from these studies demonstrated that maternal infection can have drastic behavioural, biochemical, and neuronal outcomes relating to ASD, on offspring that extend into adulthood.

However, understanding the mechanism by which maternal infection influences offspring behaviour is also important (Ashdown et al., 2005). The two distinct possibilities appear to be either through a direct infection of the foetus, or through an interaction to the maternal immune response. It is the latter of these two possibilities that appears to be supported by the most evidence. The fact that various pathogens have been linked to both ASD and schizophrenia suggests that the particular type of pathogen may be irrelevant and instead the explanatory mechanism be one that underlies all infections – a maternal immune response/activation (Gilmore & Fredrik Jarskog, 1997; Libbey et al., 2005). In addition, research using two immune activating agents that elicit a maternal immune activation but *do not* contain a live virus or bacterial pathogens, provide support for the hypothesis that the consequences of a maternal infection stem from a reaction to the maternal immune response (to be explained below). The primary reason for this support is that these agents produce many of the same ASD-related deficits created by prenatal influenza models, and appear sufficient for achieving these outcomes (Meyer, Feldon, & Fatemi, 2009).

Poly (I:C) and LPS

The first immunogen is polyriboinosinic-polyribocytidilic acid, or Poly (I:C); a synthetic substance that evokes an antiviral immune response but does not contain a virus. Offspring of pregnant mice injected with Poly (I:C) display behavioural deficits related to

ASD, including deficits in social behaviour, communication, repetitive behaviour, and pre-pulse inhibition (Malkova, Yu, Hsiao, Moore, & Patterson, 2012; Shi et al., 2003). In addition, offspring of Poly (I:C) injected mothers exhibited cerebellar pathology resembling that found in human patients with ASD (Shi et al., 2009), abnormalities in the hippocampus (Ohkawara, Katsuyama, Ida-Eto, Narita, & Narita, 2014; Zuckerman, Rehavi, Nachman, & Weiner, 2003), and atypical behavioural and pharmacological responses relevant to schizophrenia (Zuckerman & Weiner, 2005). These findings indicate that the ASD-related behavioural consequences of a maternal infection do not stem from direct viral infection of the foetus, but rather from a reaction to the maternal immune response.

These results in rodents also extend to non-human primate models of maternal immune activation. An adapted version of Poly (I:C) for primates was administered to pregnant rhesus macaques and the behaviour of their offspring was extensively analysed. The offspring of mothers exposed to an immune activation displayed deficits relative to controls along numerous behavioural measures related to ASD, including social interaction, communication, and repetitive behaviour (Bauman et al., 2014).

The second immunogen that has helped answer the question of how maternal infection influences offspring is lipopolysaccharide (LPS). LPS is an important component of the outer membrane of gram-negative bacteria and is used to mimic a bacterial infection without exposure to live bacteria. Among its varied biological effects, it causes the production and release of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α (Schletter, Heine, Ulmer, & Rietschel, 1995). Research has also found ASD-like outcomes in the offspring of rats administered LPS during pregnancy, including reduced play behaviour in juveniles, decreased social interaction in adults, reduced communication, increased repetitive behaviour, and deficits in pre-pulse inhibition (Baharnoori, Bhardwaj, & Srivastava, 2012; Borrell, Vela, Arévalo-Martin, Molina-Holgado, & Guaza, 2002; Fortier, Luheshi, & Boksa, 2007; Kirsten et al., 2012; Kirsten, Taricano, Maiorka, Palermo-Neto, & Bernardi, 2010). Like the findings from Poly (I:C) research, the data from LPS research support the claim that maternal infection likely influences offspring behaviour through a maternal immune activation.

Moreover, the impact of these immunogens is unlikely to be due to their direct action on the foetus. Indeed, when pregnant Sprague-Dawley rats were injected with LPS, maternal tissue examination detected LPS in the placenta, blood, liver, and kidney; and pro-inflammatory cytokines were detected in the maternal plasma and placenta. However,

foetal tissue examination detected no LPS or pro-inflammatory cytokine induction (Ashdown et al., 2005). Similarly, another study found maternal administration of LPS increased cytokine mRNA in the placenta but did not affect cytokine mRNA in the foetal brain (Gayle et al., 2004). These data suggest that the effects of maternal exposure to LPS on offspring are not a result of the direct action of LPS on the foetus, but rather a result of the indirect action of LPS and its downstream consequences that occur at placental sites. In summary, the fact that maternal infection seems to be mediated by a maternal immune activation and its downstream consequences suggests Poly (I:C) or LPS can serve as effective models in animals for mimicking the key aspect of a maternal infection, as it pertains to ASD.

Maternal Immune Activation Mechanism

The process of how maternal immune activation influences offspring has also been investigated. Using Poly (I:C) to model maternal immune activation, typical ASD-related deficits associated with maternal Poly (I:C) administration were observed in offspring (Smith et al., 2007). When the cytokine IL-6 from the maternal immune response system was blocked, through administration of an anti-IL-6 antibody or by using IL-6 knockout mice, the ASD-like behavioural abnormalities were not observed. In contrast, when administering blocking antibodies to other cytokines, such as IL-1β and IFNγ, the behavioural deficits were not prevented. These results indicate the cytokine IL-6 is central to the process of how a maternal immune response influences offspring (Smith et al., 2007). Interestingly, a recent report found elevated plasma concentrations of IL-6 in autistic patients and argued that levels of IL-6, in combination with levels of 5-HT, hold great promise as a potential biomarker for ASD (Yang, Liu, Sang, Zhu, & Du, 2015).

In summary, the data from human and non-human animals suggest maternal infection is a clear risk factor for the development of ASD, likely acting through the maternal immune response that releases pro-inflammatory cytokines. More specifically, it is possible that the crucial cytokine in this process is IL-6. In addition, considering the important role of genetics in the development of ASD, it is likely any effect of a maternal immune response does not work in isolation but rather in combination with a susceptible genotype.

An interaction between genetic predisposition and environmental factors taking place during the sensitive perinatal period likely contributes to the development of ASD. Given the importance of 5-HT in ASD and the evidence linking a maternal immune activation to

this disorder, the current thesis aimed to combine a maternal immune activation model with a SERT knockout genetic animal model to assess whether the impact of a maternal immune activation depends on SERT genotype in rats.

Prenatal Exposure to Valproate

The second environmental risk factor investigated in this thesis is prenatal exposure to valproate. Valproic acid (valproate, VPA, 2 propylpentanoic acid), is an anti-convulsant and mood stabiliser used predominantly to treat epilepsy, bipolar disorder, and migraine (Lloyd, 2013; Mulleners, McCrory, & Linde, 2014; Trinka, Höfler, Zerbs, & Brigo, 2014). However, its usefulness has also been trialled in Alzheimer's disease, cancer, and HIV treatments (Avallone et al., 2014; Brodie & Brandes, 2014; Grishina et al., 2015; Hu et al., 2011; Lehrman et al., 2005; Qing et al., 2008). VPA exerts a broad range of activities via a multitude of different mechanisms, some known and some unknown, and for this reason the VPA literature is both remarkably complex and equivocal in nature (Douma et al., 2014; Kwan, Sills, & Brodie, 2001; Patterson, 2011).

In addition to its prophylactic properties, VPA is a known teratogen (Diav-Citrin et al., 2008; Koren, Nava-Ocampo, Moretti, Sussman, & Nulman, 2006; Meador, Reynolds, Crean, Fahrbach, & Probst, 2008; Morrow et al., 2006; Wyszynski et al., 2005). A systematic review of the literature concluded that taking VPA during pregnancy was associated with a 3.77 fold increased risk of major congenital malformations in offspring relative to healthy women, a 2.59 fold increased risk relative to women treated with other anti-epileptic medication, and a 3.16 fold increased risk relative to those with untreated epilepsy (Koren et al., 2006). Together, the literature investigating VPA exposure and congenital malformations indicate an approximate 3 fold increase in major malformations in children exposed prenatally to VPA (Ornoy, 2009).

A teratogen is predominantly defined as an agent that causes malformation to, and/or disrupts the development of, the embryo or foetus. What constitutes a teratogenic *outcome* is somewhat less clear. Typically, discussion of outcomes surrounds congenital malformations such as neural tube defects present from birth. However, in addition to the increased likelihood of congenital malformations, there is strong evidence that children exposed prenatally to VPA are significantly more likely to experience developmental delay and develop ASD, thus making it a key environmental risk factor for this disorder (Adab et al., 2004; Christensen et al., 2013; Christianson, Chester, & Kromberg, 1994; Dean et al., 2002; Moore et al., 2000; Rasalam et al., 2005; Roullet, Lai, & Foster, 2013;

Smith & Brown, 2014; Viinikainen et al., 2006; Williams & Hersh, 1997). Most recently, data from a Danish population-based study showed that prenatal exposure to VPA was associated with an almost 3 fold increased risk for ASD. Specifically, those exposed to VPA had an absolute risk factor of 4.42% for ASD, whereas controls not exposed to VPA had an absolute risk factor of 1.53% (Christensen et al., 2013). As will be discussed later in the chapter, there is convincing evidence to suggest that the congenital malformations and the ASD-like pathology depend, to a large degree, on similar mechanistic processes including alterations in epigenetics and oxidative stress (Dufour-Rainfray et al., 2011).

Prenatal VPA as an Animal Model for ASD

Prenatal exposure to VPA has been thoroughly assessed as an animal model for ASD. In fact, prenatal exposure to VPA is now a well-established model for ASD and has been used in cell-cultures, tadpoles, zebrafish, and rodents (Bauman, Crawley, & Berman, 2010; Jacob et al., 2014; James et al., 2015; Kim et al., 2011; Miyazaki, Narita, & Narita, 2005; Patterson, 2011; Rodier, Ingram, Tisdale, & Croog, 1997; Schneider & Przewlocki, 2005). The typical method of creating this model is to inject pregnant rats with a single dose of VPA around the time of the foetal neural tube closure, approximately GD 12 (Kim et al., 2011). However, the exact dose, method of injection, day of exposure, and whether the exposure is acute or chronic, can vary from study to study; often leading to varying outcomes (Cohen, Varlinskaya, Wilson, Glatt, & Mooney, 2013; Štefánik, Olexová, & Kršková, 2015). In fact, it has been proposed that the dose of VPA determines the mechanism of influence and thus the outcome (Johannessen & Johannessen, 2003).

Numerous behavioural and biochemical outcomes associated with ASD in humans have been produced by this model. Importantly, these outcomes are observed in a variety of species, including 'outbred' genetically heterogeneous rodent strains with stronger translational validity to the genetically diverse human population.

Specifically, prenatal exposure to VPA can produce the following behavioural abnormalities that are associated with ASD in humans: lower sociability, deficits in communication, increased repetitive behaviour/stereotypies, pre-pulse inhibition deficits, lowered sensitivity to pain, increased anxiety, and hyperlocomotor activity (Choi et al., 2014; Dufour-Rainfray et al., 2010; Gandal et al., 2010; James et al., 2015; Mehta, Gandal, & Siegel, 2011; Schneider & Przewlocki, 2005; Schneider et al., 2008).

In addition, prenatal exposure to VPA produces the following biochemical, anatomical or neuronal deficits, many of which are associated with ASD in humans: a reduction in Purkinje cells, cerebellar and gastrointestinal abnormalities (Ingram, Peckham, Tisdale, & Rodier, 2000; Kim, Choi, et al., 2013; Rodier et al., 1997), deficits in the Akt/mTOR pathway (Nicolini et al., 2015), increased cortical thickness and number of neurons in the neocortex (Sabers, Bertelsen, Scheel-Krüger, Nyengaard, & Møller, 2015), an increase in the basolateral nucleus of the amygdala (Loohuis et al., 2015), a reduction in spine density in the hippocampus (Takuma et al., 2014), and decreased cortical brain-derived neurotrophic factor (BDNF) mRNA (Roullet, Wollaston, Decatanzaro, & Foster, 2010).

Of particular importance to the current study, prenatal exposure to VPA has also been demonstrated to significantly impact the serotonergic system. Specifically, exposure to VPA on GD 9 modified the normal migration of 5-HT neurons in the dorsal raphe nuclei of rats in one study (Miyazaki et al., 2005), and led to hyperserotonemia, the most consistently replicated neurochemical finding in human patients, in another study (Narita et al., 2002). Mimicking the non-specific dysregulation of 5-HT in ASD, both increased *and* decreased hippocampal 5-HT levels have been observed at postnatal day (PND) 50, following VPA exposure at GD 9 (Dufour-Rainfray et al., 2010; Narita et al., 2002). In addition, zebrafish models of foetal VPA exposure observed that VPA blocked 5-HT neuronal differentiation, but failed to effect cerebellar Purkinje neuronal differentiation, indicating a particular sensitivity to 5-HT (Jacob et al., 2014). (See Table 1 for a summary of these findings).

Table 1. Behavioural, biochemical, and neuroanatomical changes observed in animal models of prenatal exposure to VPA.

Reference	**Effect**	**Species**
(Choi et al., 2014)	Hyperlocomotor activity	Rat (Sprague-Dawley)
(Gandal et al., 2010)	↓ social interaction ↓USV ↑rep. behav.	Mice
(Mehta et al., 2011)	↑ anxiety ↑rep. behav.	Mice
(Schneider & Przewlocki, 2005)	↓ sensitivitity to pain ↓ p.p.i ↓ social behav. ↑ stereotypies	Rat (Wistar)
(Schneider et al., 2008)	↓ sensitivity to pain ↑ rep. behav. ↑ anxiety ↓ social behav.	Rat (Wistar)
(James et al., 2015)	Abnormal social behaviour	Xenopus laevis Tadpoles
(Ingram et al., 2000)	↓ purkinje cells	Rat (Long Evan)
(Kim, Choi, et al., 2013)	Gastrointestinal abnormalities	Rat (Sprague-Dawley)
(Rodier et al., 1997)	Cerebellar abnormalities	Rat
(Nicolini et al., 2015)	Deficits in the Akt/mTOR pathway	Rat (Wistar Han)
(Sabers et al., 2015)	↑ cortical thickness ↑ neurons neocortex	Rat (Wistar)
(Loohuis et al., 2015)	↑ in the basolateral nucleus of the amygdala	Rat (Wistar)
(Takuma et al., 2014)	↓ spine density in hippocampus	Mice
(Roullet et al., 2010)	↓ BDNF mRNA	Mice

(Miyazaki et al., 2005)	Abnormal 5HT differentiation, migration and maturation	Rat (Wistar)
(Narita et al., 2002)	Hyperserotonemia ↑ 5HT in hippocampus	Rat (Sprague-Dawley)
(Dufour-Rainfray et al., 2010)	↓ 5HT in hippocampus	Rat (Wistar)
(Jacob et al., 2014)	Failure of 5HT expression	Zebrafish

The VPA model has even been able to replicate the male-female imbalance found in ASD. VPA exposure in animals has a more detrimental impact on behaviour, morphology and the immune system in males than it does on females (Kataoka et al., 2013; Kim, Kim, et al., 2013; Mowery et al., 2015; Schneider et al., 2008). The reasons for this differential impact of VPA are uncertain (Mowery et al., 2015; Schneider et al., 2008). However, the preponderance of evidence suggests the likely answer is that natural differences between the sexes exacerbate or protect against the teratogenic impact of VPA. In other words, female-specific biochemical patterns during critical developmental periods may protect against VPA (Schneider et al., 2008). Indeed, female oestrogen has been described as protective against harmful toxins implicated in the onset of ASD (Geier, Kern, & Geier, 2010).

Despite the preponderance of evidence supporting VPA as a valid animal model for ASD, recent evidence has found that prenatal VPA administration actually *increased* social behaviour in VPA-treated rats, relative to controls (Cohen et al., 2013; Štefánik et al., 2015). These contrary findings suggest two important points: first, that more detailed analysis of the VPA model is needed and second, that the experimental details involved in the VPA model are of critical importance to the outcome. This second point is particularly important and will now be discussed in greater detail.

Experimental Variables in VPA Models

When a wider examination of the literature on the outcomes and mechanisms of VPA is undertaken (not just related to models of ASD) the most evident theme is the wide-scale heterogeneity in the data. It can be safely concluded that VPA is somewhat of a ‘dirty’ drug in that it potentially works via multiple mechanisms and leads to many different outcomes, making it very difficult to understand. It is therefore vitally important to

understand the factors contributing to the variability of the effects of VPA (Roullet et al., 2013).

Cell, animal, and human research on VPA has demonstrated several key points: first, response to VPA differs as a function of the developmental age, brain region, and sex investigated (Bittigau et al., 2002; Kataoka et al., 2013). For instance the apoptotic effects of VPA in 14 different brain regions was studied in rat pups exposed to VPA at various developmental stages. Results revealed the response to VPA differed as a function of both developmental age and brain region (Bittigau et al., 2002). Regional specific neuronal cell loss has also been observed in mouse models of VPA (Kataoka et al., 2013). In addition, mice administered VPA at GD 12.5 led to social interaction deficits in male, but not female mice, highlighting the importance of sex in VPA exposure (Kataoka et al., 2013).

The second key point is that response to VPA is both differentiation stage and cell-type dependent (Fujiki, Sato, Fujitani, & Yamashita, 2013; Wang et al., 2011). For instance, VPA was found to have a proapoptotic effect on embryonic stem cell-derived neural progenitor cells of glutamatergic neurons, but this effect was not observed in their neuronal progeny (Fujiki et al., 2013). Moreover, a neuron-astrocyte culture mix treated with VPA induced apoptotic effects that were not observed in a simple neuron-enriched culture, implicating the importance of cell-type in VPA-induced neurodegeneration (Wang et al., 2011).

The third key point is that even seemingly small experimental changes can lead to not just different but opposing findings. A clear example of this comes from two different research groups investigating VPA exposure at GD 9 and measuring hippocampal 5-HT at PND 50 (Dufour-Rainfray et al., 2010; Narita et al., 2002). Whereas one paper found an *increase* in 5-HT in Sprague Dawley rats following 800 mg/kg VPA (Narita et al., 2002), the other found a 46% *decrease* in 5-HT in Wistar rats following 600 mg/kg VPA (Dufour-Rainfray et al., 2010). The discrepant findings were hypothesised to be a result of the differences in the experimental procedure (Dufour-Rainfray et al., 2010).

The final key point is that VPA-induced outcomes are highly dependent on dosage and timing, or the window of exposure, to the drug (Jeong et al., 2003; Johannessen & Johannessen, 2003; Takuma et al., 2014). The *amount* of VPA administered has been repeatedly shown to affect the outcome of the drug in humans, with higher doses associated with higher rates of teratogenicity (Diav-Citrin et al., 2008; Koren et al., 2006; Meador et al., 2008). *When* VPA is administered also has significant implications for the response to the drug. For instance, mice administered VPA at GD 12.5, but not GD 9 and

GD 14.5, exhibited ASD-like symptomatology, including deficits in social interaction (Kataoka et al., 2013). Another, particularly striking, example of the role of timing in VPA-induced-outcome was seen in a paper exposing mice prenatally to VPA and then treating these same mice with VPA postnatally. VPA exposed mice had deficits in novel object recognition, and decreased spine density in the hippocampus. Remarkably, postnatal chronic treatment of VPA attenuated both deficits (Takuma et al., 2014). In other words, the very drug that created the deficits prenatally attenuated the deficits when given postnatally.

Taken together, the data suggest strongly that VPA has a particularly high sensitivity for experimental variables and therefore even slightly different experimental models can produce very different results. It is clear that the timing, dosage, cell-type, differentiation stage, strain-type, sex, and brain region studied can all have a meaningful impact on the outcome of research using VPA. These factors likely explain some of the diversity in the VPA literature.

Mechanisms of Action

We have seen that prenatal exposure to VPA is a well-established animal model for ASD, but by what mechanisms does VPA exert this ASD-related influence on animals? Although numerous research teams have investigated the mechanisms of action for VPA, a consensus remains elusive (Bollino, Balan, & Aurelian, 2015; Fathe, Palacios, & Finnell, 2014; Fujiki et al., 2013; Jeong et al., 2003). The following sections of this chapter will focus on the two major mechanisms for which there is the most compelling evidence: Histone Deacetylase-Inhibition (HDAC-I), and oxidative stress. A discussion of the mechanisms underlying VPA's prophylactic properties is beyond the scope of this thesis.

Histone Deacetylase Inhibition

HDAC-I has been proposed as one of the major mechanisms through which VPA exerts its teratogenic influence (Eikel, Lampen, & Nau, 2006; Fujiki et al., 2013; Lloyd, 2013; Menegola et al., 2005; Phiel et al., 2001; Tung & Winn, 2010). Deoxyribonucleic-acid (DNA) molecules are surprisingly long. In order for DNA to fit within the small confines of the cell nucleus, it has to be dramatically compressed. This compression is accomplished by tightly wrapping the DNA molecule around proteins known as histones, to form nucleosomes: the repeating units of chromatin (Kornberg, 1977; Li & Reinberg,

2011). However, this high degree of compression makes it more difficult for gene transcription to occur, as this involves large proteins (such as transcription factors) binding to DNA before ribonucleic acid (RNA) polymerase can bind and initiate gene transcription. Thus, gene expression is regulated, among others, by how tightly DNA is wrapped around the core histones. Several different modifications of the core histones are known to influence this wrapping, including histone methylation, histone phosphorylation, and histone acetylation. This latter process is regulated by two classes of enzymes: histone acetyltransferases (HATs), and histone deacetylases (HDACs). Put simply, HATs attach acetyl groups to lysine residues on histones, leading to a slight uncoiling of the DNA around the histones, thus allowing transcription factors to bind more easily. HDACs, on the other hand, remove acetyl groups from histones, leading to a more compressed DNA-histone package and hence limit the binding of transcription factors. Functionally, histone acetylation enhances, while histone de-acetylation inhibits gene transcription (Ivanov, Barragan, & Ingelman-Sundberg, 2014; Lloyd, 2013).

VPA has been shown to inhibit HDAC, (Fujiki et al., 2013; Menegola et al., 2005; Phiel et al., 2001), thus preventing HDACs from removing acetyl groups. Therefore, functionally HDAC-Is result in hyperacetylation and *gene activation*. By disturbing the natural HAT, HDAC dynamic process, VPA has the ability to impact many different genes at the same time (Lloyd, 2013). These HDAC-Inhibitory properties are thought to underlie the teratogenic influence of VPA. Indeed, like VPA, other HDAC-Is such as trichostatin A (TSA) and sodium butyrate demonstrate teratogenic effects. Interestingly, whereas VPA analogues that retain HDAC inhibitory activity also induce teratogenicity, analogues that lack this effect do not (Gurvich et al., 2005).

Histone modifications that can alter gene expression come under the umbrella term of epigenetics. Epigenetics is defined as *the study of changes of function to the genome that modify the expression of genes but do not change the nucleotide sequence* (Ivanov et al., 2014; Tordjman et al., 2014). It is now believed that specific environmental factors can induce changes in gene expression via epigenetic mechanisms. These changes in gene expression are functionally expressed by the affected organism and can be responsible for a variety of phenotypes, both positive and negative. Importantly, histone modifications can be long-lasting, thus affecting gene transcription for prolonged periods of time. Epigenetics plays an important role in the broader explanatory model of gene-environment interactions and therefore VPA can be viewed as an environmental

agent that has the capability to interact with specific genes that lead to an ASD phenotype.

The main question that follows from VPA's influence on HDAC is: why does HDAC-I induced *gene activation* lead to teratogenic effects? At the moment it is virtually impossible to answer this question for a number of reasons. First, depending on the type of histone that is acetylated (there are four basic histone (H2A, H2B, H3, and H4) that make up the nucleosome, plus H1 that connects nucleosomes together), histone acetylation can induce different effects. Second, the histone acetylation can occur in many different genes, thus leading to an increased transcription of many genes. Third, epigenetic changes, such as histone acetylation may be very local, i.e., differ between different brain regions, or within a single brain region, or even between different types of cells. Altogether, the puzzle of which genes are activated by VPA and when and where has not been solved. However, the types of genes that were activated can be inferred from the behavioural or biochemical outcomes. In other words, if we observe a teratogenic outcome, we can infer that the types of genes that lead to this outcome were the ones activated in this instance. The field of toxicogenomics has the potential to make significant strides in our understanding of VPA-responsive genes.

Reactive Oxygen Species and Oxidative Stress

Reactive Oxygen Species (ROS) may also play an important role in the mechanism of VPA-induced teratogenesis (Tung & Winn, 2011). ROS are highly reactive molecules that, in excess, have the capacity to damage many elements of a cell (Andersen, 2004; Wells et al., 2009). There are a variety of endogenous mechanisms by which ROS are generated, including mitochondrial respiration and the immune response system (Lloyd, 2013; Wells et al., 2009), but ROS can also be enhanced exogenously by xenobiotics, including VPA (Defoort, Kim, & Winn, 2006; Kawai & Arinze, 2006; Na, Wartenberg, Nau, Hescheler, & Sauer, 2003; Tung & Winn, 2011; Wells et al., 2009). Although generation of ROS is both normal and beneficial, excessive generation of ROS can have major detrimental effects through either disruption of signal transduction and/or oxidative damage to lipids, DNA, RNA, proteins, and carbohydrates (Wells et al., 2009). A variety of defence mechanisms exist that help fight against the excess generation of ROS and regulate this environment, including: antioxidant enzymes and compounds, and direct and indirect repair systems (Davies, 2000; Sies, 1997). When the generation of ROS overwhelms the multi-tiered defence mechanisms a state of oxidative stress ensues and

deleterious effects to the host can occur. The balancing act of ROS generation vs. the host's defence mechanisms to keep a regulated and healthy ROS environment reflects what is referred to as the *oxygen paradox* – the concept that although aerobic life requires oxygen to survive, oxygen is also intrinsically dangerous to its existence (Davies, 1995).

Importantly, the embryo and foetus have lower antioxidant enzyme levels, and in turn, a lowered defence system against excess generation of ROS (Wells et al., 2009; Winn & Wells, 1999). This lowered defence system theoretically leaves the embryo and foetus with a higher susceptibility to the effects of ROS generating mechanisms or xenobiotics, such as VPA (Zaken, Kohen, & Ornoy, 2000). Numerous studies have demonstrated that exposure to VPA increases the production of ROS and has negative consequences for cell survival and development (Defoort et al., 2006; Na et al., 2003; Tung & Winn, 2011). One mechanism whereby this may be achieved is through enzymatic bioactivation (Winn & Wells, 1997). Xenobiotics can be bioactivated by certain enzymes that *are* highly prevalent in the embryo, such as prostaglandin H synthase (PHS) and lipoxygenase (LPO) and converted to free radical reactive intermediates which commence ROS generation (Wells et al., 1997). If the excess ROS generation overwhelms the host's defence mechanisms and oxidative stress results, then adverse developmental effects may be produced (Wells, Lee, McCallum, Perstin, & Harper, 2010). Put simply, the teratogenic effect of VPA could result from a combination of an undeveloped defence mechanism and VPA's ability to initiate ROS production.

Furthermore, VPA has the ability to interfere with the defence mechanisms themselves. Superoxide dismutase (SOD) and glutathione (GSH) are two important antioxidants involved in the defence against ROS, and a downregulation of both SOD and GSH has been observed following VPA exposure (Hsieh, Wang, Tsai, Peng, & Peng, 2012; Zhang, Wang, & Nazarali, 2010). GSSG (glutathione in its oxidised form) and its ratio with GSH can be used as a measure of oxidative stress, with increases in GSSG: GSH ratio indicative of increased oxidative stress. Dose-dependent increases in embryonic GSSG: GSH ratio have been observed following VPA exposure at doses ≥ 100 μg/ml (Zhang et al., 2010). Together, these data suggest VPA's ability to alter antioxidant homeostasis in the embryo may play an important role in VPA's teratogenic influence.

ROS can directly induce DNA double strand breaks (Winn, 2003). Homologous Recombination (HR) is a DNA repair mechanism that can repair DNA double strand breaks (Haber, 1999). However, HR is not an entirely error-free procedure and has the potential to contribute to detrimental genetic changes. Thus, increased levels of HR

theoretically would increase the odds of important genes in the developmental process being disrupted at critical time points, possibly resulting in teratogenesis (Defoort et al., 2006). Interestingly, VPA has been demonstrated to cause oxidative stress and, in turn, increase HR levels *in vitro*. Furthermore, the antioxidative enzyme *catalase*, one of the cellular defence mechanisms against oxidative stress, completely blocked the increased HR following VPA treatment (Defoort et al., 2006). These data suggest HR could be an underlying mechanism of VPA-induced teratogenesis and that oxidative stress plays an important role (Defoort et al., 2006).

Finally, the role of oxidative stress and ROS in the mechanism of VPA-induced teratogenesis is further supported by data demonstrating the attenuating effects of certain antioxidants in prenatal VPA animal models. For instance, green tea extract was found to exhibit neuroprotective effects, possibly due to its antioxidant properties (Banji et al., 2011). Likewise, the antioxidant Vitamin E attenuated the VPA-induced teratogenic effects in mice (Al Deeb, Al Moutaery, Arshaduddin, & Tariq, 2000). Embryonic models have suggested the main mechanisms of Vitamin E in attenuating VPA-induced teratogenicity are through the inhibition of ROS and the restoration of GSH (Hsieh, Chen, Lin, Peng, & Peng, 2014). In addition, the neuroprotective antioxidant Astaxanthin (Liu & Osawa, 2009) was seen to improve ASD-related behavioural outcomes in mice, an effect also attributed to its antioxidant properties (Al-Amin, Rahman, Khan, Zaman, & Reza, 2015).

Taken together, the data from human and non-human animals indicate that prenatal exposure to VPA is both an environmental risk factor and an established animal model for ASD that most likely exerts its influence through its HDAC-I and ROS-inducing properties. However, it appears no one has investigated this established animal model in combination with a genetic animal model in order to determine if the effects of VPA depend on a particular genotype. Conceptually, research of this nature has at least three particular end-goals: first, to understand at a theoretical level why only some foetuses exposed to VPA develop ASD. The second goal is to identify a biomarker that will assist doctors in identifying pregnant women at particular risk from taking VPA. Understanding which women are vulnerable, or indeed resistant, to the detrimental influence of VPA will allow safer prescription practices, and hopefully fewer cases of VPA-induced ASD. Finally, research of this nature aims for a better understanding of the pathological mechanisms of VPA in order to be able to test potential new drugs for ASD. The current thesis aimed to play a role in achieving these goals by combining the prenatal exposure to

VPA animal model with the SERT genetic animal model to assess whether the impact of VPA depends on SERT genotype.

Animal Models

Following this in-depth consideration of each of the three risk factors to be investigated in this thesis, the paper will now continue with an overview of animal modelling in general and, more specifically, how to model ASD in rats. Finally, in this section there will be a discussion on the benefits of combining these specific risk factors and investigating the interaction.

Animal models play a major role in the research process of any disorder/disease, as they allow for the testing of specific hypotheses and the identification of novel therapies (Bauman et al., 2010). Although there is some disagreement on what constitutes a *good* animal model for a given disorder, a model's suitability is most often evaluated for three kinds of validity: construct, face, and predictive validity (Nestler & Hyman, 2010; Willner, 1984). Construct validity is determined by the extent to which the construction of the animal model reflects the aetiological processes of the disorder in humans. Ideal construct validity would be obtained if you were modelling a disorder (X), with only one determinant (Y), and you created a model for X by replicating Y in an animal. Of course, when modelling complicated multi-factorial disorders such as ASD, achieving perfect construct validity is improbable, so instead investigators traditionally aim as high as possible. Ideal animal models of disorders should be constructed using evidence-based, plausible risk factors and/or agents believed to play a causal role in the disorder in humans (Nestler & Hyman, 2010). It has been argued that construct validity is the most important kind of validity for animal models as it relates to the underlying theory of the entire model (van der Staay, Arndt, & Nordquist, 2009).

Face validity is determined by the extent to which the model produces behavioural, biochemical, and neuronal outcomes relevant to the disorder in question. When modelling neuropsychiatric disorders, achieving suitable face validity poses several challenges. First, few neuropsychiatric disorders in humans have well understood neurobiological hallmarks of the disorder. Second, some neuropsychiatric disorders such as depression have an extremely diverse set of symptoms; in fact, two humans can be clinically depressed whilst sharing no common symptoms, or even expressing opposing symptoms such as weight gain vs. weight loss. The diverse nature of symptoms can make modelling certain disorders troublesome. Third, many symptoms of disorders do not appear to be

easily modelled in animals, for example an animal model of schizophrenia would have difficulty modelling hallucinations. Fortunately, modelling ASD in animals has significantly less difficulty with this last problem than some other disease models, such as depression or schizophrenia. The reason for this is that the core symptoms of ASD (impaired social behaviour, impaired communication, and repetitive behaviour) can be reasonably mimicked in animals, even if their correlates are only an approximation (Nestler & Hyman, 2010; Patterson, 2011). Assessing face validity in an animal model must be done with caution, as inferences surrounding animal behaviour are often clouded with anthropomorphic reasoning (Holmes, 2003; van der Staay et al., 2009).

Finally, predictive validity is determined by the extent to which an animal model responds to pharmacological treatments in a way that would be expected in humans with the disorder. Ideal predictive validity would be obtained if a model produced behavioural outcomes relevant to a human disorder, and administering a proven treatment in humans for that disorder reversed these behaviours in the animal model. Achieving suitable predictive validity is problematic when generating an animal model for ASD as there are no known effective treatments (Nestler & Hyman, 2010).

In conclusion, an *ideal* animal model for ASD would possess three characteristics: first, it would be generated by mimicking both genetic *and* environmental causation; second, it would produce behavioural and biochemical outcomes relevant to ASD; and finally, pharmaceutical options beneficial in humans would also reduce symptomatology in the animals. However, there are several major challenges for developing an ideal ASD animal model, including the complex aetiology, the heterogeneity of the symptomatology, a lack of diagnostic biomarkers, and no existing pharmacological treatment, leaving it difficult to achieve ideal construct, face, and predictive validity. In fact, creating an *ideal* animal model of any human disease and especially in the field of brain disorders has been notoriously difficult, so a ‘best-we-can-do’ approach is traditionally taken. Whether this traditional approach is appropriate or indeed desirable will be discussed in the final chapter of this thesis.

Modelling ASD in Rats

It is now possible to model a variety of autistic features in rodents, including both symptoms and risk factors (Patterson, 2011). The current thesis aims to take advantage of a unique genetic animal model in order to mimic the 5-HTTLPR in Wistar rats. The SERT knockout rat was developed via ENU (N-ethyl-N-nitrosourea) mutagenesis and

resulted in a rat model with a premature stop-codon in the SERT. The resulting rat model is now a powerful tool for researchers studying a variety of diseases in which 5-HT plays an important role (Smits et al., 2006). Whereas homozygous SERT knockout rats have a 100% reduction in SERT function, heterozygous SERT knockout rats have an approximately 40% reduction in SERT function (Homberg, Olivier, et al., 2007), making the heterozygous SERT knockout rat ideal for modelling the S variant of the 5-HTTLPR human polymorphism. In addition, as wild-type rats have normal SERT function they are ideal for modelling the L variant of the 5-HTTLPR. Thus, there is now a way to investigate the 5-HTTLPR genotype using rats: by comparing heterozygous SERT knockout rats with wild-type rats; and this is exactly the method the current research will employ. Homozygous SERT knockout rats have no human equivalent and for this reason are not the main focus of this research (Kalueff, Ren-Patterson, & Murphy, 2007). The two environmental risk factors under investigation: maternal infection and prenatal exposure to VPA will be mimicked via administration of LPS or VPA to pregnant mothers at relevant gestational periods. The process of modelling ASD's aetiology in this way, combined with the plausibility of the theoretical underpinnings previously described will determine the construct validity of this model.

Combining the Risk Factors

Although animal models for each of these environmental risk factors exist in isolation, no one has ever combined these two risk factors with a genetic rat model of any kind. Despite the knowledge that ASD is caused by both genetic and environmental factors, most existing animal models mimic only one part of the aetiology for this disorder and have largely ignored combining a multitude of aetiological contributors (Belzung et al., 2005; Ranger & Ellenbroek, 2015). The current investigation attempts to combine the multiple risk factors previously described with the aim of identifying a possible gene-environment interaction.

There are four reasons to investigate a possible gene-environment interaction in these circumstances. First, we know that ASD is caused by both genetic and environmental determinants. Second, the data suggest 5-HTTLPR genotype likely plays a role, but not in a well understood manner; and it seems one of the major reasons for this ambiguity is the failure of the majority of linkage studies to take into account environmental influences. Third, prenatal exposure to a maternal infection or VPA increases the risk of ASD, but not all exposed individuals develop ASD. Fourth, an interaction between these particular

environmental and genetic risk factors seems plausible as both LPS and VPA have been shown to interact with the serotonergic system (De Theije et al., 2014; Hrupka & Langhans, 2001; Jacob et al., 2014; Macchi et al., 2013; Miyazaki et al., 2005; MohanKumar, MohanKumar, & Quadri, 1999; Narita et al., 2002). The primary objective in aiming to develop an animal model using both genetic and environmental determinants is that it could provide a novel model for ASD with superior construct validity and explanatory power than older models, as the generation of this novel model would more closely reflect the aetiological process that occurs in humans.

The fact that existing models have already demonstrated ASD-like effects with each of these risk factors in isolation provides an important question in need of solving: how can we identify an interaction whilst at the same time rule out that it is just the combined impact of two main effects? The key to identifying an interaction (if there is one to be found), is in using the right *degree* of genetic and environmental insult. Many previous animal studies have used VPA and LPS doses that may simply be too high to identify an interaction because of a ceiling effect – in other words, the environmental insult is simply too large. In addition, previous studies involving the SERT genetic animal model have used animals with a *complete* ablation of the SERT (i.e., a homozygous knockout), possibly making the genetic effect too large and masking any effect of the environment. Conversely, in order to avoid a floor effect, neither the genetic nor environmental effect can be too small. Thus, in order to be able to detect an interaction the environmental insult and genetic effect should both be moderate. Therefore, the current experiments used moderate doses of VPA and LPS, and focused on heterozygous knockout rats, rather than homozygous knockout rats.

Objectives and Hypotheses

There is a distinct lack of research investigating the interaction between genetic and prenatal environmental risk factors in ASD, making these types of investigations a critical priority for research in this field (Chaste & Leboyer, 2012; Kinney et al., 2008; Tordjman et al., 2014). In fact, when investigations of this kind have been performed, their results have been informative (Schwartzer et al., 2013). Data from human and animal research indicate that three particularly good candidates for investigation are the 5-HTTLPR polymorphism, a maternal infection that generates a maternal immune response, and prenatal exposure to VPA. It is now possible to model these three risk factors in rodents.

Overall, the goal of the current thesis was to develop a novel animal model for ASD with construct validity superior to traditional models. In turn, this thesis investigated the hypothesis that rats with a genetically compromised SERT function will be more vulnerable to the impacts of different environmental risk factors. Specifically, chapter 2 investigated whether the association between a maternal immune response and ASD-like behaviour previously observed in animal models was influenced by SERT genotype. In addition, chapters 3 and 4 investigated whether the association between prenatal exposure to VPA and ASD-like symptomatology previously observed in animal models was influenced by SERT genotype. In these endeavours, the aforementioned genetic and environmental risk factors were modelled in Wistar rats and their behaviour was investigated in multiple behavioural paradigms designed to detect the core symptoms of ASD. These risk factors were modelled using the SERT knockout rat model in combination with prenatal administration of LPS or VPA. In addition to these behavioural experiments, biochemical changes in the BDNF gene and immunological changes in IL-6 were investigated. Together, these investigations sought to identify if the proposed manipulations resulted in behavioural, biochemical, or immunological profiles in accordance with those observed in humans with ASD.

There existed one overriding hypothesis in this thesis: rats with a genetically compromised SERT function would show an increased vulnerability to the impacts of both a maternal immune activation and prenatal VPA exposure. On the basis of human and animal research, it was expected that rats exposed to the environmental risk factors in this study would display behaviour in accordance with ASD symptomatology on one or more of the behavioural or biochemical assays, and that these effects would be exaggerated if they also had a compromised SERT genotype.

CHAPTER 2: MATERNAL IMMUNE ACTIVATION AND SOCIAL BEHAVIOUR

Data from both humans and non-human animals suggest maternal infection is a risk factor for the development of ASD (Atladóttir et al., 2010; Bauman et al., 2014; Shi et al., 2003). Maternal infection most likely exerts its influence via the maternal immune response and the corresponding release of pro-inflammatory cytokines such as IL-6 (Smith et al., 2007). Thus, maternal immune activation appears to be the principle mechanism underlying maternal infection and its association with ASD.

LPS is an immunogen used to mimic a bacterial infection without exposure to live bacteria. LPS evokes strong immune responses when administered to animals and has been used to model ASD in animals (Kirsten et al., 2012). In this chapter subcutaneous administration of LPS to a pregnant rat was used to induce a maternal immune response.

The three major behavioural hallmarks of ASD are deficits in social behaviour and communication, and an increase in repetitive behaviour (Levy et al., 2009). The purpose of this chapter was to conduct an extensive investigation of the effects of LPS on social behaviour in particular; more specifically, to analyse the behavioural consequences of a 0.5 mg/kg dose of LPS administered subcutaneously at GD 10 and again at GD 11 and to see if the behavioural response was dependent on SERT genotype. Multiple social behaviour paradigms were performed throughout various points of the animals' lives, namely: juvenility, adolescence, and adulthood. These experiments were designed to assess the presence of ASD-like social deficits in rats.

When evaluating an animal model for its validity, it is good practice to evaluate each domain of interest (in this case social behaviour) in more than one paradigm. The underlying theory is that if there truly is a behavioural deficit of importance to ASD it should be robust enough to be detected across a variety of experiments designed to measure the same concept but in different ways. In addition, behaviour such as social behaviour is complex and different paradigms can assess different aspects of this construct; thus by using more than one paradigm one can obtain a better understanding about which aspect(s) are affected. The data from these experiments helped determine the face validity of a *SERT reduction-maternal immune activation* gene-environment model for ASD. These experiments tested the hypothesis that rats with a genetically compromised SERT function would show an increased vulnerability to the impact of a maternal immune activation encountered during foetal development.

Animals

All experimental animals were Wistar rats, bred in the animal laboratory on the 7th floor of Easterfield building, Victoria University. The experimental animals varied according to their SERT genotype. The genotypes of interest were wild-type and heterozygous SERT knockout animals. All animals were group housed in a temperature-controlled room (temperature: 21± 2°C; 55% humidity) and kept on 12 hr light-dark cycles (lights on at 07:00) with food and water available ad libitum. All pups were weaned at PND 21, and then housed with sex-matched littermates. For most experiments males were kept for the behavioural experiments, whilst females were euthanized; however, females were used when breeding difficulties resulted in low numbers of males. There were two reasons for focusing primarily on male animals: first, in humans ASD is a male dominant disorder that is 4-5 times more likely in males than females (Wingate et al., 2014), and second, male rats spend more time engaging in a variety of social behaviours than females (Meaney & Stewart, 1981), making them better candidates to observe potential social deficits. Animals were housed in rectangular Plexiglas cages (40 cm x 25 cm x 12 cm), with pine bedding. An animal technician cared for the rats and performed euthanasia on the animals when their role in the experiment had come to an end. All procedures in this chapter were approved by the Victoria University of Wellington Animal Ethics Committee.

Breeding

The current thesis employed two distinct breeding procedures, one for each environmental risk factor (the second breeding procedure will be described in the next chapter on VPA). When modelling the first environmental risk factor, maternal infection, wild-type Wistar mothers were mated with either wild-type males *or* homozygous SERT knockout males. Once mated (defined as the day a vaginal plug was discovered) the females were removed from the males, housed individually, and subcutaneously injected with either LPS (0.5 mg/kg) or saline on GD 10 and 11. Thus, this created four groups of offspring for experimentation (Table 2):

Table 2. Experimental groups used in chapter 2.

Group	Genotype	Treatment
1	Wild-type	SAL
2	Wild-type	LPS
3	Heterozygous	SAL
4	Heterozygous	LPS

In all of the following experiments, multiple litters were used to create the sample size for each experimental group. The reason for this was to reduce the chance of potentially abnormal litters representing an entire group. The sample sizes reported in these experiments indicate the number of individual rats, rather than litters.

Data analysis

IBM SPSS statistics version 22 was used for all statistical analyses in this chapter. The alpha level for statistical significance was set at $p < .05$.

Experiment I (EXP-I): Social approach and novelty seeking in juveniles

The purpose of this experiment was to investigate social approach and social novelty seeking behaviour in juvenile rats. Typically, rats are highly social creatures with the tendency to approach social cues, and prefer novel conspecifics over familiar ones (Lehman & Adams, 1977; Smith, Wilkins, Mogavero, & Veenema, 2015). The current paradigm aimed to exploit this typical behaviour in an experimental setting to see if the proposed model led to deficits in such behaviour – thus, the experiment was designed to be able to detect one of the three core behavioural hallmarks of ASD: a reduction in social behaviour. Juvenile animals were used for this experiment as ASD is a disorder detectable at a very young age and thus observing a social deficit in juveniles would more closely mimic the behavioural profile seen in patients.

Method

Animals

EXP-I was performed on male and female juvenile rats (PND 24-30). In this experiment, *stranger* rats were also used; these strangers were untreated, Sprague Dawley, female juveniles (approximately PND 30), used solely for the purposes of a novel social cue. All

animals were moved into the experimental room 30 min before the experiment began. Lighting and temperature conditions were the same in the experimental room as they were in the housing room. The sample sizes for the experimental groups in this paradigm were as follows: wild-type + saline = 10; wild-type + LPS = 16; heterozygous + saline = 17; heterozygous + LPS = 7.

Apparatus

The experiment took place in a standard open field circular arena (80 cm diameter, 45 cm height) with black flooring and walls. Two cylindrical small cages (11 cm diameter, 14 cm height) were used to house the *stranger* rats during the appropriate phases (two and three) of the experiment. Ethovision XT v9.0 video tracking software was used to determine the duration and frequency animals spent in the relevant areas of the open field. The floor and walls were wiped down with 75% ethanol before each new experimental animal was put into the open field in order to minimise scent carry-over from previous animals.

Procedure

The experiment employed an adapted version of the social approach and social novelty paradigm described in Jones et al (2010) and Nadler et al (2004). The current experiment consisted of three main phases: habituation, the social approach phase, and the social novelty phase. In the first phase of the experiment, animals were placed individually into the empty open field arena and left for 10 min to freely explore; this was done in order to habituate the animals to the new environment.

The second phase of the experiment began immediately after habituation and its primary aim was to measure social approach behaviour. First, the rat was removed from the open field following habituation and two cylindrical wire mesh cages were placed in the open field (30 cm apart from each other), with one of the cylinders (A) containing a *stranger* pup, and the other cylinder (B) containing nothing at all and acting as a control, novel object. Next, the experimental rat was released back into the open field, now faced with the two new environmental stimuli: a social cue (A) and a non-social cue (B). The rat was then left for 10 min to freely explore its environment and time spent with its nose or mid-point within 5 cm of each cylinder was recorded. Degree of sociability was calculated as the time spent next to the social cylinder (A) relative to the time spent investigating both cylinders (A + B). Spending time next to the social cylinder was

inferred as a sign of sociability and the greater the amount of time spent, the greater the degree of sociability.

The third phase of the experiment began immediately after the end of phase two, and its primary purpose was to measure preference for a novel social stimulus. The reason there was no time delay between phase two and three is that an earlier experiment, using the same paradigm, conducted in our laboratory utilised a one hour gap between phase two and three but found no evidence of social novelty preference in any of the experimental groups (to be described later in EXP-III). Hypothesising that the lack of novelty preference may have been due to the one hour time delay, the delay was eliminated in the current experiment. The set-up of the open field in phase three was almost the same as in phase two, except this time in cylinder (B), which previously contained nothing, a *new* stranger pup was placed. Thus, cylinder (A) now housed the *familiar* pup from phase two, and cylinder (B) housed a *new* stranger pup. The experimental rat was then released back into the open field and again given 10 min to freely explore the arena and time spent in the proximity of each cylinder was recorded. Degree of social novelty seeking was calculated as the time spent next to the new-social cylinder (B) relative to the time spent investigating both cylinders (A + B). Spending time next to cylinder (B) was inferred as a sign of social novelty seeking behaviour.

Experimental sessions were run in the morning, from 09:00 till 12:00. The primary outcomes of interest were: the percentage of total investigatory time spent investigating cylinder (A) in phase two and the percentage of total investigatory time spent investigating cylinder (B) in phase three. However, distance moved during habituation (phase one) was also calculated to control for any effect locomotion may have had on the social behaviour data.

Results

As this experiment involved both males and females, a one-way analysis of variance (ANOVA) was used to test the effect of sex on each of the three primary outcomes of interest. Results revealed there was no main effect of sex on distance moved in phase one: $F(1, 48) = 1.218$, $p = .275$; the mean percentage of time exploration occurring near cylinder (A) during phase two: $F(1,48) = 1.509$, $p = .225$; or the mean percentage of exploration occurring near cylinder (B) during phase three: $F(1,48) = 3.002$, $p = .090$. Thus, both sexes were pooled together for all future analyses.

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on distance moved during phase one. Results indicated a significant main effect of genotype, $F(1, 46) = 10.769$, $p = .002$, with wild-types moving significantly more than heterozygous animals. There was no main effect of treatment type $F(1, 46) = .065$, $p = .801$. However, there was a significant interaction between genotype and treatment type $F(1, 46) = 17.218$, $p < .001$. Specifically, LPS exposure significantly increased locomotion in wild-types ($p = .008$) but significantly decreased locomotion in heterozygous animals ($p = .006$). (See Fig. 1).

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the mean percentage of exploration time occurring near cylinder (A) during phase two. Results indicated there was no main effect of genotype, $F(1, 46) = .524$, $p = .473$, or treatment type $F(1, 46) = .028$, $p = .867$. In addition, there was no significant interaction between genotype and treatment type $F(1, 46) = 1.884$, $p = .176$. (See Fig. 2). One sample t-tests were performed to determine whether the experimental groups displayed a significant preference for the social cue (preference was inferred when the percentage of exploration next to (A) was significantly higher than 50%). Results indicated that all groups except wild-type saline-treated animals displayed a significant preference for the social cue.

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the mean percentage of exploration time occurring near cylinder (B) during phase three. Results indicated a significant main effect of genotype, $F(1, 46) = 6.205$, $p = .016$, with wild-types exploring cylinder (B) a higher percent of the time than heterozygous animals. However, there was no main effect of treatment type $F(1, 46) = .1.273$, $p = .265$, and no significant interaction between genotype and treatment type $F(1, 46) = .002$, $p = .965$. (See Fig. 3). One sample t-tests were performed to determine whether the experimental groups displayed a significant preference for the novel social cue (preference was inferred when the percentage of exploration next to (B) was significantly higher than 50%). Results indicated that only wild-type LPS-treated animals displayed a significant preference for social novelty.

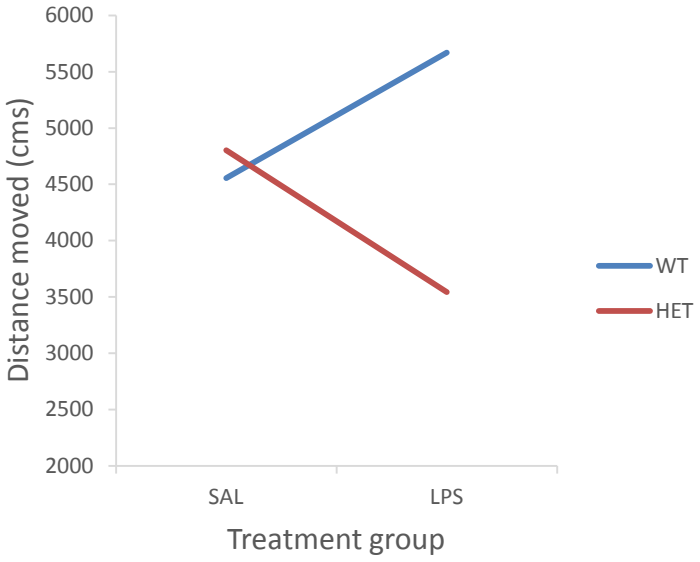

Figure 1. EXP-I: Effects of SERT genotype and LPS treatment in juveniles on the distance moved during habituation. Lines represent the mean.

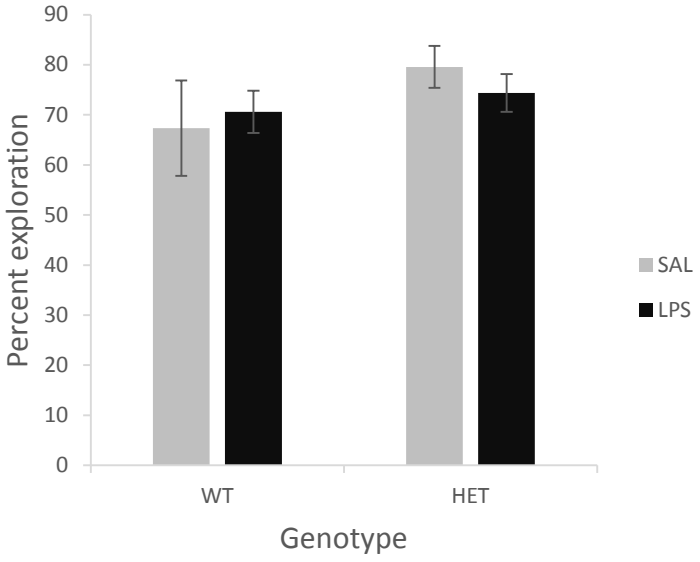

Figure 2. EXP-I: Effects of SERT genotype and LPS treatment in juveniles on the mean percentage of exploration time near the social cylinder (A) in phase two. Bars represent the mean (+SEM).

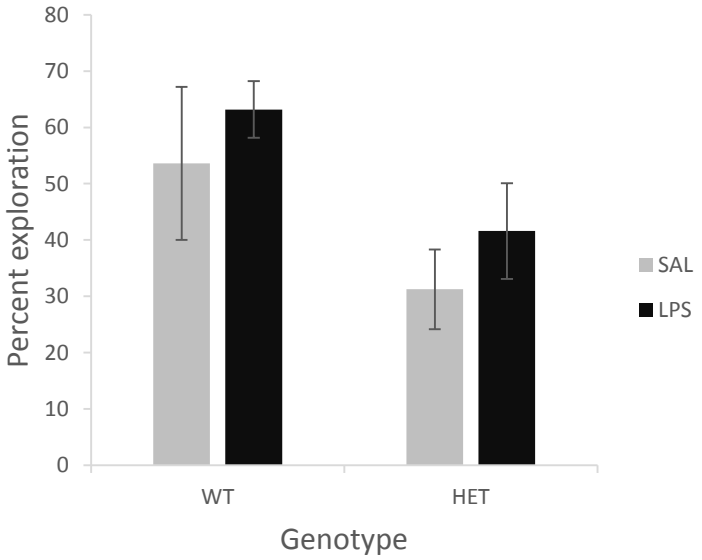

Figure 3. EXP-I: Effects of SERT genotype and LPS treatment in juveniles on the mean percentage of exploration time near the novel social cylinder (B) in phase three. Bars represent the mean (+SEM).

Experiment II (EXP-II): Social motivation in adolescents

The purpose of this experiment was to investigate social motivation in adolescent rats. Typically, rats are highly social animals that find socialisation rewarding (Peartree et al., 2012; Thiel, Okun, & Neisewander, 2008). The current paradigm aimed to exploit this typical behaviour in an experimental setting to see if the proposed model led to deficits in social motivation – thus, the experiment was designed to be able to detect one of the three core behavioural hallmarks of ASD: a reduction in social behaviour.

Method

Animals

EXP-II was performed using male adolescent rats (PND 35-45). All animals were moved into the experimental room 30 min before the experiment began each day. The sample sizes for the experimental groups in this paradigm were as follows: wild-type + saline = 9; wild-type + LPS = 9; heterozygous + saline = 13; heterozygous + LPS = 15.

Apparatus

The experiment took place in a conditioned place preference box (Panlab/Harvard instruments) comprised of two main chambers (40 cm x 34 cm x 45 cm) and a connecting starting chamber (25 cm x 13 cm x 45 cm). The two main chambers differed both visually (spotted vs. stripy walls; black vs. grey floor colour) and texturally (smooth vs. rough flooring); moreover, olfactory cues (essential oils) were added to the original box so that one chamber (A) smelled like rosemary and the other (B) like lemon (The Aromatherapy Company, New Zealand). Three drops of oil were added to the far corner of the respective chambers to make them even more distinguishable from each other. Rosemary and lemon smells were used because pilot research in our lab had previously found that although Wistar rats can distinguish easily between the two they tend to have no preference for either smell (data not shown). In short, chamber (A) had spotted walls, a black, smooth floor, and smelled like rosemary; whereas chamber (B) had stripy walls, a grey, rough floor, and smelled like lemon. The box was cleaned thoroughly and wiped down with 75% ethanol before each trial. Temperature conditions were the same as the housing room; however, lighting conditions differed. The room was dimly lit with only a lamp in order to promote locomotor activity and to make it easier to track the animals with the Ethovision software. Ethovision XT v9.0 video tracking software was used to determine the duration and frequency animals spent in each compartment.

Procedure

The experiment used a conditioned place preference (CPP) paradigm based on the protocol described by Peartree et al (2012). The paradigm consisted of three main stages: habituation, conditioning, and testing. Each experimental block took 6 days to complete. In the first stage of the experiment animals were placed individually in the CPP box and given three habituation sessions of 10 min per day for 3 consecutive days. Each habituation session began at approximately the same time in the afternoon, at 13:30. The habituation sessions gave the rats a chance to freely explore the box and allowed the researcher to determine if there was a baseline preference for a particular chamber. Preference was calculated using the average time spent in each chamber across the last two habituation days – the least preferred chamber was calculated for each experimental animal for the purposes of the next stage in the experiment.

The second stage of the experiment began on day 4 with 2 days of conditioning. Conditioning consisted of two 10 min sessions per day with a 6 hr interval separating the morning (beginning 09:30) and afternoon (beginning 15:30) sessions. During the conditioning stage, both of the main chambers were sealed off from one another in order to prevent free exploration throughout the whole box. In one session rats were paired with another rat (a foreign rat of the same sex and age) in their *least* preferred compartment (established during habituation). The pairing animals used for the social reward were not used for any other purpose in the experiment. In the other session, rats were placed alone in their preferred compartment. Session type (paired vs. alone) was counterbalanced, so that half the rats were paired in the morning session and the other half were alone in the morning session, and vice versa for the afternoon session. Moreover, animals that were paired in their morning session on the first day were conditioned alone in the morning session on the second day; likewise, animals conditioned alone in the first morning session of conditioning were paired in the second morning session of conditioning on the second day. Thus, each animal had a morning-alone, morning-social, afternoon-alone, and afternoon-social conditioning session.

The third stage of the experiment was performed on day 6 when the animals were tested for a possible change in preference compared to the habituation stage. The rats were once again placed individually in the box for 10 min with the ability to freely explore both chambers and time spent in each chamber was recorded. An increased preference for their least preferred chamber following conditioning was interpreted as a

sign of social motivation, i.e., socialising was rewarding, and the larger the increase in preference, the more strongly rewarding socialising was to that animal.

The primary outcome of interest was the degree to which social conditioning influenced subsequent time spent in an animal's least preferred chamber. This outcome was measured by taking the percentage of time spent in the least preferred chamber during habituation and comparing this number to the percentage of time spent in that particular chamber in the third and final test stage of the experiment that followed conditioning. Thus, the *change* in percentage of time spent in the least preferred chamber was the outcome of most importance. In addition, distance moved during the first habituation session on day one was calculated.

Results

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the total distance moved during the first session of habituation. Results indicated there was no main effect of genotype, $F(1, 42) = 1.92$, $p = .214$, or treatment type $F(1, 42) = .665$, $p = .419$. However, there was a significant interaction between genotype and treatment type $F(1, 42) = 13.634$, $p = .001$. Specifically, LPS exposure significantly reduced locomotion in wild-types ($p = .001$), and increased locomotion in heterozygous animals, albeit not significantly ($p = .054$). This pattern of locomotion in adolescent rats was in contrast to the effect LPS had on juveniles' locomotion in the previous experiment. (See Fig. 4).

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the influence of social conditioning, as measured by the change in the percentage of time animals spent in their least preferred chamber (established during habituation) following conditioning. Results indicated there was no main effect of genotype, $F(1, 42) = .040$, $p = .843$, or treatment type $F(1, 42) = .014$, $p = .905$. Similarly, there was no significant interaction between genotype and treatment type $F(1, 42) = 2.064$, $p = .158$. (See Fig. 5). In addition, one sample t-tests were performed to determine whether the experimental groups displayed a significant social conditioning effect (successful conditioning was inferred when the change in percentage of time spent in their least preferred chamber following conditioning was significantly higher than zero). Results indicated that only the heterozygous LPS-treated animals displayed a significant effect of social conditioning.

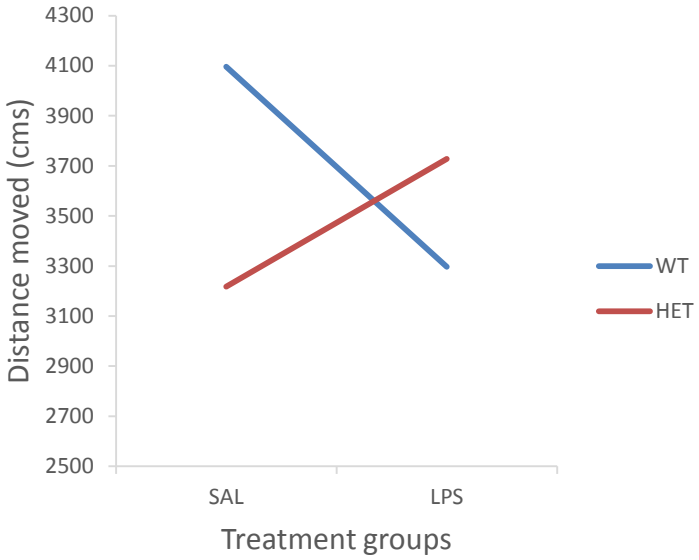

Figure 4. EXP-II: Effects of SERT genotype and LPS treatment on distance moved during the first habituation session. Lines represent the mean.

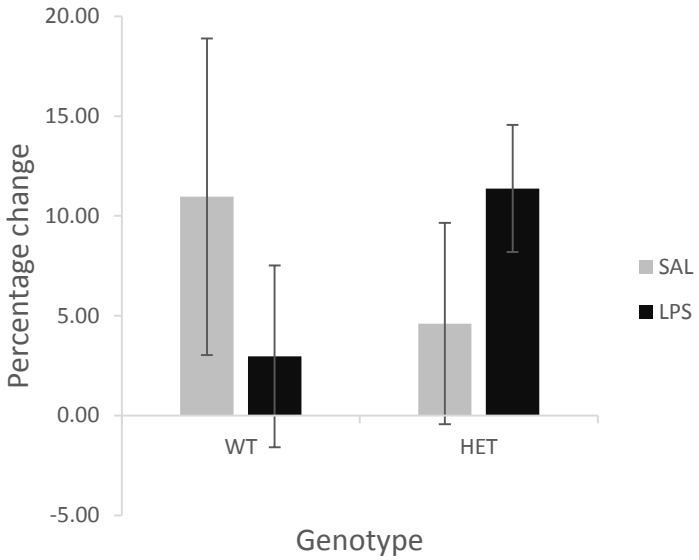

Figure 5. EXP-II: Effects of SERT genotype and LPS treatment on the rewarding properties of social conditioning. Data are expressed as the change in percentage of time spent in an animal's least preferred chamber following a two day social-conditioning period. Bars represent the mean (+SEM).

Experiment III (EXP-III): Social approach and novelty seeking in adults

The purpose of this experiment was to investigate social approach and social novelty seeking behaviour in adult rats. This experiment was designed to be able to detect the same social deficits in adult rats that EXP-I aimed to identify in juveniles. The reason this experiment was performed in adults as well as juveniles was to assess whether any social deficits extended throughout the lifespan of the animal and thus mimic the profile of behavioural deficits observed in patients.

Method

Animals

EXP-III was performed on adult male rats (PND 65-75). As in EXP-I *stranger* rats were also used; these *strangers* were untreated, Sprague Dawley, female juveniles (approximately PND 30). All animals were moved into the experimental room 30 min before the experiment began. Lighting and temperature conditions were the same in the experimental room as they were in the housing room. The sample sizes for the experimental groups in this paradigm were as follows: wild-type + saline = 12; wild-type + LPS = 5; heterozygous + saline = 9; heterozygous + LPS = 14.

Apparatus

The experiment used the same open field arena, cylindrical wire mesh cages, and Ethovision video tracking software as EXP-I.

Procedure

EXP-III employed the same protocol described in EXP-I, but with a one hour delay between phase two (social approach phase) and phase three (social novelty phase). The primary outcomes of interest were: the percentage of total investigatory time spent investigating cylinder (A) in phase two and the percentage of total investigatory time spent investigating cylinder (B) in phase three. In addition, distance moved during habituation (phase one) was calculated.

Results

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on distance moved during phase one. Results indicated there was no main effect of genotype, $F(1, 36) = .001$, $p = .973$, or treatment type $F(1, 36) = 1.045$, $p = .314$.

Similarly, there was no significant interaction between genotype and treatment type $F(1, 36) = .629$, $p = .433$. (See Fig. 6).

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the mean percentage of exploration time occurring near cylinder (A) during phase two. Results indicated there was no main effect of genotype, $F(1, 36) = 1.158$, $p = .289$, or treatment type $F(1, 36) = 1.334$, $p = .256$. Similarly, there was no significant interaction between genotype and treatment type $F(1, 36) = .023$, $p = .881$. (See Fig. 7). One sample t-tests were performed to determine whether the experimental groups displayed a significant preference for the social cue (preference was inferred when the percentage of exploration next to A was significantly higher than 50%). Results indicated that all groups except heterozygous saline-treated animals displayed a significant preference for the social cue.

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the mean percentage of exploration time occurring near cylinder (B) during phase three. Results indicated there was no main effect of genotype, $F(1, 36) = .255$, $p = .617$, or treatment type $F(1, 36) = .000$, $p = .995$. Again, there was no significant interaction between genotype and treatment type $F(1, 36) = .441$, $p = .511$. (See Fig. 8). One sample t-tests were performed to determine whether the experimental groups displayed a significant preference for the novel social cue (preference was inferred when the percentage of exploration next to B was significantly higher than 50%). Results indicated that no experimental groups displayed a significant preference for social novelty.

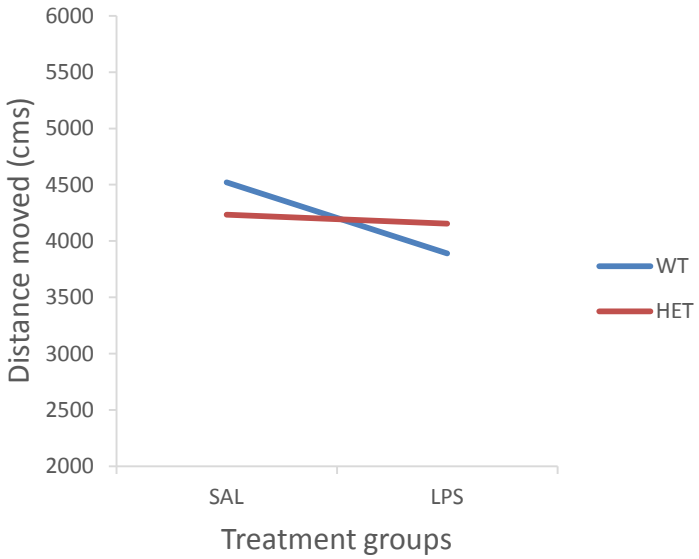

Figure 6. EXP-III: Effects of SERT genotype and LPS treatment in adults on distance moved during habituation. Lines represent the mean.

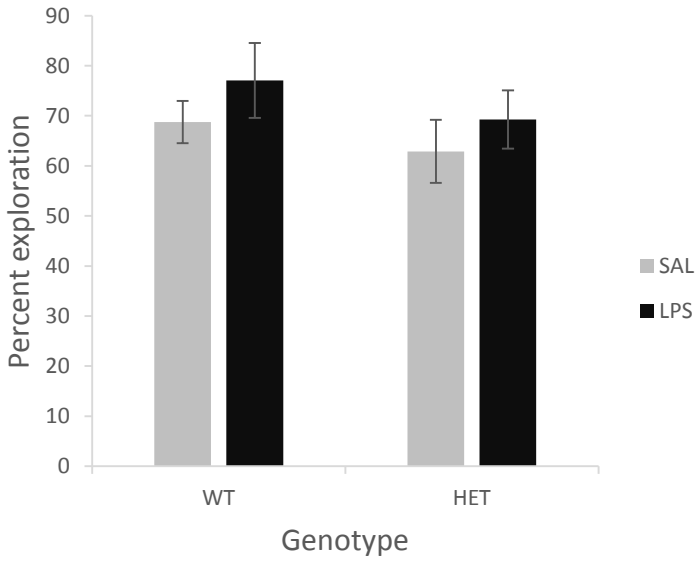

Figure 7. EXP-III: Effects of SERT genotype and LPS treatment in adults on the mean percentage of exploration time near the social cylinder (A) in phase two. Bars represent the mean (+SEM).

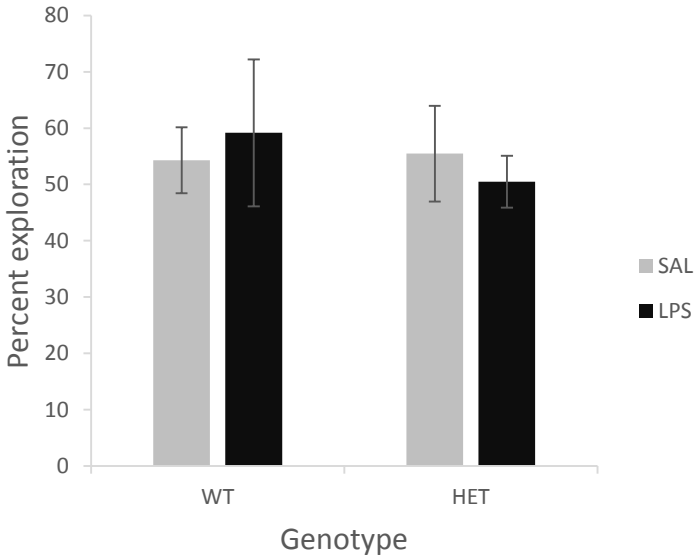

Figure 8. EXP-III: Effects of SERT genotype and LPS treatment in adults on the mean percentage of exploration time near the novel social cylinder (B) in phase three. Bars represent the mean (+SEM).

Experiment IV (EXP-IV): Olfactory-choice paradigm in adults

The purpose of this experiment was to investigate preference for social olfactory cues in adult rats. Typically, rats are highly social animals that will vigorously investigate a social cue and the smell of a novel rat (Lehman & Adams, 1977). The current paradigm aimed to exploit this typical behaviour in an experimental setting to see if the proposed model led to deficits in such behaviour – thus, the experiment was designed to be able to detect one of the three core behavioural hallmarks of ASD: a reduction in social behaviour.

Method

Animals

EXP-IV was performed on male adult rats (PND 90+). As in EXP-II, the experimental room was dimly lit with a lamp and was thus darker than their housing room. Temperature conditions were the same as their housing room. All animals were moved to the experimental room 30 min prior to any testing. The sample sizes for the experimental groups in this paradigm were as follows: wild-type + saline = 9; wild-type + LPS = 6; heterozygous + saline = 10; heterozygous + LPS = 12.

Apparatus

The experiment took place in a clean cage identical to the standard housing cages of the animals except that no food or water was accessible in the testing cage. Before the introduction of any new rat to the paradigm, fresh bedding was placed in the clean cage and the roof of the cage was wiped down with 75% ethanol to remove all prior olfactory cues. Cotton swabs (15 cm) suspended from the roof of the cage were used as the source of social and non-social olfactory cues. The social odour was obtained using soiled bedding from a cage housing foreign male rats – the swab was dragged in a zig-zag pattern along the bottom of the dirty cage. The non-social odour was obtained using banana extract (Hansells, Auckland, NZ; 1:100 dilution). A stop watch was used during the session to measure the time spent investigating each cue. Testing sessions were also recorded with a video camera in order to double-check measured times.

Procedure

The experiment employed an adapted version of the olfactory habituation/dishabituation paradigm described by Crawley et al (2007). In essence, the experiment is an olfactory-

choice paradigm, giving rats the choice between a social olfactory cue and a non-social olfactory cue and measuring the time spent investigating each of the cues. The experiment consisted of two phases: habituation and the test session.

In the first phase of the experiment, animals were taken from their home cage and placed in a fresh cage by themselves. The new cage was the same as their housing cage, except with fresh bedding, no food or drink to access, and two 15 cm cotton swabs suspended from the roof of the cage. Both these swabs were fresh and contained no traces of odour. The experimental animal was left to freely explore the cage for 5 min.

The second phase of the experiment began immediately after habituation and its primary aim was to investigate sociability, as measured by time-spent sniffing social or non-social odours. The two swabs during the habituation phase were discarded and replaced with two new swabs, one of which contained a banana odour (non-social) and the other the smell of another cage of animals (social). In order to obtain the banana odour, the swab was dipped into a 1:100 solution of banana extract. In order to obtain the social smell, the swab was dragged in a zig-zag pattern along the bottom of a dirty, freshly-soiled cage which had previously housed a litter of foreign males. The animal was then allowed 5 min to freely roam around the cage, and time spent investigating each cue was recorded. Investigatory behaviour was defined as the rat's nose being within approximately 2 cm of the head of the cotton swab, or if the rat grabbed the head of the swab with a forepaw. Time spent next to each cotton swab was interpreted as a measure of interest in that particular odour, thus spending time sniffing the social odour was inferred as a sign of sociability – the higher the amount of time spent, the greater the degree of sociability. If experimental groups exhibited an ASD-related phenotype they would be expected to show a reduced preference for the social swab. Experimental sessions were run in the morning, from 09:00 till 12:00. The primary outcomes of interest were: the total time spent investigating both olfactory cues and the percentage of total investigatory time spent sniffing the social cue.

Results

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the total time investigating both olfactory cues combined. Results indicated there was a main effect of genotype $F(1, 33) = 7.960$, $p = .008$, with wild-types spending significantly more cumulative investigatory time than heterozygous animals.

There was no main effect of treatment type $F(1, 33) = .110$, $p = .742$. Similarly, there was no significant interaction between genotype and treatment type $F(1, 33) = .000$, $p = .990$.

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the total time investigating the social olfactory cue. Results indicated there was no main effect of genotype, $F(1, 33) = 1.492$, $p = .231$, or treatment type $F(1, 33) = 1.439$, $p = .239$. Similarly, there was no significant interaction between genotype and treatment type $F(1, 33) = 1.062$, $p = .310$. (See Fig. 9).

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the total time investigating the banana olfactory cue. Results indicated there was a main effect of genotype, $F(1, 33) = 7.203$, $p = .011$, with wild-types spending more time sniffing the banana swab than heterozygous animals. There was no main effect of treatment type $F(1, 33) = 1.010$, $p = .322$. In addition, there was no significant interaction between genotype and treatment type $F(1, 33) = .264$, $p = .611$. (See Fig. 9).

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the *percentage* of total investigatory time spent sniffing the social swab. Results indicated there was a main effect of genotype, $F(1, 33) = 4.395$, $p = .044$, with wild-types spending a lower percentage of time investigating the social swab than heterozygous animals. There was a main effect of treatment type $F(1, 33) = 4.697$, $p = .038$, with LPS-treated animals spending a lower percentage of time investigating the social cue than saline-treated animals. There was no significant interaction between genotype and treatment type $F(1, 33) = .093$, $p = .763$. (See Fig. 10). In addition, one sample t-tests were performed to determine whether the experimental groups displayed a significant preference for the social cue (preference was inferred when the percentage of total investigatory time next to the social cue was significantly higher than 50%). Results indicated that no experimental group statistically preferred the social olfactory cue over the banana olfactory cue.

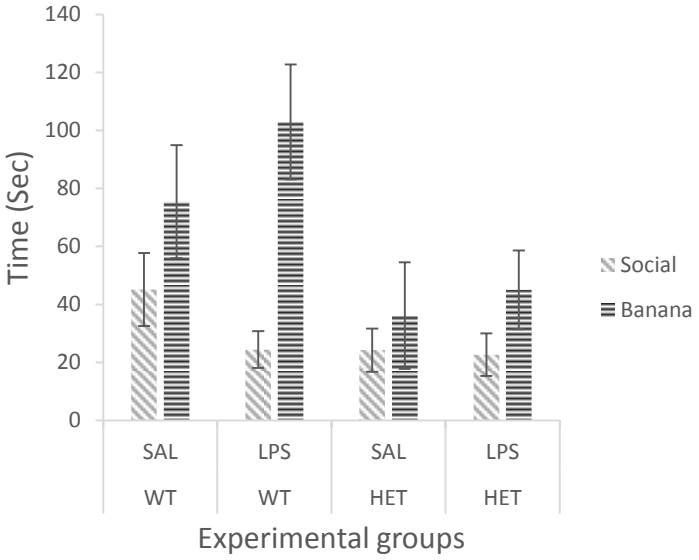

Figure 9. EXP-IV: Effects of SERT genotype and LPS treatment in adults on the total time spent investigating each of the olfactory cues. Bars represent the mean (+SEM).

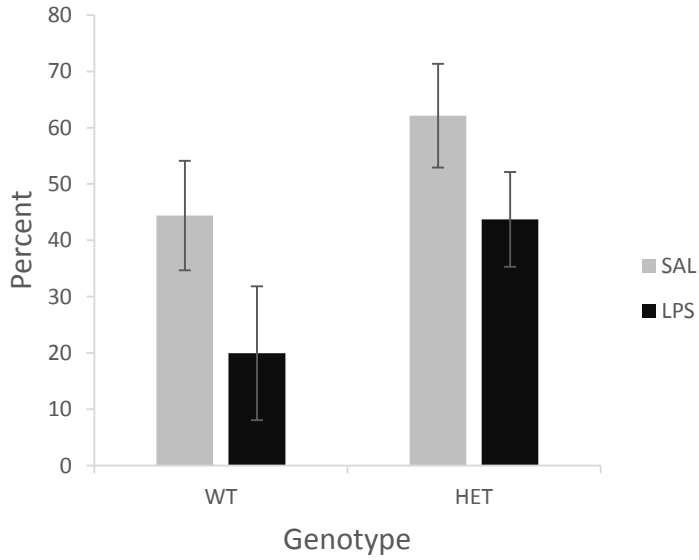

Figure 10. EXP-IV: Effects of SERT genotype and LPS treatment in adults on the percentage of total sniffing exploration spent investigating the social cue. Bars represent the mean (+SEM).

Discussion

The purpose of this chapter was to investigate the effects of LPS on social behaviour in Wistar rats and to test the hypothesis that rats with a genetically compromised SERT function would show an increased vulnerability to the impact of a maternal immune activation encountered during foetal development. Four paradigms were run at different stages of the animals' lives with the aim of detecting ASD-like social deficits. Collectively, the data from this chapter produced three key findings: first, no gene-environment interactions were observed for any measure of social behaviour across the four paradigms. Second, both SERT genotype and LPS treatment influenced social behaviour on at least some measures of social behaviour. Third, several gene-environment interactions were found in relation to locomotor activity.

The most important finding in the current chapter was that no gene-environment interaction was observed on any measure of social behaviour. Thus, the data provide support for the null hypothesis: that there is no interaction between SERT genotype and maternal immune activation on social behaviour in rats. This conclusion presents two distinct interpretations: first, it is in fact true there is no interaction; or second, the experimental conditions failed to detect real interactions at play (a type II error). The failure to detect a real interaction at play could occur for a number of reasons, including a ceiling or floor effect of either the gene or environmental risk factor being used, or the experimental paradigms themselves not being subtle enough to detect an interaction.

The interpretation that seems most consistent with the data is the first one: that there is no interaction between SERT genotype and maternal immune activation on social behaviour in rats. First, although the four paradigms described in this chapter investigated social behaviour in different ways and at different developmental age points in the animals, the data consistently demonstrated there was no gene-environment interaction on any of the measures of social behaviour. The fact that all the data across a wide range of measures points to the same conclusion is strong evidence that there is no gene-environment interaction at play.

Second, a ceiling/floor effect of either the genotype or the environmental factor seems unlikely given the fact only moderate genetic and environmental insults were used. For instance, these experiments focused on heterozygous SERT rats, which only have a 40% reduction in SERT proteins (Homberg, Olivier, et al., 2007; Kalueff, Ren-Patterson, et al., 2007). In behavioural studies published so far, heterozygous rats produce either no or only small effects compared to wild-types (Olivier et al., 2008). Moreover, in the present

study, when a significant genotype effect was found (EXP-I: heterozygous animals displayed lower novelty seeking than wild-types) the effect was only small, leaving room for environmental influences. Likewise, with respect to the environmental risk factor, two injections of LPS (0.5 mg/kg s.c.) spaced over 24 hr were used – this dose represents a moderate dose compared to others used in the literature. For instance, Kirsten, Taricano, Maiorka, et al. (2010) used a much lower dose (0.1 mg/kg i.p. once), while Basta-Kaim et al. (2012) used much higher doses (1 mg/kg s.c every second day from GD 7 until birth). In addition, when main effects of treatment were found (EXP-IV: LPS-treated rats demonstrated less interest in a social olfactory cue than saline-treated rats), these effects were only moderate and left room for the potential influence of genotype.

Finally, both in EXP-I and II a significant gene-environment interaction was found, albeit in relation to locomotion, indicating these two variables do interact on at least some behavioural measures. Overall, it seems likely that SERT genotype does not interact with prenatal LPS exposure, at least in the paradigms used in the current chapter (which are well validated paradigms for investigating social behaviour) (Bambini-Junior et al., 2011; Crawley, 2007; McFarlane et al., 2008; Moy et al., 2007; Peartree et al., 2012; Silverman, Yang, Lord, & Crawley, 2010).

There were two experiments investigating social approach and social novelty seeking in this chapter, one in juveniles (EXP-I) and one in adults (EXP-III). Both experiments demonstrated similar patterns of social approach behaviour in phase two, with almost universal preference for the social cue over the empty cylinder and very little difference between the experimental groups. However in phase three, EXP-I showed an effect of genotype, with heterozygous animals demonstrating significantly less social novelty seeking behaviour than wild-types. In contrast, there was no genotype effect on social novelty seeking in EXP-III, as heterozygous animals displayed the same degree of social novelty seeking as wild-types. Although this study is the first to investigate social behaviour in heterozygous rats, previous research has shown that juvenile homozygous SERT knockout rats show a reduction in play behaviour (Homberg, Schiepers, et al., 2007). Thus, the results from EXP-I support the idea that a genetic reduction in SERT functioning can reduce certain aspects of social behaviour in young rats. The fact that there was no genotype effect in EXP-III indicates that the genotype effect is transient and that heterozygous animals have normal levels of social behaviour in adulthood. These findings are supported by Moy et al. (2009) who investigated adult SERT knockout mice in the social approach/novelty seeking paradigm and found no difference between wild-

type and heterozygous SERT mice in phase two or three. In addition, the results from EXP-IV further support the idea that adult heterozygous rats do not show a deficit in social behaviour. In fact, in this experiment heterozygous animals actually spent a higher percentage of time investigating the social cue than wild-types.

It should be noted that in addition to the animals' age there was another difference between EXP-I and III. Specifically, EXP-III had a one hour gap between phase two and three whereas EXP-I did not. However, it seems unlikely that the lack of a genotype effect was due to the one hour delay.

Interestingly, there was a lack of preference for social novelty found in both EXP-I and III. In fact, across both experiments only wild-type LPS-treated juveniles displayed a significant preference for the novel social cue over the familiar social cue in phase three. Although preference for social novelty has been demonstrated in both mice and rats (Bambini-Junior et al., 2011; McFarlane et al., 2008; Moy et al., 2004; Moy et al., 2007), it is by no means a universal finding and there is significant precedent for the observations in this thesis (Moy et al., 2009; Moy et al., 2007; Roullet et al., 2010). The initial paradigm performed (EXP-III) included a one hour gap between phases two and three. This gap was considered as a potential explanation for the low degree of social novelty seeking and was thus excluded in the paradigm investigating juveniles (EXP-I). However, even EXP-I found a low degree of social novelty seeking, suggesting the one hour delay was not the reason for the low preference for social novelty. Previous research has demonstrated preference for social novelty to be related to the strain of animal used in the experiment, with some strains exhibiting no preference for social novelty (Moy et al., 2007). Thus, the lack of social novelty observed in the current thesis may be accounted for by the strain or species of animals used, and/or the differences in protocol compared with previous studies using a similar paradigm. A key difference in protocol is that the current thesis used a round open field with the cylinders at opposing ends, whereas other studies have used a three-chambered box with the cylinders separated by a middle chamber.

In addition, previous research has indicated that the parameters used for assessing social approach/novelty seeking can have an important influence on the overall conclusion. Thus, while many studies use 'time spent in the chamber' others have more specifically assessed 'time spent sniffing the cylinder.' It was suggested that 'time spent sniffing the cylinder' may be the more sensitive measure of social approach behaviour (Moy et al., 2007). For instance, measuring 'time spent sniffing the cylinder' allowed for

the detection of social approach behaviour in the BALB/cByJ, BTBR T+tf/J, and 129S1/SvImJ inbred mouse strains in one study, and the male and female Slc6a4-null mice and female heterozygous Slc6a4 mice in another study, that was not detected using the 'time spent in the chamber' measure (Moy et al., 2009; Moy et al., 2007).

However, these authors found no evidence for an increased sensitivity when measuring social novelty seeking via the 'time spent sniffing' calculation. The current thesis did not use chambers, but instead measured time spent (nose-point and mid-point of the animal) in the immediate proximity (within 5 cm) of the cylinder and thus represents a measure more in line with the sniffing measure used in previous studies.

EXP-II investigated the rewarding properties of social behaviour in a CPP paradigm. Aside from the fact EXP-II did not find a genotype effect, treatment effect, or gene-environment interaction, the experiment also found limited evidence for the rewarding properties of social behaviour in a CPP paradigm, with only heterozygous LPS-treated animals displaying evidence of social conditioning. These findings are in contrast to previous research that has observed social behaviour to be rewarding in a CPP paradigm (Calcagnetti & Schechter, 1992; Peartree et al., 2012; Van den Berg et al., 1999). Several factors may have contributed to the surprising CPP results in EXP-II.

First, the animals were not socially isolated before the conditioning phase due to limited cage space in the laboratory and thus social behaviour may not have been as rewarding as possible. The data provided by Kirsten, Taricano, Maiorka, et al. (2010) support this idea – these authors found that only by increasing the motivation for social behaviour through isolation were the differences between control animals and LPS-treated animals able to be identified.

Second, the 'pairing' animal is important to the social value of the social conditioning and EXP-II could not always use an ideal social pair (Trezza, Damsteegt, & Vanderschuren, 2009). In this experiment, animals were paired with foreign rats of the same sex, strain, and age, but breeding issues meant the paired rat was not always the same treatment and genotype as the experimental rat, which would have been ideal. The reason the pairing rat is important is that the interaction between two rats is key to the rewarding value of a social interaction. For example, if the pairing rat is unresponsive to play then social conditioning will not be as rewarding for the experimental rat and will likely not induce a positive change in preference in the CPP box. Considering EXP-I found social deficits in heterozygous rats it is possible that pairing wild-type rats with

heterozygous rats during the conditioning phase may have been less rewarding for the wild-types than expected.

Finally, despite the experiment being performed in dim lighting conditions, it was also performed during the animals' 'light phase' and not during the peak social activity that occurs in their 'dark phase.' These described limitations may partly explain why no significant social conditioning was found in wild-type rats. In turn, this lack of effect on the control group may partly explain why no significant main effects or an interaction was observed, as our genetic and environmental risk factors were expected to decrease the degree of social conditioning. Nonetheless, it is surprising to find that heterozygous LPS-treated animals were the only group to display evidence of social conditioning and the reason for this finding remains unclear. Future research using a CPP paradigm to assess social behaviour would benefit from social isolation, appropriate pairing of animals, and experimentation during the animals' dark phase.

In EXP-IV the preference of rats for a social vs. non-social odour was investigated, based on similar paradigms in mice (Crawley et al., 2007; Ryan, Young, Crawley, Bodfish, & Moy, 2010). Surprisingly, none of the experimental groups showed a significant preference for the social over the banana olfactory cue. This is in contrast to prior research observing several different strains of mice to be significantly more interested in novel social olfactory cues than novel banana olfactory cues (Crawley et al., 2007). However, these previous experiments presented the cues sequentially whereas in EXP-IV both cues were deliberately presented simultaneously to more directly assess choice behaviour in the rats. Perhaps presenting the cues sequentially might have led to a different outcome. Moreover, to the best of our knowledge, this is the first time a study of this kind was performed using rats and thus it is at least possible that rats show less preference for a social olfactory cue, or, alternatively, that banana may not have been the ideal non-social cue in rats that it was in mice. The difference in protocol, combined with the different species of animals used across the experiments, may partially account for these divergent findings.

Collectively, the data presented in this chapter suggest that our LPS treatment protocol provided only a small effect on social behaviour. Although EXP-IV observed that LPS had detrimental effects on social behaviour in adulthood, this was the only experiment in the chapter to find such an effect. In fact, the remaining three experiments (I, II, and III) found no effect of LPS on social behaviour. These findings are not too surprising considering a moderate dose of LPS was deliberately chosen in order to detect

an interaction. The findings reported here support those by Xuan and Hampson (2014) who similarly observed social behaviour equivalent to controls in rodents prenatally treated with LPS. Nonetheless, many studies have reported a reduction in social behaviour following foetal exposure to a maternal immune response (Bauman et al., 2014; Kirsten, Taricano, Maiorka, et al., 2010; Shi et al., 2003).

Evidently, the question of whether a maternal immune activation influences social behaviour provides diverse answers. The explanation for this diversity is likely that social behaviour is a nuanced class of behaviours, made up of several subcomponents, prone to subtle differences in experimental design and thus contrasting findings from experiments that are not exact replications are to be expected. Therefore, contrasting findings are unlikely to be due to one specific reason, but instead may be a combination of the type of social behaviour being investigated (social approach, social novelty, etc), experimental animals being used (age, strain, species, and sex), immunogen being administered (type, method, timing, and dose), and the paradigm and protocol utilised. Protocol differences that may provide the biggest influence on social behaviour data may be the method of housing the animals (grouped vs. individual housing), and whether the animals are investigated in their light or dark phase.

Interestingly, the data from this chapter indicated LPS treatment produced alterations in locomotor activity that depended on both SERT genotype and the age of the animals. Briefly, while in juveniles LPS increased locomotion in wild-types but decreased locomotion in heterozygous animals (EXP-I), no such effect was seen in adult animals (EXP-III). Interestingly, in EXP-II in adolescents, LPS decreased locomotion in wild-types and increased locomotion in heterozygous animals. However, EXP-II had a completely different experimental design (three compartments that differed in texture, smell, and visual cues) and thus it is difficult to compare this with the other two experiments. Nonetheless, EXP-I and III show that the LPS dosage was active enough to influence some aspects of behaviour, that these risk factors can in fact interact, and that the influence of LPS on locomotion depends on both the age and genotype of the animal but that these effects extinguish in adulthood.

Although this is the first study to investigate prenatal LPS in heterozygous rats, the influence prenatal LPS had in wild-types in EXP-I and III (an increase in locomotor activity in juveniles, but no effect in adults) is consistent with previous literature (Lin, Lin, & Wang, 2012; Wischhof, Irrsack, Osorio, & Koch, 2015). However, it should be acknowledged that a wide range of different effects on locomotion following prenatal

LPS have been reported, likely due to the large diversity in LPS treatment schedules (Harvey & Boksa, 2014; Kirsten, Taricano, Flório, Palermo-Neto, & Bernardi, 2010; Stigger et al., 2013).

Conclusion

Taken together, the results of these four experiments indicate that there was no interaction between SERT genotype and maternal immune activation on social behaviour in rats. Although the data show that both SERT genotype and LPS treatment can independently influence social behaviour on at least some measures, the effect of genotype was dependent on the specific parameter and the majority of data suggested LPS had little effect. These small/moderate effects were not too surprising considering moderate genetic and environmental insults were deliberately chosen for the purposes of detecting an interaction. Thus, these findings lend only some support to previous literature suggesting both a compromised SERT genotype and maternal immune activation/maternal infection lead to social deficits in animals (Bauman et al., 2014; Homberg, Schiepers, et al., 2007; Shi et al., 2003; Shi et al., 2009). However, the results in this chapter do seem to resemble human research in this field i.e., there is an inconsistent 5-HTTLPR genotype association with ASD, and although maternal infection increased the likelihood of ASD in children, the overall odds of developing ASD are still low (Atladóttir et al., 2010; Devlin et al., 2005). Interestingly, there was evidence to suggest LPS treatment produced behavioural outcomes relating to locomotion that depended on both SERT genotype and the age of the animal.

In summary, the data from this chapter did not provide initial face validity for a *SERT reduction-maternal immune activation* gene-environment model for ASD, but importantly, the data did demonstrate that these two risk factors can interact on certain behavioural measures and have independent effects on specific social parameters. Claims of the risks provided by SERT genotype and maternal immune activation in the development of ASD should be made with caution.

CHAPTER 3: PRENATAL VALPROATE AND BEHAVIOUR

VPA is a mood-stabilizing drug used for epilepsy, migraine, bipolar, and other mood disorders. Moreover, its uniquely diverse properties mean it is also being trialled as a treatment in a variety of diseases including cancer, HIV, and Alzheimer's disease (Avallone et al., 2014; Brodie & Brandes, 2014; Grishina et al., 2015; Hu et al., 2011; Lehrman et al., 2005; Qing et al., 2008). A complete explanation of the molecular mechanisms underlying VPA's wide-ranging effects is currently lacking; however, the preponderance of evidence suggests HDAC-I and oxidative stress are two important mechanisms through which VPA exerts its teratogenic influence (Ranger & Ellenbroek, 2015). The reason VPA's mechanistic action is unclear is that VPA works via many different mechanisms, produces a multitude of outcomes, interacts with a variety of neurotransmitters, enzymes, and proteins, and the mechanism of influence can change depending on the dose and timing of usage (Bollino et al., 2015; Fathe et al., 2014; Jeong et al., 2003; Johannessen & Johannessen, 2003; Lloyd, 2013).

Despite its prophylactic properties, VPA is a teratogenic agent that can damage developing foetuses and potentially lead to severe consequences throughout life, including congenital malformations and neurodevelopmental deficits (Christensen et al., 2013; Diav-Citrin et al., 2008; Koren et al., 2006; Morrow et al., 2006). Indeed, an increasing amount of data from both humans and animals suggest that prenatal exposure to VPA is a clear environmental risk factor for ASD (Christensen et al., 2013; Moore et al., 2000; Rasalam et al., 2005; Schneider & Przewlocki, 2005). The most recent large-scale population-based study conducted in Denmark found that individuals prenatally exposed to VPA had a risk factor of 4.42% for ASD, whereas controls not exposed to VPA had a risk factor of 1.53% – indicating a near 3 fold increased risk for ASD in those prenatally exposed to VPA (Christensen et al., 2013).

The purpose of this chapter was to investigate the behavioural consequences of a 400 mg/kg dose of VPA administered subcutaneously at GD 12 and to see if the behavioural response was dependent on SERT genotype. This VPA schedule was chosen for two reasons: first, it has been used before (Kim et al., 2011), and second it represents a moderate dose within the context of the literature – an important point when attempting to identify a potential interaction. ASD is characterised by three core deficits: a reduction in social behaviour, impaired communication, and an increase in repetitive behaviour/stereotypy. Multiple behavioural paradigms aimed at investigating all three of

these core dysfunctions are included in this chapter. These experiments were designed to be able to detect ASD-like phenotypes in rats, using species-relevant behaviours. The data from these experiments helped determine the face validity of a *SERT reduction-prenatal exposure to VPA* gene-environment model for ASD. These experiments tested the hypothesis that rats with a genetically compromised SERT function would show an increased vulnerability to the impact of prenatal exposure to VPA.

Animals

The types of animals used in this chapter were identical to the animals used in chapter 2. In brief, all experimental animals were Wistar rats that varied according to their genotype. The genotypes of interest were wild-type and heterozygous SERT knockout rats. All rats were group housed in a temperature-controlled room (temperature: 21± 2^{o}C; 55% humidity) and kept on 12 hr light-dark cycles (lights on at 07:00) with food and water available ad libitum. Pups were weaned at PND 21, and then housed with sex-matched littermates in standard Plexiglas cages (40 cm x 25 cm x 12 cm), with pine bedding. An animal technician cared for the rats and euthanised the animals when their role in the experiment had come to an end. All procedures in this chapter were approved by the Victoria University of Wellington Animal Ethics Committee.

Breeding

Rather than mating wild-type mothers with wild-type or homozygous males as in chapter 2, instead heterozygous mothers and heterozygous fathers were mated. The reason for using this new breeding method was primarily the low success rate of breeding using the first technique. The switch to using heterozygous parents appeared to increase the success rate of breeding.

Once a heterozygous mother successfully mated (defined as the day a vaginal plug was discovered, GD 0) she was removed from the male, housed individually, and subcutaneously injected with either VPA (400 mg/kg) or saline on GD 12. This new technique created the four experimental groups for this chapter (Table 3):

Table 3. Experimental groups used in chapter 3.

Group	Genotype	Treatment
1	Wild-type	SAL
2	Wild-type	VPA
3	Heterozygous	SAL
4	Heterozygous	VPA

In addition, because this new technique did not guarantee one particular genotype in the offspring as with the first technique, within a few days of weaning (PND 21) rats were ear-punched and the tissues subsequently genotyped by a commercial genotyping company (Transnetyx, Cordova, TN, USA).

In all of the following experiments, multiple litters were used to create the sample size for each experimental group. The reason for this was to reduce the chance of potentially abnormal litters representing an entire group. The sample sizes reported in these experiments are indicative of individual rats (except where otherwise indicated).

Data analysis

IBM SPSS statistics version 22 was used for all statistical analyses in this chapter. The alpha level for statistical significance was set at $p < .05$.

Experiment V (EXP-V): Ultrasonic vocalisations in pups

The purpose of this experiment was to investigate ultrasonic vocalisation (USV) communication in PND 7 rats. Although rat communication is not perfectly understood, much is known about the way they communicate. Rats emit USVs as a means of communication and the frequency of these USVs depends on a variety of factors, including the age of the animal, its emotional state, and environmental variables (Brudzynski, 2013; Brudzynski, Kehoe, & Callahan, 1999; Insel, Hill, & Mayor, 1986). Various situations throughout a rat's life can induce the rat to emit USVs, including: separation from the nest, interaction with a predator, social, sexual, and aggressive behaviour, engagement in cooperative behaviour with a conspecific, courtship, and exploration (Barfield, Auerbach, Geyer, & McIntosh, 1979; Blanchard, Blanchard, Agullana, & Weiss, 1991; Burgdorf et al., 2008; Knutson, Burgdorf, & Panksepp, 1998; Łopuch & Popik, 2011; Wöhr & Scattoni, 2013; Wöhr & Schwarting, 2007).

In pups, USVs are a vital means of communication and play a significant role in survival. Previous research has demonstrated that pups separated from their mothers will reliably emit USVs, most likely as a way of helping her to locate them and return them to the safety and warmth of the nest. The isolation-induced USVs emitted by the pup indicate a negative affective experience and may be similar to human infant cries for help (Shair et al., 2015). These 'retrieval' or 'distress' calls are effective in eliciting maternal search behaviour both when the pup emits the call in real time and when it is simply an audio play-back of a call (Allin & Banks, 1972; Brunelli, Shair, & Hofer, 1994; Farrell & Alberts, 2002; Smotherman, Bell, Starzec, Elias, & Zachman, 1974; Wöhr & Scattoni, 2013). The current paradigm aimed to exploit this typical method of communication in an experimental setting to see if the proposed model led to deficits in this type of communication – thus, the experiment was designed to be able to detect one of the three core behavioural hallmarks of ASD: impaired communication.

Method

Animals

EXP-V was performed using male and female pups at PND 7. As this age was prior to the weaning process (PND 21), the animals were still housed with their mother and siblings. Thus, the entire home cage including the mother and litter was moved to the experimental room 30 min prior to experimentation. Lighting and temperature conditions were the same in the experimental room as in the housing room. Food and water were available ad libitum. The sample sizes for the experimental groups in this paradigm were as follows: wild-type + saline = 7; wild-type + VPA = 6; heterozygous + saline = 17; heterozygous + VPA = 11.

Apparatus

EXP-V took place in a cylindrical wire mesh cage (11 cm diameter, 14 cm height) lined with fresh pine bedding and a specialised ultrasonic microphone (Ultravox) placed directly above the cage, approximately 15 cm from the animal. Noldus Ultravox XT software and technology was used to record pup USVs and Raven Pro 1.5 was used to detect the calls.

Procedure

Essentially, this experiment involved separating a pup from its mother and then measuring the pup's USVs for a 5 min period. Specifically, an individual pup was taken from its home cage and placed inside the wire mesh cage lined with pine bedding. After a delay of 10 s, the ultrasonic microphone placed above the cage was turned on and recorded USVs for the next 5 min. The 10 s delay was used to control for the immediate effects of handling. During the 5 min period of USV recording, the experimenter left the room to ensure minimal noise was picked up by the microphone. Once this period was completed, the pup was removed from the wire mesh cage and its role in the experiment was complete. The primary outcomes of interest were the total number of USV emissions, or 'calls,' produced by the pup during the experiment, and the mean duration of these calls.

Results

As this experiment involved both males and females, initially one-way ANOVAs were used to test the effect of sex on the primary outcomes of interest. Results revealed there was no main effect of sex on the number of total calls: $F(1, 40) = .499$, $p = .484$; or the mean duration of these calls: $F(1, 40) = 1.224$, $p = .275$. Thus, both sexes were pooled together for all future analyses.

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the total number of calls. Results indicated there was no main effect of genotype, $F(1, 38) = .002$, $p = .961$, or treatment type $F(1, 38) = .266$, $p = .609$. Similarly, there was no significant interaction between genotype and treatment type $F(1, 38) = 3.200$, $p = .082$. (See Fig. 11).

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the mean duration of calls. Results indicated there was no main effect of genotype, $F(1, 38) = 2.636$, $p = .113$, or treatment type $F(1, 38) = 1.140$, $p = .292$. Similarly, there was no significant interaction between genotype and treatment type $F(1, 38) = .006$, $p = .940$. (See Fig. 12).

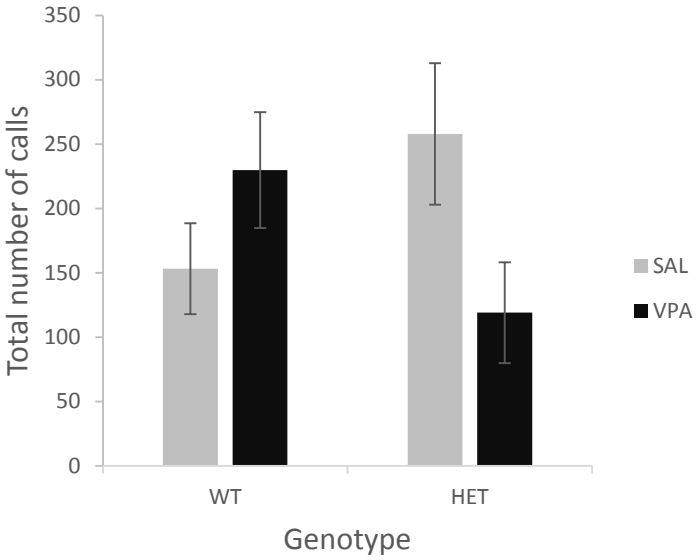

Figure 11. EXP-V: Effects of SERT genotype and VPA treatment on the total number of USV calls emitted by PND 7 pups during a 5 min isolation period. Bars represent the mean (+SEM).

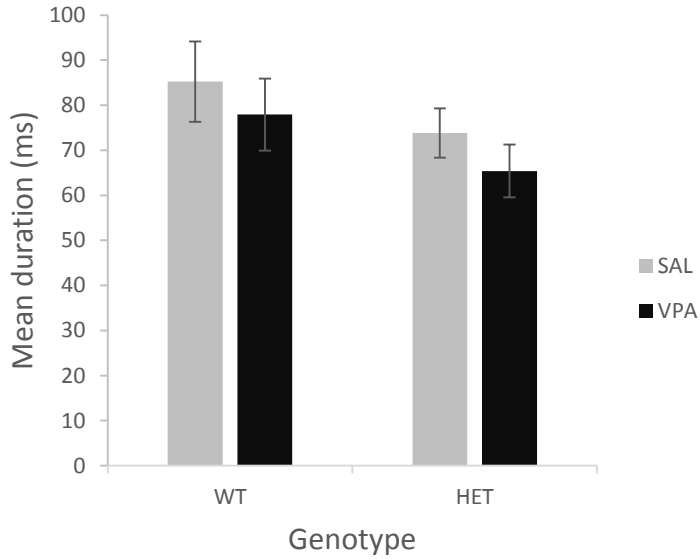

Figure 12. EXP-V: Effects of SERT genotype and VPA treatment on the mean duration of USV calls emitted by PND 7 pups during a 5 min isolation period. Bars represent the mean (+SEM).

Experiment VI (EXP-VI): Social interaction following isolation in adults

The purpose of this experiment was to investigate social interaction in adult rats following a brief period of isolation. Typically, rats engage in social interaction and will actively interact with a novel conspecific (Grant, 1963; Lehman & Adams, 1977), particularly after a period of short-term isolation (Niesink & Van Ree, 1982). The current paradigm aimed to exploit this typical behaviour in an experimental setting to see if the proposed model led to deficits in social interaction. Thus, the experiment was designed to detect one of the three core behavioural hallmarks of ASD: a reduction in social behaviour.

Method

Animals

EXP-VI was performed using adult male rats (PND 90+). It is important to note that this experiment investigated pairs of animals, rather than individual animals. For this paradigm each rat was taken from its home cage and individually housed in a clean cage in the experimental room the day before experimentation to optimise social interaction. Specifically, rats were isolated at midday and experimentation began at 10:00am the following day. Food and water were available ad libitum during this period of isolation. Lighting and temperature conditions were the same in the experimental room as they were in the housing room. This paradigm used 5 wild-type saline pairs, 4 wild-type VPA pairs, 11 heterozygous saline pairs, and 11 heterozygous VPA pairs.

Apparatus

The experiment took place in the same standard open field circular arena (80 cm in diameter, 45 cm in height) with black flooring and walls as used in previous experiments. Ethovision XT v9.0 video tracking software was used to investigate animal social interactions. The arena was washed with warm water and wiped down with 75% ethanol before each new pair of experimental animals was put into the open field; this was done in order to minimise scent carry-over from previous animals.

Procedure

Essentially, this experiment involved pairing animals of identical genotype and prenatal treatment, but from different litters, following a period of isolation (approximately 24 hr) and examining their social interaction for 30 min.

Animals were placed at opposite ends of the open field and video was recorded for 30 min. Active and passive social behaviours were manually scored using Ethovision XT v9.0 software. Active social behaviours were defined as: at least one animal in the pair actively investigating the other, either through sniffing, following, passing, mounting, grooming, or attacking its partner. Passive social behaviour was defined as both animals disengaged from active social behaviours but still within close proximity of each other, suggesting they still prefer social contact even if they are not directly interacting. A pair was defined as being in close proximity if a rat's body was within touching distance of the other rat's tail. The primary outcomes of interest were: total duration of both active and passive social behaviours, and the frequency of both active and passive social behaviours.

Results

Active Duration

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the total duration of active social behaviour. Results indicated there was no main effect of genotype, $F(1, 27) = .034$, $p = .856$, or treatment type $F(1, 27) = 1.458$, $p = .238$. However, there was a significant interaction between genotype and treatment type $F(1, 27) = 4.674$, $p = .040$. (See Fig. 13).

Active Frequency

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the frequency of active social behaviour. Results indicated there was no main effect of genotype, $F(1, 27) = .077$, $p = .783$, or treatment type $F(1, 27) = .641$, $p = .430$. However, there was a significant interaction between genotype and treatment type $F(1, 27) = 6.614$, $p = .016$. (See Fig. 14).

Passive Duration

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the total duration of passive social behaviour. Results indicated there was no main effect of genotype, $F(1, 27) = .690$, $p = .414$, or treatment type $F(1, 27) = 3.317$, $p = .080$. Similarly, there was no significant interaction between genotype and treatment type $F(1, 27) = .403$, $p = .531$. (See Fig. 15).

Passive Frequency

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the frequency of passive social behaviour. Results indicated there was no main effect of genotype, $F(1, 27) = .002$, $p = .961$, or treatment type $F(1, 27) = 1.701$, $p = .203$. Similarly, there was no significant interaction between genotype and treatment type $F(1, 27) = .265$, $p = .611$. (See Fig. 16).

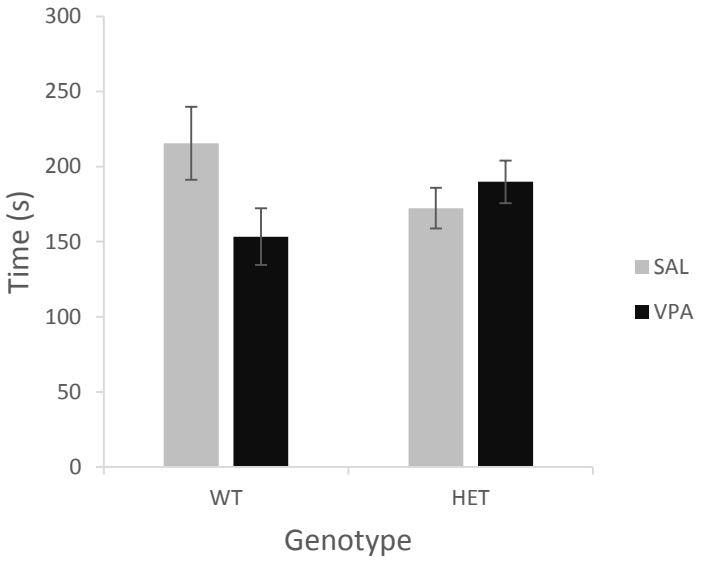

Figure 13. EXP-VI: Effects of SERT genotype and VPA treatment on the total duration of active social behaviour in adult pairs following a period of isolation. Bars represent the mean (+SEM).

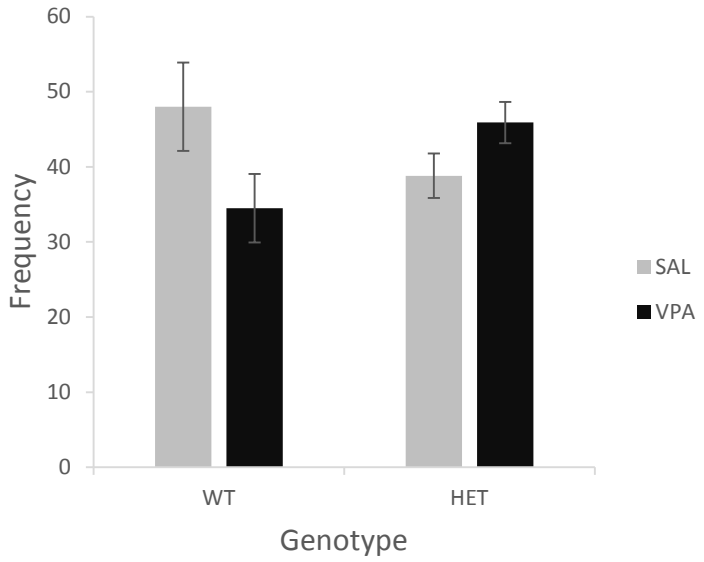

Figure 14. EXP-VI: Effects of SERT genotype and VPA treatment on the frequency of active social behaviour in adult pairs following a period of isolation. Bars represent the mean (+SEM).

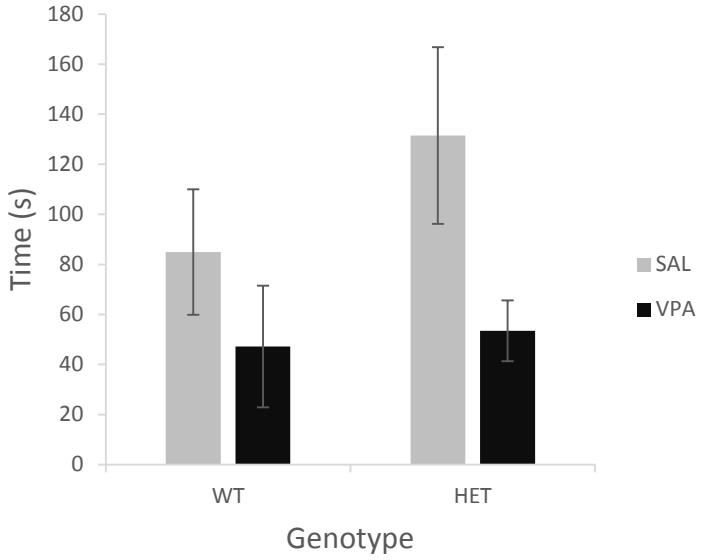

Figure 15. EXP-VI: Effects of SERT genotype and VPA treatment on the total duration of passive social behaviour in adult pairs following a period of isolation. Bars represent the mean (+SEM).

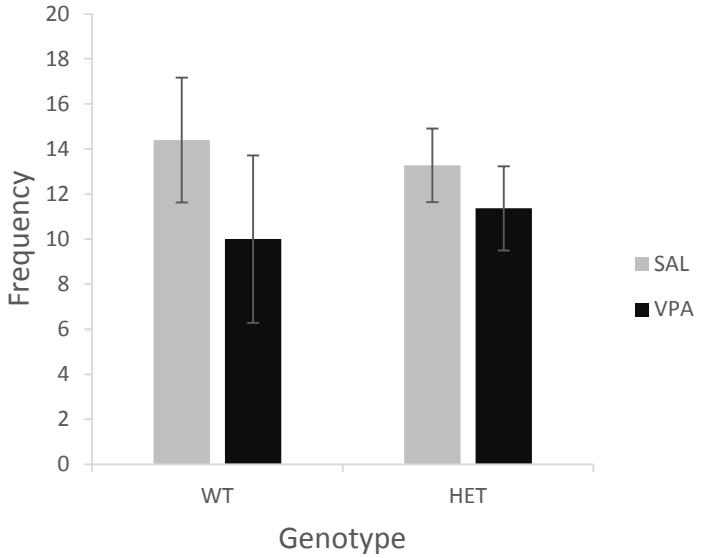

Figure 16. EXP-VI: Effects of SERT genotype and VPA treatment on the frequency of passive social behaviour in adult pairs following a period of isolation. Bars represent the mean (+SEM).

Experiment VII (EXP-VII): Scent marking in adults

The purpose of this experiment was to investigate scent marking communication in adult rats. In addition to the emission of USVs, rodents can communicate through olfactory signals, or scent marking (Arakawa, Blanchard, Arakawa, Dunlap, & Blanchard, 2008; Wöhr & Scattoni, 2013). Scent marking is a form of communication whereby the animal deposits urinary pheromones, used to convey information in a variety of domains including territorial and fighting behaviour, sexual and social behaviour, and predator identification. Typically, male rodents will produce scent marks in the presence of a female urinary cue (Adams, 1976; Wöhr, Roullet, & Crawley, 2011; Wöhr & Scattoni, 2013). The current paradigm aimed to exploit this typical behaviour in an experimental setting to see if the proposed model led to deficits in this type of communication. Thus, the experiment was designed to be able to detect one of the three core behavioural hallmarks of ASD: impaired communication.

Methods

Animals

EXP-VII used adult male rats as experimental animals (PND 90 +). However, female adult rats were also used in the socialisation phase that preceded the experimental phase. The sample sizes for the experimental groups in this paradigm were as follows: wild-type + saline = 8; wild-type + VPA = 5; heterozygous + saline = 8; heterozygous + VPA = 7.

Apparatus

The experiment took place in a standard open field circular arena (80 cm in diameter, 45 cm in height) with black flooring and walls. A clean, regular housing polycarbonate cage was used during the socialisation phase in which male rats were exposed to foreign female animals. Lemon essence (The Aromatherapy Company, New Zealand) was used as the novel smell competing with the social smell of female urine; both of these odours were pipetted onto pieces of circular standard filter paper. The open field was cleaned with a 70% ethanol solution before new experimental animals were introduced to the paradigm. Ninhydrin spray was used to identify scent markings left by the animals during the experimental procedure.

Procedure

The procedure for this experiment was adapted from the scent marking paradigm described by Wöhr et al. (2011). The experiment was divided into three distinct phases: the socialisation, habituation, and experimental phases. The socialisation phase consisted of placing an individual male rat in a fresh cage with a foreign female rat of an identical strain and leaving them there for 5 min. The purpose of this phase was to ensure the experimental male rats had been directly exposed to female smell before the next phase of the experiment. It must be remembered that male rats had only been housed with other males since weaning at PND 21.

The habituation phase came 6-9 days later. Experimental rats were placed in an open field for 20 min in order to habituate to the novel environment. The purpose of this phase was to reduce the potential effect of anxiety in the experimental phase. Following 20 min free exploration, the rat was removed temporarily whilst the open field was cleaned of urine and faeces.

Immediately following the cleaning of the open field, the experimental phase commenced. Two pieces of filter paper were placed on either side of the open field, one containing 30 µl of lemon essence, and the other containing 30 µl of adult female urine. The rat was then placed back into the open field and left to freely explore for 5 min. At the end of this 5 min exploration period the rat was removed and placed back in its home cage – its role in the experiment complete. The filter paper was then removed and left to dry for an hour so it could be analysed later for scent markings. Scent markings near the cues were interpreted as signs of communication, thus the more scent markings there were, the more communicative that animal was interpreted as being. The primary outcomes of interest were: the percentage of scent marks made next to the urinary cue and the total number of scent marks.

Scent marking analysis

After the experimental phase the filter paper was removed and left to dry for an hour. Once dried, the filter paper was sprayed with ninhydrin spray and left for 24 hr in order to illuminate the scent markings on the paper. Ninhydrin spray reacts with the ammonia in urine to produce a distinguishable purple colour that can be observed for analysis (Laskar, Bhattacharya, & Basak, 1991). The ninhydrin spray illuminated scent markings, pools of urine, sneeze markings, and footprints. The scent markings were counted using transparent grid paper, each of the squares 1 cm x 1 cm. Scent marks were defined as

marks smaller than four 1 cm grids, unless they had obvious visual characteristics of footprints or sneezes. Marks larger than four 1 cm grids were defined as urine pools and not included in the analysis.

Urine Collection

Urine was collected from female strain-matched rats in the oestrus phase of the reproductive cycle. To determine whether the females were in the oestrus phase, they were picked up gently by the base of the tail and the genital region was visually inspected. A female was defined as being in the oestrus phase when a reddish and relaxed vagina was observed. In order to collect urine, the female rat was held over a container and its stomach was rubbed to encourage urination. Fresh urine was always used for experimentation – no more than 60 min old.

Results

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the percentage of overall scent marks made next to the urinary cue. Results indicated that there was no main effect of genotype, $F(1, 24) = 3.834$, $p = .062$, or treatment type $F(1, 24) = .781$, $p = .386$. Similarly, there was no significant interaction between genotype and treatment type $F(1, 24) = 2.663$, $p = .116$. (See Fig. 17). One sample t-tests were performed to determine whether the experimental groups displayed a significant preference for marking next to the urinary cue (preference was inferred when the percentage of scent marks was significantly higher than 50%). Results indicated that no group displayed statistical preference for scent marking next to the urinary cue.

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the total number of scent marks. Results indicated there was no main effect of genotype, $F(1, 24) = .000$, $p = .983$. However, there was a main effect of treatment type $F(1, 24) = 4.763$, $p = .039$, with saline-treated animals marking significantly more than VPA-treated animals. There was no significant interaction between genotype and treatment type $F(1, 24) = .835$, $p = .370$. (See Fig. 18).

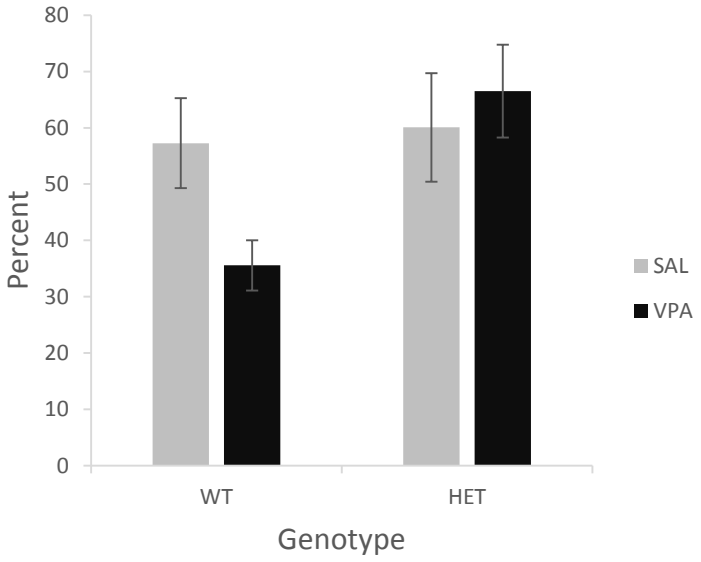

Figure 17. EXP-VII: Effects of SERT genotype and VPA treatment on the percentage of scent markings produced next to the urinary social cue. Data are expressed as a percentage of the number of scent markings produced next to both cues combined. Bars represent the mean (+SEM).

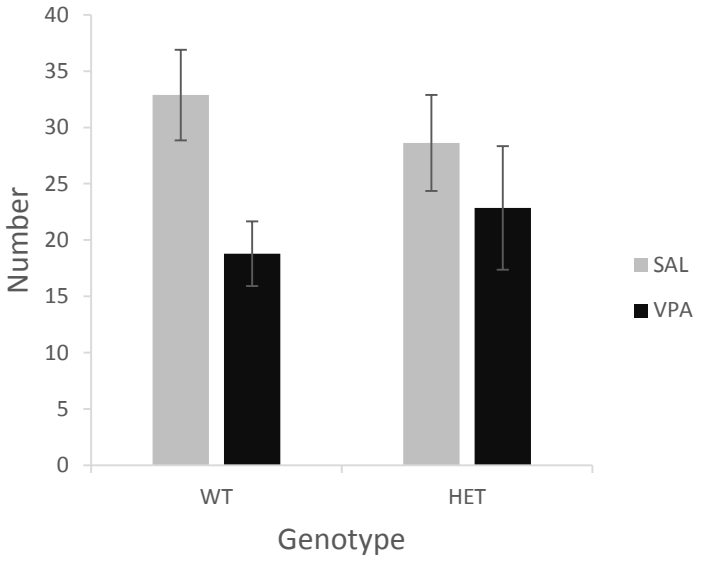

Figure 18. EXP-VII: Effects of SERT genotype and VPA treatment on the total number of scent marks produced during the experimental phase. Bars represent the mean (+SEM).

Experiment VIII (EXP-VIII): Social approach and novelty seeking in adolescents

The purpose of this experiment was to investigate social approach and social novelty seeking behaviour in adolescent rats. This experiment was designed to be able to detect the same social deficits in adolescent rats that EXP-I and III aimed to identify in juveniles and adults, respectively. EXP-VIII was performed in adolescents so that these social behaviours could be evaluated at three different developmental stages.

Method

Animals

EXP-VIII was performed using adolescent male rats (PND 35-60). As in EXP-I *stranger* rats were also used; these *strangers* were untreated, Sprague Dawley, female juveniles (approximately PND 30). All animals were moved into the experimental room 30 min before the experiment began. Lighting and temperature conditions were the same in the experimental room as they were in the housing room. The sample sizes for the experimental groups in this paradigm were as follows: wild-type + saline = 7; wild-type + VPA = 8; heterozygous + saline = 10; heterozygous + VPA = 18.

Apparatus

EXP-VIII used the same open field arena, cylindrical wire mesh cages, and Ethovision XT v9.0 video tracking software as EXP-I and III, described in chapter 2. In addition, a tennis ball was used as an inanimate novel object in phase two.

Procedure

EXP-VIII employed the same protocol described in EXP-I, with one key difference: there was a novel inanimate object placed in the empty cylindrical cage (B) during phase two. The reason for this adaptation was to control for the possibility of a ceiling effect of the social cue in phase two. As both EXP-I and III did not observe a social deficit in phase two, it is possible that the difference between a novel rat in a cage and an empty cage was simply too large, meaning a social deficit would need to be drastic to reveal a difference in an experimental group. Thus, in the current experiment the comparison was between a novel rat and a novel inanimate object (a tennis ball).

The primary outcomes of interest were: the percentage of total investigatory time spent investigating cylinder (A) in phase two and the percentage of total investigatory

time spent investigating cylinder (B) in phase three. Distance moved during habituation (phase one) was also calculated.

Results

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on distance moved during phase one. Results indicated there was no main effect of genotype, $F(1, 39) = .563$, $p = .458$, or treatment type $F(1, 39) = .028$, $p = .867$. Similarly, there was no significant interaction between genotype and treatment type $F(1, 39) = 2.050$, $p = .160$.

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the mean percentage of exploration time occurring near cylinder (A) during phase two. Results indicated there was no main effect of genotype, $F(1, 39) = .771$, $p = .385$, or treatment type $F(1, 39) = .073$, $p = .789$. Similarly, there was no significant interaction between genotype and treatment type $F(1, 36) = 1.506$, $p = .227$. (See Fig. 19). One sample t-tests were performed to determine whether the experimental groups displayed a significant preference for the social cue (preference was inferred when the percentage of exploration time next to (A) was significantly higher than 50%). Results indicated that all groups except the heterozygous saline-treated animals displayed a significant preference for the social cue.

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the mean percentage of exploration time occurring near cylinder (B) during phase three. Results indicated there was no main effect of genotype, $F(1, 39) = 2.378$, $p = .131$, or treatment type $F(1, 39) = 1.472$, $p = .232$. Again, there was no significant interaction between genotype and treatment type $F(1, 39) = 1.271$, $p = .266$. (See Fig. 20). One sample t-tests were performed to determine whether the experimental groups displayed a significant preference for the novel social cue (preference was inferred when the percentage of exploration time next to (B) was significantly higher than 50%). Results indicated that no experimental groups displayed a significant preference for the novel social cue.

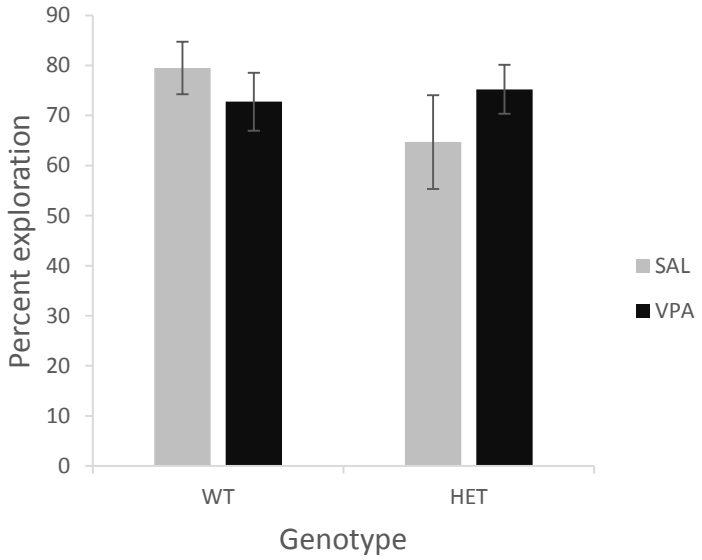

Figure 19. EXP-VIII: Effects of SERT genotype and VPA treatment in adolescents on the mean percentage of exploration time near the social cylinder (A) in phase two. Bars represent the mean (+SEM).

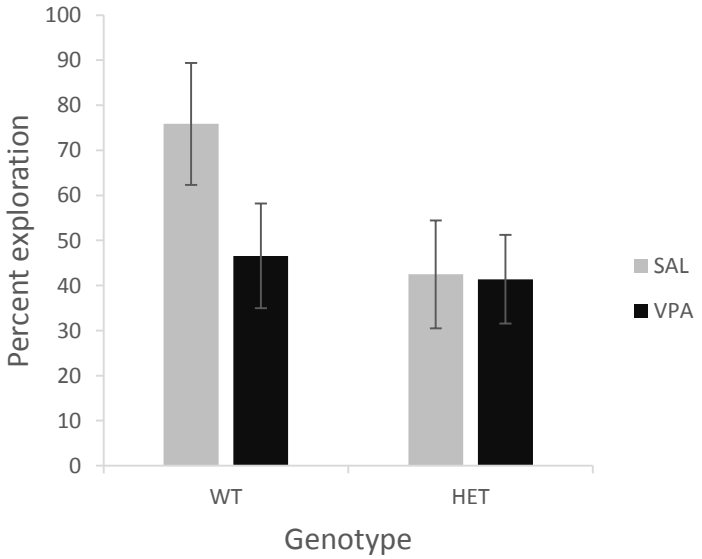

Figure 20. EXP-VIII: Effects of SERT genotype and VPA treatment in adolescents on the mean percentage of exploration time near the novel social cylinder (B) in phase three. Bars represent the mean (+SEM).

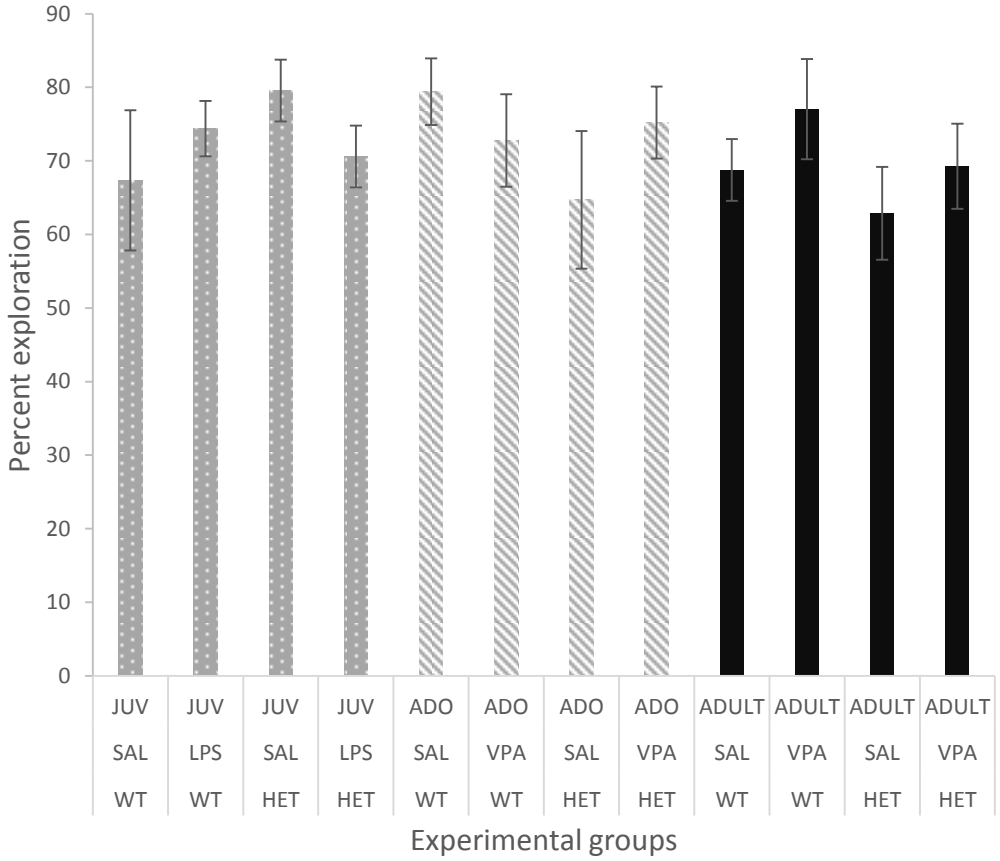

Figure 21. Summary of EXP-I, III, and XIII: mean percentage of exploration spent near the social cylinder in phase two for juvenile, adolescent, and adult animals, treated with saline, LPS, or VPA. Bars represent the mean (+SEM).

Experiment IX (EXP-IX): Repetitive behaviour in juveniles

The purpose of this experiment was to investigate repetitive behaviour in juvenile rats. Typically, rats are highly exploratory animals with a tendency to investigate all novel cues in a new environment (Sara, Dyon-Laurent, & Hervé, 1995). The current paradigm aimed to exploit this typical behaviour in an experimental setting to see if the proposed model led to deficits in such behaviour. Thus, the experiment was designed to be able to detect one of the three core behavioural hallmarks of ASD: increased repetitive behaviour. Juvenile animals were used as ASD is a disorder detectable at a very young age and thus observing repetitive behaviour in juveniles would more closely mimic the behavioural profile seen in patients.

Methods

Animals

EXP-IX was performed using juvenile (PND 22-30) male and female rats. Animals were habituated to the experimental room for 30 min before beginning the experiment. The sample sizes for the experimental groups in this paradigm were as follows: wild-type + saline = 9; wild-type + VPA = 13; heterozygous + saline = 31; heterozygous + VPA = 15.

Apparatus

The experiment took place inside a standard Plexiglas locomotor activity box (42 cm x 42 cm x 30 cm), with a 16-hole steel hole-board apparatus placed on the floor of the activity box (Med Associates). Effectively, the hole-board apparatus provided a novel floor with 16 identical holes (20 mm in diameter, in a square 4 x 4 orientation) for the rat to be able to poke its nose inside. Each time the rat poked its nose inside a hole, the event was recorded by a computer program (Med Associates). The activity boxes were cleaned with 70% ethanol before the introduction of any new rat to the paradigm.

Procedure

First, rats were placed inside the locomotor activity boxes *without* the hole-board apparatus inside and left to freely explore their novel experimental environment for 5 min. The purpose of this stage was to habituate the rats to the locomotor boxes to reduce any effect anxiety may play in the experiment. Next, the rats were removed from the box, the hole-board apparatus was inserted, and the rats were reintroduced to the box and left to freely explore for 15 min. Nose-pokes into each hole were recorded. Re-entries (nose-

pokes) into a previously explored hole were interpreted as a measure of repetitive behaviour and/or stereotypy. Behaviourally, a rat with an ASD phenotype would be expected to produce more re-entries than typical rats.

The primary outcomes of interest were: the number of novel nose-pokes and the number of re-entries into a previously explored hole. Locomotor activity was unable to be measured due to a technical fault in the computer program monitoring the activity boxes.

Results

As this experiment involved both males and females, initially one-way ANOVAs were used to test the effect of sex on the primary outcomes of interest. Results revealed there was no main effect of sex on the number of novel nose-pokes: $F(1, 66) = 1.903$, $p = .172$; the number of re-entries: $F(1, 66) = .005$, $p = .944$; or the total number of nose-pokes: $F(1, 66) = .194$, $p = .661$. Thus, both sexes were pooled together for all future analyses.

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the number of novel nose-pokes. Results indicated there was no main effect of genotype, $F(1, 64) = 1.789$, $p = .187$, or treatment type $F(1, 64) = 2.191$, $p = .144$. Similarly, there was no significant interaction between genotype and treatment type $F(1, 64) = 2.357$, $p = .130$. (See Fig. 22).

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the number of re-entries into a previously explored hole. Results indicated there was no main effect of genotype, $F(1, 64) = .742$, $p = .392$, or treatment type $F(1, 64) = .172$, $p = .680$. However, there was a significant interaction between genotype and treatment type $F(1, 64) = 7.897$, $p = .007$. Specifically, prenatal exposure to VPA increased the number of re-entries in wild-types ($p = .055$), but significantly decreased the number in heterozygous animals ($p = .050$). (See Fig. 23).

A 2 x 2 (gene x environment) ANOVA tested the effects of the SERT genotype and treatment type on the total number of nose-pokes. Results indicated there was no main effect of genotype, $F(1, 64) = 1.126$, $p = .293$, or treatment type $F(1, 64) = .567$, $p = .454$. However, there was a significant interaction between genotype and treatment type $F(1, 64) = 7.002$, $p = .010$. (See Fig. 24). Specifically, prenatal exposure to VPA significantly increased the total number of nose-pokes in wild-types ($p = .050$), and decreased the total number of nose-pokes in heterozygous animals, albeit not significantly ($p = .113$).

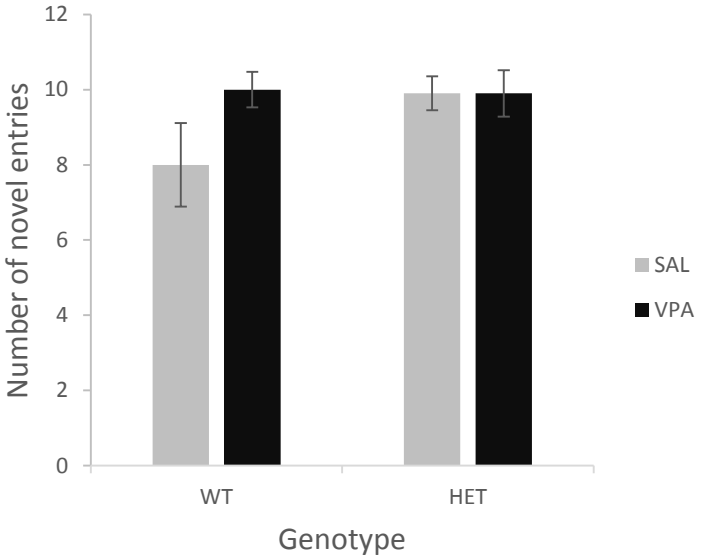

Figure 22. EXP-IX: Effects of SERT genotype and VPA treatment in juveniles on the number of novel nose-pokes performed during a 15 min period. Bars represent the mean (+SEM).

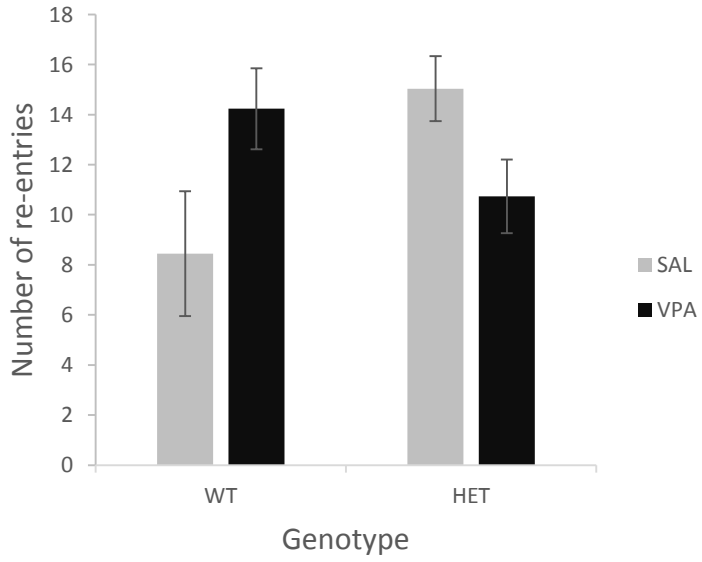

Figure 23. Effects of SERT genotype and VPA treatment in juveniles on the number of re-entries into a previously explored hole performed during a 15 min period. Bars represent the mean (+SEM).

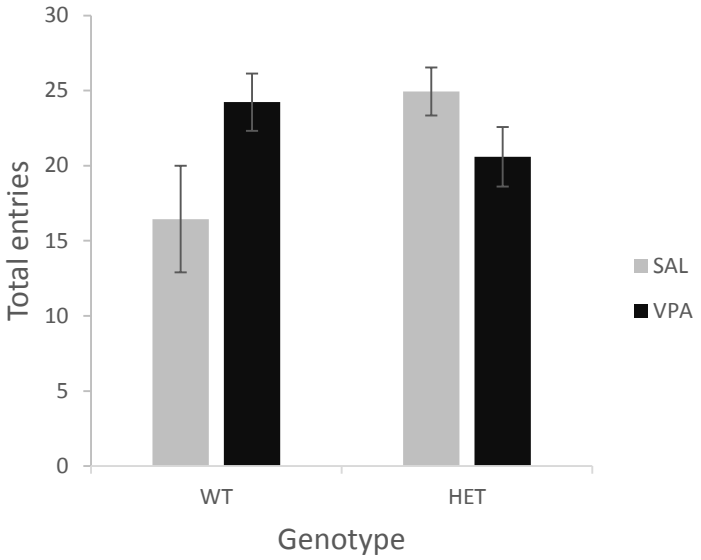

Figure 24. EXP-IX: Effects of SERT genotype and VPA treatment in juveniles on the total number of nose-pokes performed during a 15 min period. Bars represent the mean (+SEM).

Discussion

The purpose of this chapter was to investigate the effects of prenatal exposure to VPA on the three core behavioural domains of ASD and to test the hypothesis that rats with a genetically compromised SERT function would show an increased vulnerability to the impact of this prenatal exposure to VPA. Five different experiments (V, VI, VII, VIII, IX), including measures of social behaviour, communication, and repetitive behaviour were performed with the aim of detecting ASD-like deficits. Collectively, the data from this chapter produced three key findings: first, gene-environment interactions were observed on some measures of social behaviour and repetitive behaviour, but not on measures of communication. Second, of the interactions observed, none were in accordance with the pattern expected by the hypothesis; in fact, heterozygous animals seemed more resilient to the effects of VPA. Third, VPA is particularly sensitive to experimental variables.

In order to identify a potential interaction, a moderate dose of VPA (400 mg/kg) was used, relative to previous literature. For instance Cohen et al. (2013) used a similar lower dose (350 mg/kg) while others have used much higher doses (800 mg/kg) (Miyazaki et al., 2005; Narita et al., 2002). VPA was administered at GD 12 as this timing is widely used throughout the literature and is a critical period in which VPA exposure can lead to ASD-like deficits in rats (Kim et al., 2011).

Social Behaviour

Two experiments (EXP-VI and VIII) were performed to investigate the first diagnostic criterion of ASD: a reduction in social behaviour. EXP-VI investigated social interaction in adult rats following a brief period of isolation. A significant gene-environment interaction between SERT genotype and prenatal exposure to VPA was found on both measures of active social behaviour. Specifically, prenatal exposure to VPA reduced the duration and frequency of active social behaviour in wild-types, but did not influence active social behaviour in heterozygous animals. The pattern of this interaction was at odds with the hypothesis that predicted an exaggerated detrimental influence of VPA on social behaviour in heterozygous animals. In fact, if anything, VPA treatment actually tended to *increase* the frequency of active social behaviour in heterozygous animals, albeit not significantly. No gene-environment interactions were observed for measures of passive social behaviour.

These findings support previous literature demonstrating that prenatal exposure to VPA can have a detrimental influence on aspects of social behaviour in rats (Bambini-Junior et al., 2011; Schneider & Przewlocki, 2005), and furthermore, EXP-VI shows for the first time that VPA's influence on social behaviour depends on SERT genotype. However, the data provide no evidence for the hypothesis that a compromised SERT function will leave animals more vulnerable to prenatal VPA exposure. Rather, the data suggest that a compromised SERT genotype may actually be protective against prenatal VPA exposure, at least with regards to active social behaviour. Although speculative, the data also indicate the possibility that VPA exposure may even be desirable for rats with a compromised SERT function, at least with regards to the frequency of active social behaviour, as VPA treatment actually led to a small (albeit non-significant) increase in active social behaviour.

That VPA can demonstrate prosocial properties given the right experimental conditions has been reported before. Several recent papers have observed increased social behaviours in rats following prenatal exposure to VPA. In fact, these prosocial effects were even demonstrated in multiple rat strains (Long Evans and Wistar) and at different doses (350 mg/kg and 600 mg/kg) (Cohen et al., 2013; Štefánik et al., 2015). The variety of outcomes in research investigating prenatal VPA treatment and social behaviour demonstrates how sensitive VPA is to experimental variables.

One more important point can be gleaned from EXP-VI: the fact there was an interaction but no main effect of gene or environment alone highlights the importance of gene-environment research. As mentioned in chapter 1, ignoring interactions can lead to both genetic and environmental factors being underestimated or, at worst, dismissed. Combining these risk factors in this experiment revealed the importance of SERT genotype in active social behaviour that may have been overlooked when looking at the genotype in isolation.

EXP-VIII was the second paradigm investigating social behaviour in this chapter. Specifically, EXP-VIII investigated social approach and social novelty seeking behaviour in adolescents. No gene-environment interaction or main effect of genotype or treatment on either of the measures of social behaviour was found. The procedure in this experiment was adapted from EXP-I and III to include an inanimate object in the opposing cylinder (B) in phase two. This adaptation was designed to control for the possibility (evoked by results in EXP-I and III) that the social cue in phase two was simply too strong, when compared to a totally empty cylinder, to detect social deficits

using moderate genetic and environmental insults. Despite the different non-social stimulus, preference remained high for the social cue amongst all animals, suggesting these animals simply do not have a deficit in social approach behaviour (See Fig. 21 for a summary of all phase two results from this thesis). In addition, previous research has detected differences in social approach behaviour in phase two without the addition of high valence cues in competition with the novel animal (Bambini-Junior et al., 2011; McFarlane et al., 2008; Moy et al., 2007).

Animals in EXP-VIII displayed a lack of social novelty. In fact, no experimental group displayed a significant preference for the novel social cue over the familiar social cue. Several studies using this approach/novelty paradigm have observed preference for social novelty in a variety of rodent strains (Bambini-Junior et al., 2011; Moy et al., 2007). However, not all studies using this paradigm have observed preference for social novelty, indicating the type of animal/strain investigated and the specific protocol used may have an important influence on the outcome (Moy et al., 2007; Roullet et al., 2010). As described in chapter 1, a vast range of even subtle experimental details can lead to differing outcomes when investigating VPA (Roullet et al., 2013). Thus, it is possible that the lack of social novelty seeking observed in EXP-VIII was a combination of the type of animals used, the dose and timing of VPA, the way VPA was administered, and the use of an open field over the more traditional three-chambered box.

That being said, the data from this thesis indicate that social approach and social novelty are two distinct aspects of social behaviour that do not necessarily correlate. This idea is in line with previous research. For instance, Moy et al. (2007) investigated these two behaviours across ten different inbred mouse strains and found that the rank order for the degree of social approach did not match up with the rank order for the degree of social novelty. Moreover, the data from this thesis show that social approach differs fundamentally from social interaction (EXP-VI) and thus, from an animal modelling perspective, it is important to include both paradigms in order to encompass multiple aspects of social behaviour.

Collectively, the data from EXP-VI and VIII demonstrate that prenatal exposure to 400 mg/kg of VPA administered subcutaneously at GD 12 can reduce certain measures of social behaviour in wild-types, but not in heterozygous animals. The influence of VPA shown here in wild-types is consistent with the majority of previous research in this field (Bambini-Junior et al., 2011; Schneider & Przewlocki, 2005). However, as this thesis is the first extensive investigation of social behaviour in heterozygous rats, the finding that

heterozygous rats appear resilient to the disruptive effect of VPA to social behaviour is novel. Overall, no main effect of SERT genotype on social behaviour was observed in this chapter. This finding is in accordance with previous research which, despite finding reductions in play behaviour in homozygous rats, actually found non-playful facets of social behaviour, such as active social behaviour, to be unaffected in homozygous rats (Homberg, Schiepers, et al., 2007). In studies published to date, heterozygous rats have produced either no or only small effects when behaviourally compared to wild-types (Olivier et al., 2008).

In summary, the experiments investigating social behaviour in this chapter suggest that there is an interaction between SERT genotype and prenatal exposure to valproate on some measures of social behaviour, although not in the pattern predicted by the hypothesis. The fact that not all measures of social behaviour were influenced by VPA demonstrates the diversity of social behaviours and the specificity of VPA's influence.

Communication

There were two experiments (EXP-V and VII) included in this chapter designed to assess the second diagnostic criterion of ASD: impaired communication. EXP-V investigated USV communication in PND 7 rats and EXP-VII investigated scent marking in adult rats. Across these two paradigms there was no interaction between SERT genotype and prenatal exposure to VPA observed on any measure of communication. Thus, the data do not support the hypothesis that a compromised SERT function will leave animals more vulnerable to the detrimental impact of prenatal VPA exposure, at least with regards to communication. There are several reasons to trust this conclusion. First, a number of different communication measures at different stages of life were investigated and all the data indicate there is no interaction between SERT genotype and prenatal exposure to VPA. Second, only moderate genetic and environmental insults were used (heterozygous animals and 400 mg/kg VPA), suggesting a ceiling/floor effect of the genetic and environmental manipulations is unlikely. In support of this point, when a main effect of treatment was found (EXP-VII: VPA-treated animals produced fewer scent marks than saline-treated animals) the effect was only moderate and left room for the potential influence of the genotype. Finally, EXP-VI demonstrated that SERT genotype and prenatal exposure to VPA *can* interact on certain behavioural measures. Taken together, the data suggest it is likely that the SERT genotype does not interact with prenatal exposure to VPA with regard to the communication paradigms used in this chapter.

Overall, the data from these communication paradigms showed no significant effect of SERT genotype. Although to the best of our knowledge no previous literature has investigated the impact of SERT genotype on communication in rats, significant evidence does implicate the role of 5-HT in rodent communication (Wöhr, van Gaalen, & Schwarting, 2015). For instance, SSRI drugs acting on the SERT have been demonstrated to significantly reduce USV calling in pups (Winslow & Insel, 1990; Zimmerberg & Germeyan, 2015). In addition, mice with genetically modified serotonergic systems have produced alterations in isolation-induced USVs. Specifically, mouse pups with a genetic deletion of the 5-HT1b receptor emit fewer isolation-induced USVs than wild-type controls (Brunner, Buhot, Hen, & Hofer, 1999; El-Khodor et al., 2004). In addition, studies investigating mouse pups with a genetic deletion of the 5-HT1a receptor have observed both decreases (Weller et al., 2003) and increases (Zanettini et al., 2010) in isolation-induced USVs. Finally, mice lacking any brain 5-HT (tryptophan hydroxylase 2 knockout mice) have shown deficits in both USV and scent marking behaviour (Kane et al., 2012; Mosienko et al., 2015).

Thus, given the importance of 5-HT in communication, it is surprising no significant effect of SERT genotype was found on any measure of communication in this chapter. One explanation for this might be the use of a more moderate genetic insult used here than in previous studies. For instance, the current chapter used heterozygous SERT rats whereas previous research used full knockouts of 5-HT1a, 5-HT1b, and tryptophan hydroxylase 2 (El-Khodor et al., 2004; Kane et al., 2012; Mosienko et al., 2015; Weller et al., 2003).

In addition, some of these previously mentioned genetic mouse models and pharmacological substances that have disrupted USVs via the serotonergic system may have done so through the anxiolytic outcome of these manipulations rather than through a true disruption in communication. Although the main interpretation of the isolation-induced USV paradigm in ASD modelling is that fewer vocalisations are indicative of a reduced ability to communicate, an alternative interpretation is that fewer vocalisations simply indicates a lowered response to a stressful environment, or, in other words, reduced anxiety (Malkova et al., 2012). Indeed, the role of anxiety in isolation-induced USVs has been demonstrated, with pup USVs decreasing following administration of anxiolytic compounds and increasing after administration of anxiogenic compounds (Hodgson, Guthrie, & Varty, 2008; Insel et al., 1986). Moreover, 5-HT1b and tryptophan hydroxylase 2 knockout mice both display *decreased* levels of anxiety, consistent with

the fact they display a decrease in isolation-induced USVs (Mosienko et al., 2012; Zhuang et al., 1999).

Thus, in the current chapter, the fact rats with a compromised SERT genotype did not have altered levels of communication, may simply reflect the fact heterozygous rats do not have a big enough genetic insult to disrupt communication on the one hand, and do not exhibit reduced levels of anxiety on the other hand. Data from the other behavioural experiments in this chapter support the claim that these experimental animals are unlikely to differ in terms of anxiety. For instance, EXP-VIII found no genotype effect on locomotor activity during habituation and EXP-IX found no genotype effect on novel nose-pokes in an exploration paradigm. If there were real differences in anxiety between the experimental groups, it is reasonable to expect they would have been detected by either one of these measures.

Collectively, the data from EXP-V and VII showed that prenatal exposure to 400 mg/kg of VPA subcutaneously administered at GD 12 reduced communication abilities in adult rats but not in pups. Specifically, VPA exposure was able to reduce overall scent marking in adults, but did not statistically influence USV emissions in pups. To the best of our knowledge, EXP-VII was the first to investigate prenatal VPA exposure in a scent marking paradigm and in this respect the data constitute a novel finding that support previous literature showing other types of communicatory deficits in VPA-exposed animals (Gandal et al., 2010). Moreover, this finding puts the VPA model in line with other well-validated models of ASD in rodents, such as maternal immune activation models and the BTBR T+tf/J mouse model, that have demonstrated reduced scent marking in similar paradigms (Malkova et al., 2012; Wöhr et al., 2011). In contrast, it was surprising VPA exposure did not alter USV calls in pups, as has been found in prior research. For instance, both Moldrich et al. (2013) and Gandal et al. (2010) found prenatal VPA exposure to reduce USVs in pups isolated from their mothers. However, both these previous papers used mice and a higher dose of VPA (600 mg/kg), which may partially explain the divergent findings.

Interestingly, EXP-VII revealed that no experimental group had a preference for marking next to the urinary cue over the lemon cue. Previous research in mice has shown preference for scent marking near urinary cues over non-social cues (Kane et al., 2012). However, there are several possible explanations for these discrepant findings: first, due to the inherent difficulties of visually identifying a female rat in oestrus it was hard to guarantee the female urine was definitely oestrus cycle urine. Second, there may be

fundamental species differences between rats and mice in their reaction for social odours. Third, Kane et al. (2012) presented the cues sequentially and used phosphate-buffered saline as the non-social cue whereas in EXP-VII the cues were presented simultaneously and lemon was used as the non-social cue. Subsequent studies will be required to address these explanations.

In summary, the experiments investigating communication in this chapter suggest that there is no interaction between SERT reduction and prenatal exposure to VPA. However, prenatal exposure to VPA did influence certain aspects of communication. That VPA did not influence all measures of communication once again demonstrates the specificity of VPA's influence and its sensitivity to experimental variables.

Repetitive Behaviour

EXP-IX was designed to assess the last diagnostic criterion of ASD: repetitive behaviour. A significant gene-environment interaction between SERT genotype and prenatal exposure to VPA was observed on the primary measure of repetitive behaviour: re-entries into nose holes previously explored. Specifically, prenatal exposure to VPA increased repetitive behaviour in wild-types, but significantly *decreased* repetitive behaviour in heterozygous animals. The pattern of this interaction was at odds with the hypothesis that predicted an exaggerated effect of VPA on repetitive behaviour in heterozygous animals. Echoing the results found in EXP-VI on social behaviour, EXP-IX suggests that a compromised SERT genotype may actually be protective against certain ASD-like phenotypes induced by VPA, in this case repetitive behaviour.

In addition to observing a novel gene-environment interaction, the data from EXP-IX were consistent with previous research analysing only one of these variables: SERT genotype or prenatal VPA. For instance, despite just missing statistical significance ($p = .055$), the influence of VPA on wild-type rats in this study appears consistent with previous research which demonstrated that prenatal exposure to VPA increased repetitive behaviour in rodents (Gandal et al., 2010; Mehta et al., 2011; Schneider et al., 2008). Moreover, when analysing just the saline-treated animals, heterozygous-saline animals performed significantly more re-entries into previously explored holes than wild-type-saline animals ($p = .022$). This finding is in accordance with previous literature investigating the impact of SERT genotype on repetitive behaviours. For instance, Kyzar et al. (2012) found heterozygous SERT mice to have increased levels of self-grooming behaviour, a common measure of repetitive behaviour in animal models of ASD.

Interestingly, the gene-environment interaction observed in EXP-IX was specific to the type of nose-poking that reflected ASD-like behaviour (re-entries), and did not occur on the novel nose-pokes measure which controlled for general exploratory behaviour and anxiety. Thus, this particular gene-environment model showed a very specific effect on one of the three behavioural hallmarks of ASD: repetitive behaviour.

Conclusion

Taken together, the results of these five experiments indicated that SERT genotype interacted with prenatal exposure to VPA on some measures of social behaviour and repetitive behaviour, but not communication. However, prenatal exposure to VPA was able to independently influence certain aspects of communication. Despite the observation of multiple gene-environment interactions, none were in accordance with the pattern predicted by the hypothesis. Instead, VPA tended to have a more detrimental effect on the wild-type animals, whereas a compromised SERT genotype seemed to offer protection against some of these effects.

There were several instances in this chapter where VPA did not exert the expected ASD-like influence (for example, EXP-VIII did not find a reduction in social approach behaviour in VPA-exposed animals). This fact suggests three important points: first, more detailed analysis of the prenatal VPA model is required; second, the selected VPA dose indeed had a moderate effect; and third, the experimental details involved in a prenatal VPA model are of critical importance to the outcome (Cohen et al., 2013; Roullet et al., 2013; Štefánik et al., 2015). In fact, a range of experimental variables have been shown to influence the outcome of VPA, including the timing and dose (Roullet et al., 2013). The work in this chapter adds SERT genotype to the expansive list of variables that can influence or determine the outcome of prenatal exposure to VPA.

In summary, this chapter provided an extensive investigation into the behavioural effects of prenatal VPA and its potential interaction with SERT genotype in rats. Taken together, the data suggest that prenatal exposure to 400 mg/kg of VPA produces a diverse set of outcomes, some of which are consistent with an ASD-phenotype, and some of which are not. These findings seem consistent with the human context, where, although prenatal VPA exposure significantly increases the risk of ASD in children (Christensen et al., 2013), the overall risk of developing ASD is still very low.

CHAPTER 4: THE BIOCHEMICAL AND IMMUNOLOGICAL CONSEQUENCES OF PRENATAL EXPOSURE TO VALPROATE

Although the three core diagnostic criteria of ASD are all behavioural, individuals with ASD often have altered biochemical and immunological profiles as well. For instance, elevated levels of pro-inflammatory cytokines and hyperserotonemia are well replicated findings among patients (Chez et al., 2007; Devlin et al., 2005; Jyonouchi et al., 2001; Lam et al., 2006; Li et al., 2009; Vargas et al., 2005). There is a significant amount of research investigating potential biomarkers for ASD and recent findings may hold promise (Yang et al., 2015). Understanding the chemical underpinnings of ASD will provide several benefits, but most importantly it will enable significant progress in the development of pharmacological treatments and allow for simpler diagnoses in patients.

This chapter focused on two different biochemical processes that have been associated with ASD: a *dysregulation* in BDNF gene expression and an *increased* level of the proinflammatory cytokine IL-6.

BDNF

BDNF is a small protein encoded by the similarly named BDNF gene. This protein belongs to the family of neurotrophins (themselves, within the class of growth factors) and plays a critical role in the development, growth, differentiation, synaptic plasticity, and survival of various neuronal populations in both the peripheral and central nervous systems (Allen et al., 2013; Binder & Scharfman, 2004; Nickl-Jockschat & Michel, 2011; Pruunsild, Kazantseva, Aid, Palm, & Timmusk, 2007; Zhang, Jiang, & Lu, 2014).

The structure of the BDNF gene is highly complex (Aid, Kazantseva, Piirsoo, Palm, & Timmusk, 2007; Homberg, Molteni, Calabrese, & Riva, 2014; Pruunsild et al., 2007; Timmusk et al., 1993). The most recent description of the BDNF gene in rodents identified at least eight 5′ noncoding exons and one protein coding 3′ exon, with all 5′ noncoding exons controlled by different promoters (Aid et al., 2007). There are 11 different transcripts of the BDNF gene, and in each transcript a 5′ exon is spliced to the protein coding exon (Aid et al., 2007; Vicario et al., 2015). Adding to the complexity, these BDNF variants show brain region-specific expression and are differentially regulated by DNA methylation and histone deacetylation (Aid et al., 2007).

With respect to the current thesis, it is important to note that BDNF plays a particularly strong role in the function of serotonergic neurons and interacts with the

SERT in the development of mood disorders (Homberg et al., 2014; Martinowich & Lu, 2008). Significantly reduced BDNF expression has been observed in adult rats with a compromised SERT function (Calabrese et al., 2013; Calabrese et al., 2010; Molteni et al., 2010). Moreover, BDNF is a known target of VPA (Almeida, Roby, & Krueger, 2014; Fukuchi et al., 2009; Jeon et al., 2006; Roullet et al., 2010; Yasuda, Liang, Marinova, Yahyavi, & Chuang, 2009). Due to its multi-functionality, BDNF has been implicated in the pathogenesis of a number of neurological diseases and psychiatric disorders, including depression, schizophrenia, Huntington's, Alzheimer's, and Parkinson's disease (Homberg et al., 2014; Martinowich & Lu, 2008; Schumacher et al., 2005; Zuccato & Cattaneo, 2009).

BDNF plays a significant role in neurodevelopment and thus has become the focus of increasing attention in ASD research (Nickl-Jockschat & Michel, 2011). Unusual patterns of brain growth in ASD patients have led to the hypothesis that early BDNF hyperactivity may play a role in the aetiology of ASD (Tsai, 2005). Specifically, patients with ASD have demonstrated abnormal overgrowth of the brain in the early stages of life followed by a period of significantly slowed growth (Courchesne, Carper, & Akshoomoff, 2003; Courchesne et al., 2001).

A wealth of research has investigated the association between BDNF expression and ASD. Following the consistent pattern of biological associations in the ASD literature, a dysregulation of BDNF, as opposed to *too much* or *too little*, is evident in ASD patients. For instance, elevated levels of BDNF have been found in the peripheral blood (Nelson et al., 2001), plasma (Bryn et al., 2015; Correia et al., 2010), and serum (Connolly et al., 2006; Miyazaki et al., 2004; Ricci et al., 2013; Zhang et al., 2014) of ASD patients, relative to healthy controls. In contrast, reduced levels of BDNF have also been found in the blood of ASD patients, relative to healthy controls (Abdallah et al., 2013; Taurines et al., 2014).

Interestingly, BDNF has been linked to both the severity of ASD and the age of the patient. Specifically, while mildly autistic patients were found to have elevated levels of serum BDNF relative to controls, severely autistic patients did not (Al-Ayadhi, 2012). With regards to age, although one study found no overall difference in serum BDNF between patients and controls, when the study population was divided by age (above and below 6 years old) it was found that BDNF was significantly elevated in ASD patients below the age of 6, and significantly decreased in ASD patients above the age of 6,

relative to their age-matched control counterparts (Mansour, Mohamed, Azam, & Henedy, 2010).

Taken together, the data investigating the association between BDNF and ASD suggest that BDNF likely plays a role in the pathogenesis of this disorder. At this stage the preponderance of evidence points towards an increased level of BDNF (at least in the early stages of life), but the heterogeneity of ASD coupled with several contrasting findings indicates that a more general *dysregulation* of BDNF may be at play.

In accordance with human findings, rodent models of ASD have observed alterations of BDNF in either direction following prenatal treatment with VPA. For instance, Almeida et al. (2014) found that a prenatal dose of VPA (400 mg/kg i.p) at GD 12.5 increased BDNF in mouse foetal whole brains. In addition, Roullet et al. (2010) found regional-specific deficits in BDNF in adult mice prenatally exposed to a 800 mg/kg dose of VPA at GD 11. Specifically, these authors found reduced BDNF in the somatosensory cortex, but no change in the hippocampus. Finally, chronic postnatal treatment with VPA led to increased BDNF in both the hippocampus and the frontal cortex (Einat et al., 2003). The goal in the current chapter was to extend these previous works and to see if VPA-induced alterations in BDNF are dependent on SERT genotype.

IL-6

Interleukins are a group of cytokines of critical importance to the immune system. IL-6 is one of the key interleukins that helps the communication process between the central nervous system (CNS) and the immune system. IL-6 is a multifunctional cytokine, capable of crossing the blood-brain-barrier, that plays a role in brain development, host defence, and numerous diseases (Banks, Kastin, & Gutierrez, 1994; Ferretti & Hollander, 2015; van der Poll et al., 1997; Wei, Alberts, & Li, 2013).

Evidence for IL-6's role in ASD has been mounting, with elevated levels of IL-6 being found in plasma, peripheral blood monocytes, lymphoblasts, and in the brain of ASD patients (Ashwood et al., 2011; Enstrom, Onore, Van de Water, & Ashwood, 2010; Li et al., 2009; Malik et al., 2011; Wei et al., 2013; Wei et al., 2012; Wei et al., 2011; Yang et al., 2015). Recent hypotheses speculate that IL-6 elevation may play a role in ASD symptomatology via its detrimental impact on neuronal plasticity and neuroanatomical structures (Wei et al., 2013; Wei et al., 2012). Taken together, the data suggest IL-6 is a potential biomarker and/or promising target for intervention in ASD.

The influence VPA has on levels of IL-6 is unclear. For instance, although treatment with VPA was seen to elevate blood levels of IL-6 in humans (Shiah, Yatham, Yeh, & Ravindran, 2005; Verrotti et al., 2001), other investigators have found both no change and decreased levels of IL-6 in response to VPA (Guenther et al., 2014; Steinborn et al., 2014). Further, VPA inhibited LPS-induced IL-6 in THP-1 cells (Ichiyama et al., 2000), and supressed expression of IL-6 following intra-cerebral haemorrhage induction in rats (Sinn et al., 2007). The differential effects of VPA on IL-6 support the previously described idea that the effects of VPA strongly depend on the specific experimental variables used in each study. That being said, to the best of our knowledge, no prior studies have investigated changes in IL-6 using a prenatal exposure to VPA rat model. In fact, only a minority of studies using the prenatal VPA model have investigated any immunological measures at all (Schneider et al., 2008). In addition, if prenatal exposure to VPA were to alter IL-6 in rats, there is reason to believe SERT genotype may modulate this alteration, since Macchi et al. (2013) found heterozygous SERT rats had an exaggerated IL-6 gene expression following a postnatal immune challenge.

The purpose of this chapter was to investigate the biochemical and immunological consequences of a 400 mg/kg dose of VPA subcutaneously administered at GD 12 and to see if the response was dependent upon SERT genotype. In addition to the three core behavioural characteristics that characterise ASD, there are several biochemical findings that are consistently observed in patients with ASD, including a *dysregulation* in BDNF gene expression and an *increased* level of the proinflammatory cytokine IL-6. Assays aimed at investigating these two particular biochemical/immunological profiles are included in this chapter. The purpose of these assays was to assess whether the proposed gene-environment model could produce biochemical/immunological changes consistent with those found in human patients with ASD. These experiments were designed to extend the behavioural data included in this thesis, thus providing a more in depth evaluation of the face validity of the *SERT reduction-prenatal exposure to VPA* model. These experiments tested the hypothesis that rats with a genetically compromised SERT function would show an increased vulnerability to the impact of prenatal exposure to VPA and that this would be reflected in their biochemical and immunological profiles.

Breeding

The same breeding procedure as chapter 3 was used. In short, this involved mating heterozygous SERT knockout females with heterozygous SERT knockout males, and

separating the females once successfully mated (defined as presence of vaginal plug, GD 0). The pregnant mothers were then administered 400 mg/kg VPA or saline subcutaneously at GD 12. The offspring of these mothers were the experimental animals for this chapter. All procedures in this chapter were approved by the Victoria University of Wellington Animal Ethics Committee.

Again, the experiments performed in this chapter used multiple litters to create the sample size for each experimental group. The reason for this was to reduce the chance of potentially abnormal litters representing an entire group. The sample sizes reported in these experiments are indicative of individual rats.

Experiment X (EXP-X): BDNF mRNA expression in the hippocampus and frontal lobe

The purpose of this experiment was to investigate BDNF gene expression changes in the brains of PND 7 rats. This experiment was conducted to evaluate whether prenatal exposure to VPA could mimic a consistently replicated finding in the biochemical profiles of ASD patients (dysregulation of the BDNF gene) and if the biochemical response depended on SERT genotype. To achieve this aim, total BDNF mRNA (exon IX) was measured in both the hippocampus and the frontal lobe. Then, due to the complex transcriptional structure of the BDNF gene (11 distinct transcripts from different promoters) (Aid et al., 2007), several prominent transcripts were measured to investigate which BDNF exon contributed to the alterations in total BDNF. Specifically, BDNF IV and BDNF VI were investigated due to their prominent expression in these brain regions, their well characterised promoter regions, and the previous work demonstrating compromised SERT genotype can alter these transcripts (Calabrese et al., 2013; Calabrese et al., 2010; Molteni et al., 2010). The collection of brain samples for this experiment was performed at Victoria University of Wellington, and the analyses of these samples were carried out during an overseas research stay at the University of Milan, Italy.

Methods

Animals

Experimental animals were infant (PND 7) male and female Wistar rats treated with either saline or VPA at GD 12. Female rats were used due to the low numbers of males in some experimental groups. Wild-type and heterozygous knockout animals were used for

the biochemical investigations. Animals were kept in standard Plexiglas cages with their mother and littermates in a temperature-controlled room (temperature: 21± 2^{o}C; 55% humidity) on 12 hr light-dark cycles (lights on at 07:00) with food and water available ad libitum. On PND 7 animals were sacrificed for brain extraction. The sample sizes for the experimental groups in this experiment were as follows: wild-type + saline = 9; wild-type + VPA = 5; heterozygous + saline = 20; heterozygous + VPA = 14.

Brain Extraction

At PND 7 animals were gassed using carbon dioxide, decapitated with a guillotine, and then had their brains extracted using standard surgical tools, including scissors, pliers, and a spatula. Their fresh brains were rinsed in distilled water and then dissected immediately on an ice-chilled glass-tray using a razor blade and forceps. The hippocampus and frontal lobe were collected and stored immediately at -80^{o}C. The remaining brain tissue was discarded. The frontal lobe was taken by making a coronal cut one quarter of the way along the brain from the anterior tip – the olfactory bulbs were removed from this resulting region and were not included as a part of the frontal lobe. Following brain extraction, a tail snip was taken from each animal's body for the purposes of genotyping (Transnetyx, Cordova, TN, USA). The frozen brain samples were then shipped under dry-ice to the Department of Pharmacological and Biomolecular Sciences at the University of Milan, Italy to be analysed using real-time polymerase chain reaction (RT-PCR).

Apparatus

The experiments were carried out at the Department of Pharmacological and Biomolecular Sciences at the University of Milan. The laboratory in this department had the necessary facilities and equipment to conduct RT-PCR experiments. In addition to the standard compounds used in the single-step guanidinium-isothiocyanate-phenol-chloroform extraction method, PureZol RNA isolation reagent (Bio-Rad Laboratories) and a tissue lyser (TissueLyser II, QIAGEN) were used during RNA isolation; a Nanodrop1000 spectrophotometer (Thermo Scientific) was used during quantification; and a TaqMan qRT–PCR instrument (CFX384 real time system, Bio-Rad Laboratories) was used for the thermal cycling and RT-PCR analysis. Probe and primer sequences for the housekeeping gene (36B4) were purchased from Eurofins MGW-Operon (Germany). The use of 36B4 as a housekeeping gene when investigating BDNF is in line with previous research (Fumagalli et al., 2012). TaqMan Gene expression assays were

purchased from Applied Biosystems, Life Technologies. IBM SPSS statistics version 22 was used for all statistical analyses.

RNA preparation and quantification of BDNF mRNA expression using RT-PCR

The following procedure was completed in accordance with the established protocols developed in the Department of Pharmacological and Biomolecular Sciences at the University of Milan (Calabrese et al., 2013; Fumagalli et al., 2012).

RNA isolation

Total ribonucleic acid (RNA) was isolated by the single-step guanidinium-isothiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987) using PureZol RNA isolation reagent (Bio-Rad Laboratories) according to the manufacturer's instructions and then quantified using a NanoDrop1000 spectrophotometer. The specifics were performed as follows:

The first step was to homogenise and disrupt the samples. The samples were homogenised and disrupted by being placed in Eppendorf tubes that included a stainless steel bead and PureZol RNA isolation reagent. The amount of PureZol in the Eppendorf tubes depended on the size of the sample (1 ml of PureZol for every 50-100 mg of tissue). These Eppendorf tubes were then put in a tissuelyser (TissueLyser II, QIAGEN) and shaken at 30 Hz for 30 s.

Next, the samples were removed from the lyser and left at room temperature for 5 min to achieve complete dissociation of the nucleoprotein complexes. Next, chloroform ($CHCl_3$) was added to the Eppendorf tubes ($1/5^{th}$ of the amount of PureZOL used in step one), and the tubes were shaken vigorously for 15 s. This was then followed by another 5 min room-temperature incubation period.

In the next step, the stainless steel beads were removed from the Eppendorf tubes and the tubes centrifuged at 12,000 RCF for 15 min at 4^{o}C. Following this centrifugation, the mixture separated into three phases: the aqueous phase, the interphase, and the organic phase. RNA is found in the aqueous phase, whilst the interphase and organic phase contain the DNA and proteins. As much of the aqueous phase as possible was then transferred into a new set of ribonuclease (RNase)-free Eppendorf tubes. In order to avoid disrupting the interphase and organic phase and thus contaminating the RNA, some of the aqueous phase was intentionally left behind.

In the next step, isopropanol was added to the aqueous phase; the amount added approximately equal to the total aqueous phase already contained in the Eppendorf tubes. These tubes were then vortexed and stored at -20^{o}C overnight. The following day the Eppendorf tubes were removed from the -20^{o}C freezer, vortexed, and then centrifuged (12,000 RCF for 20 min at 4^{o}C). After centrifugation the RNA appeared as a white pellet on the bottom of each Eppendorf tube. The supernatant was carefully discarded using a vacuum and beaker.

In the next step the pellets were washed with 75% ethanol – 1 ml of 75% ethanol for every 1 ml of PureZol used in step one was added to the Eppendorf tubes. The solution was then centrifuged, (12,000 RCF for 10 min at 4^{o}C) and again the supernatant was carefully discarded using a vacuum and beaker.

The final step in the RNA isolation process was to resuspend the pellets in RNase-free diethylpyrocarbonate (DEPC)-treated water. Approximately 20 µl of DEPC-treated water was added to the Eppendorf tubes and pipetted up and down 15 times to ensure the resuspension of the pellets. The samples were then stored at -20^{o}C to await quantification.

Quantification and DNAse Treatment

Samples were taken from the -20^{o}C freezer, vortexed, and then quantified to assess the concentration of RNA and their purity using a NanoDrop1000 spectrophotometer. The samples were then processed for RT-PCR to assess the levels of BDNF mRNA. Deoxyribonuclease (DNAse) treatment was performed on an aliquot of each sample that contained 5 µg of RNA (determined per sample via the results of the initial quantification) in order to destroy any possible remaining elements of DNA that may be in the RNA sample. Briefly, a 30 µl mixture of DNase (3 µl), 10x reaction buffer with magnesium chloride ($MgCl_2$) (3 µl), DEPC-treated H_20 (24 µl – size of the sample), and the required aliquot of the sample were added to a new Eppendorf tube. This solution was centrifuged briefly at 10,000 RCF (to get all the contents to the bottom of the Eppendorf tube) then put in a thermoblock for 30 min at 37^{o}C. Next, 3 µl of ethylenediaminetetraacetic acid (EDTA) was added to the Eppendorf tube, after which it was centrifuged and then once again put in the thermoblock – this time for 10 min at 65^{o}C. This final step combined with the EDTA inactivated the DNase. Following DNase treatment, a second quantification using the NanoDrop1000 spectrophotometer was performed on the samples. This quantification provided the final average concentration figures with which to create the sample dilution plate for RT-PCR.

The samples were diluted with sterile and apyrogen (RNase-free) H_20 so that each sample had a ratio of 10 ng of RNA per 1 µl of sample. RNA was then analysed by a TaqMan qRT–PCR instrument (CFX384 real time system, Bio-Rad Laboratories) using the iScriptTM one-step RT–PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384-well plates that were sealed, briefly vortexed, and centrifuged. Samples were run in triplicate as multiplexed reactions with a normalising internal control, or housekeeping gene (36B4). Each well contained 1 µl of sample and 1.5 µls of 'mix.' The 'mix' was a different recipe for each gene being investigated but the core components of the recipe were: the primers and probe for the housekeeping gene, the primers and probe for the target gene, 2x reaction mix, and Iscript reverse transcriptase. Probe and primer sequences for the 36B4 housekeeping gene were bought from Eurofins MWG-Operon (Germany). TaqMan gene expression assays (Applied Biosystems, Life Technologies) were used to analyse some of the most prominent transcripts of BDNF; their IDs are as follows (See Table 4): BDNF IV = Rn01484927_m1; and BDNF VI = Rn01484928_m1. Probe and primers for total BDNF were designed in Milan with the following specifics:

Probe: TGTGGTTTGTTGCCGTTGCCAAG
Forward Primer: AAGTCTGCATTACATTCCTCGA
Reverse Primer: GTTTTCTGAAAGAGGGACAGTTTAT

The TaqMan qRT-PCR instrument that conducted the thermal cycling used the following protocol: a 10 min incubation period at 50^{o}C (RNA retrotranscription), followed by 5 min at 95^{o}C (TaqMan polymerase activation). Next, 39 cycles of PCR were performed with each cycle consisting of heating the samples at 95^{o}C for 10 s to enable melting, and then at 60^{o}C for 30 s to enable the annealing and extension reactions.

Four different RT-PCR experiments were performed. Three of these experiments used RNA isolated from the hippocampus samples and calculated total BDNF, BDNF IV, and BDNF VI. The last experiment used RNA isolated from the frontal lobe samples and calculated total BDNF. Transcripts IV and VI were not investigated for the frontal lobe due to the time constraints on my overseas research-stay in Milan. The primary outcome of interest was gene expression change for the specific target gene being investigated. A comparative cycle threshold (Ct) method was used to calculate the relative target gene expression.

Table 4. Gene expression assays for total BDNF, BDNF IV, and BDNF VI.

Gene	**Assay ID**
Total BDNF	
Probe	TGTGGTTTGTTGCCGTTGCCAAG
Forward Primer	AAGTCTGCATTACATTCCTCGA
Reverse Primer	GTTTTCTGAAAGAGGGACAGTTTAT
BDNF IV	Rn01484927_m1
BDNF VI	Rn01484928_m1

Results

A 2 x 2 (gene x environment) ANOVA tested the effects of SERT genotype and treatment type on total BDNF mRNA expression in the hippocampus of PND 7 rats. Results indicated there was no main effect of genotype, $F(1, 44) = 3.624$, $p = .064$. There was a main effect of treatment type $F(1, 44) = 9.238$, $p = .004$, with VPA decreasing total BDNF mRNA expression. However, there was no significant interaction between genotype and treatment type $F(1, 44) = .566$, $p = .456$. (See Fig. 25).

A 2 x 2 (gene x environment) ANOVA tested the effects of SERT genotype and treatment type on BDNF IV mRNA expression in the hippocampus of PND 7 rats. Results indicated that there was a main effect of genotype, $F(1, 44) = 4.694$, $p = .036$, with heterozygous animals exhibiting higher levels of BDNF IV. In addition, there was a main effect of treatment type $F(1, 44) = 32.381$, $p < .001$, with VPA increasing BDNF IV mRNA expression. Lastly, there was no significant interaction between genotype and treatment type $F(1, 44) = 1.498$, $p = .228$. (See Fig. 26).

A 2 x 2 (gene x environment) ANOVA tested the effects of SERT genotype and treatment type on BDNF VI mRNA expression in the hippocampus of PND 7 rats. Results indicated there was no main effect of genotype, $F(1, 44) = 1.665$, $p = .204$, or treatment type $F(1, 44) = .269$, $p = .607$. In addition, there was no significant interaction between genotype and treatment type $F(1, 44) = 1.241$, $p = .271$. (See Fig. 27).

A 2 x 2 (gene x environment) ANOVA tested the effects of SERT genotype and treatment type on total BDNF mRNA expression in the frontal lobe of PND 7 rats. Results indicated there was no main effect of genotype, $F(1, 44) = 1.628$, $p = .209$.

However, there was a main effect of treatment type $F(1, 44) = 15.155$, $p < .001$, with VPA increasing total BDNF mRNA expression. Lastly, there was no significant interaction between genotype and treatment type $F(1, 44) = .903$, $p = .347$. (See Fig. 28). (See Table 5 for a summary of these results; for the RT-PCR amplification graphs from the total BDNF analyses see figures 30-33 in appendix).

Table 5. Two-way ANOVA results of the effects of SERT genotype and VPA treatment on various BDNF transcripts in both the hippocampus and the frontal lobe. * $p < .05$.

Target	**SERT Genotype**	**VPA Treatment**	**GxE Interaction**
Hippocampus			
Total BDNF	$F(1, 44) = 3.624$, $p = .064$	$F(1, 44) = 9.238$, **p = .004***	$F(1, 44) = .566$, $p = .456$
BDNF IV	$F(1, 44) = 4.694$, **p = .036***	$F(1, 44) = 32.381$, **p < .001***	$F(1, 44) = 1.498$, $p = .228$
BDNF VI	$F(1, 44) = 1.665$, $p = .204$	$F(1, 44) = .269$, $p = .607$	$F(1, 44) = 1.241$, $p = .271$
Frontal Lobe			
Total BDNF	$F(1, 44) = 1.628$, $p = .209$	$F(1, 44) = 15.155$, **p < .001***	$F(1, 44) = .903$, $p = .347$

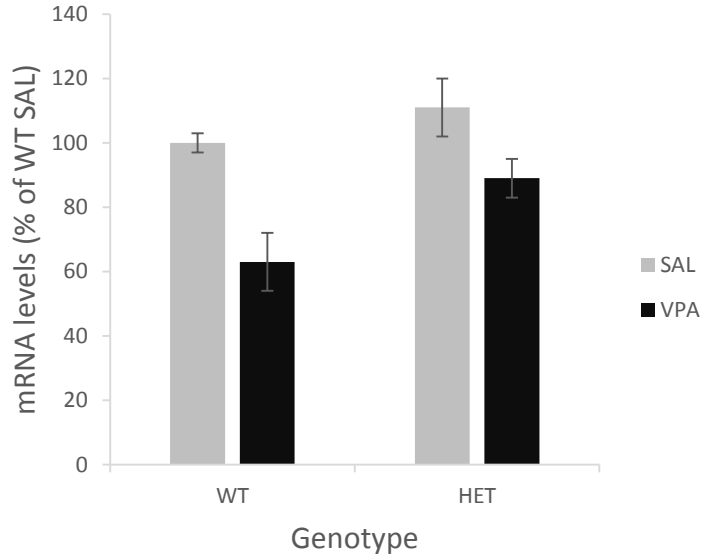

Figure 25. EXP-X: Effects of SERT genotype and VPA treatment on total BDNF mRNA levels in the hippocampus of PND 7 rats. Data are expressed as a percentage of control (wild-type saline-treated rats) values. Bars represent the mean (+SEM).

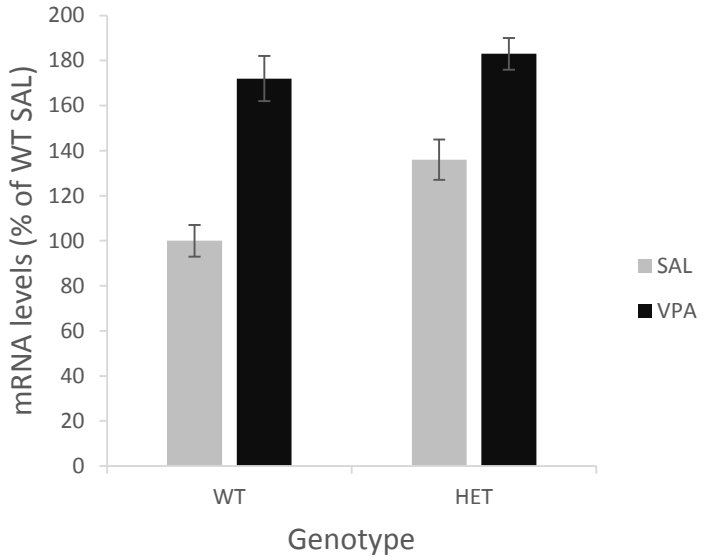

Figure 26. EXP-X: Effects of SERT genotype and VPA treatment on BDNF IV mRNA levels in the hippocampus of PND 7 rats. Data are expressed as a percentage of control (wild-type saline-treated rats) values. Bars represent the mean (+SEM).

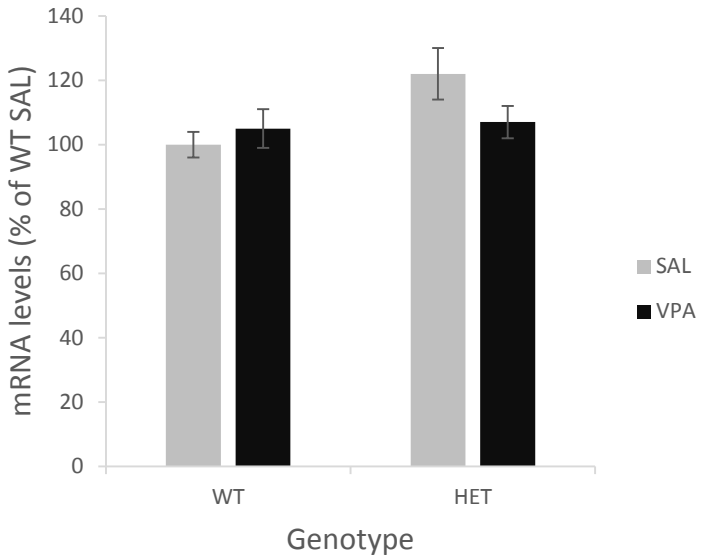

Figure 27. EXP-X: Effects of SERT genotype and VPA treatment on BDNF VI mRNA levels in the hippocampus of PND 7 rats. Data are expressed as a percentage of control (wild-type saline-treated rats) values. Bars represent the mean (+SEM).

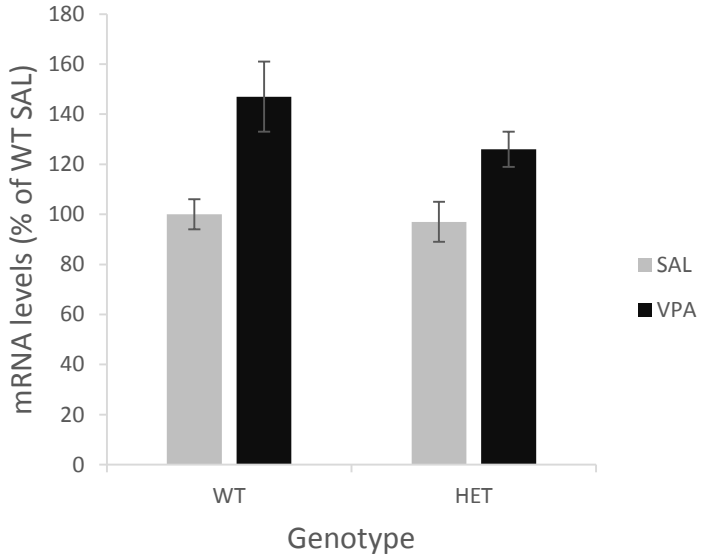

Figure 28. EXP-X: Effects of SERT genotype and VPA treatment on total BDNF mRNA levels in the frontal lobe of PND 7 rats. Data are expressed as a percentage of control (wild-type saline-treated rats) values. Bars represent the mean (+SEM).

Experiment XI (EXP-XI): IL-6 in serum

The purpose of this experiment was to investigate IL-6 levels in the serum of PND 7 rats. This experiment was conducted to evaluate whether prenatal exposure to VPA could mimic a consistently replicated finding in the immunological profiles of ASD patients (elevated levels of IL-6) and if the immunological response depended on SERT genotype.

Methods

Animals

Experimental animals were infant (PND 7) male and female Wistar rats treated with either saline or VPA at GD 12. Wild-type and heterozygous SERT knockout animals were used for this immunological investigation. Animals were kept in standard Plexiglas cages with their mother and littermates in a temperature-controlled room (temperature: 21± 2^{o}C; 55% humidity) on 12 hr light-dark cycles (lights on at 07:00) with food and water available ad libitum. On PND 7 animals were sacrificed to obtain a serum sample.

Serum collection

Animals were decapitated and as much blood as possible was collected from the neck and placed into an Eppendorf tube. A tail snip was taken from each animal's body for genotyping. The blood was then left at room temperature for 24 hr for the coagulation process to take place. Following this, the blood was then centrifuged at 2000 RPM for 10 min to fully separate the serum from the rest of the blood-matter. Next, the serum was pipetted out of the Eppendorf tube and into a new Eppendorf tube and immediately stored at -80^{o}C. The serum samples were used specifically for the immunological analysis of IL-6.

Apparatus

The experiment was performed using a commercial LEGEND MAX Rat IL-6 sandwich ELISA kit with precoated plates (Biolegend, CA, USA). An Enspire 2300 microplate reader (PerkinElmer) was used to measure the absorbance rate of the samples.

Procedure

The ELISA kit was performed in accordance with the manufacturer's instructions (to be described below). The unknown IL-6 concentrations in the serum samples were calculated using a standard curve generated from known reference IL-6 concentrations

('standards') provided in the kit. Samples and IL-6 standards were run in duplicate in a 96-well ELISA plate.

An initial optimisation pilot experiment demonstrated that the provided rat IL-6 standards (beginning at 1200 pg/ml) were too large to detect the levels of IL-6 in the current experimental animals. Thus, these standards were diluted further with the aim of providing a more accurate detection of IL-6. These new rat IL-6 standard concentrations were prepared in separate tubes so that there were eight different concentrations to eventually be placed in the final 96-well plate: 20 pg/ml, 10 pg/ml, 5 pg/ml, 2.5 pg/ml, 1.25 pg/ml, .625 pg/ml, .3125 pg/ml, and 0 pg/ml (the blank).

To begin, the plate was washed four times with 300 µl of wash buffer per well. Remaining buffer was blotted by tapping the plate upside down on absorbent paper. This washing technique was the standardised technique during this experiment.

Next, 50 µl of Matrix C was added to the wells designated for the standards and 50 µl of Assay Buffer A was added to the wells designated for the samples. Subsequently, 50 µl of standard or serum sample was added to the wells containing Matrix C or Assay Buffer A, respectively. The plate was then left to incubate at room temperature for 2 hr. Next, the contents of the plate were discarded and the plate was washed four times as previously described.

Next, 100 µl of rat IL-6 detection antibody solution was added to each well. The plate was then left to incubate at room temperature for 1 hr. Again, the contents of the plate were discarded and the plate washed four times as previously described.

Next, 100 µl of Avidin-HRP A solution was added to each well and the plate left to incubate at room temperature for 30 min. Again, the contents of the plate were discarded and the plate washed five times as previously described. In order to minimise background noise, in this washing cycle the wells were soaked for approximately 45 s in the wash buffer during each wash.

Next, 100 µl of substrate solution F was added to each well and left to incubate in the dark for 10 min. Wells containing IL-6 turned blue – the more intense the colour the higher the concentration of IL-6. This colour reaction was stopped by adding 100 µl of stop solution to each well. This solution changed the colour from blue to yellow. Finally, the plate was analysed by a microplate reader (PerkinElmer) reading at 450 nm within a few minutes of adding the stop solution.

Results

The level of IL-6 in the serum samples was too low to be reliably detected using this ELISA kit. As seen by the standard curve (See Fig. 29), accurate measurements of IL-6 below 5 pg/ml were not possible and as all the samples fell below this range (See Table 6, appendix) all that can be concluded is that the samples produced very low levels of IL-6, somewhere within the range of 0-5 pg/ml.

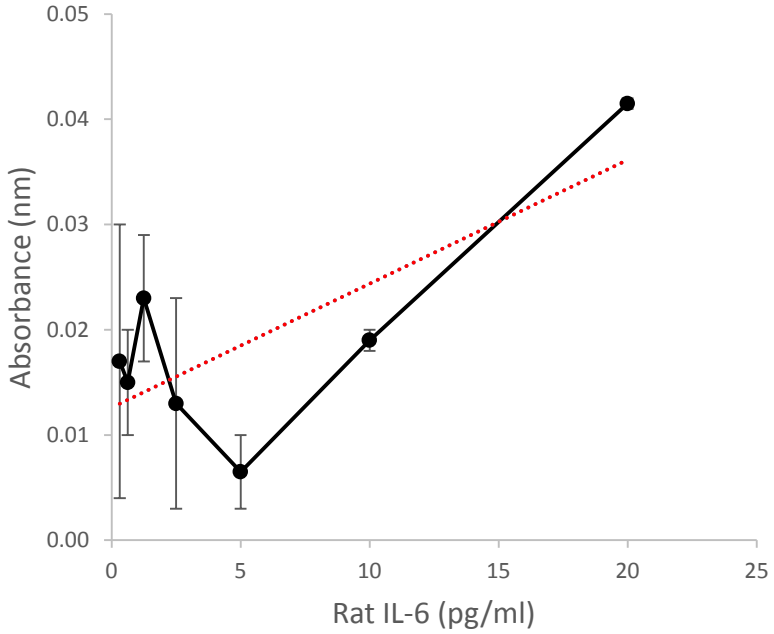

Figure 29. Standard curve. Black line represents the mean absorbance rate of each standard dilution (+SEM). The red line is the trend line. Values below 5 pg/ml are undetectable by the kit.

Discussion

The purpose of this chapter was to investigate the effects of prenatal exposure to VPA on the biochemical and immunological profiles of Wistar rats and to test the hypothesis that rats with a genetically compromised SERT function would show an increased vulnerability to the impact of this prenatal exposure. BDNF gene expression levels were measured in brain samples (EXP-X) and IL-6 levels were measured in serum (EXP-XI), both with the aim of detecting ASD-like biochemical and immunological profiles. EXP-X produced several key findings: first, no gene-environment interactions were observed with regard to BDNF expression levels. Second, compromised SERT genotype did not impact total BDNF levels, but did influence specific transcripts. Third, region-specific effects of VPA were found on total BDNF. Fourth, VPA differentially impacted BDNF transcripts within the same brain region. Together, these results are indicative of BDNF's complex transcriptional regulation and the extreme specificity with which VPA interacts with the brain. In EXP-XI the levels of IL-6 were undetectably low, suggesting this model may not produce physiologically relevant changes in IL-6.

The most important finding in the current chapter was that no gene-environment interactions were observed with regard to BDNF expression levels in either the hippocampus or frontal lobe. Thus, the data provide support for the null hypothesis: that there is no interaction between SERT genotype and prenatal exposure to VPA on BDNF expression. There are several reasons to trust this conclusion. First, multiple variants of BDNF in multiple different brain regions were investigated, and all of the data suggest that there is no interaction between SERT genotype and prenatal exposure to VPA. Second, there does not appear to be a ceiling/floor effect of the genetic or environmental factor considering only moderate genetic and environmental insults were used (heterozygous animals and 400 mg/kg VPA). In support of this idea, when main effects of genotype and treatment were found (for instance the main effect of VPA on total BDNF and main effect of SERT genotype on BDNF IV in the hippocampus), these effects were not extreme and left room for the potential influence of the other variable. Third, data from the current thesis have demonstrated that these gene and environmental risk factors *can* in fact interact, albeit on behavioural measures. Overall, it seems likely that SERT genotype does not interact with prenatal exposure to VPA with regard to BDNF gene expression at PND 7.

EXP-X found no genotype effect on total BDNF mRNA in either the hippocampus or the frontal lobe of PND 7 rats. With regards to the lack of genotype effect in the

hippocampus, this result mirrors previous literature that also found no significant difference in total BDNF between wild-types and heterozygous rats, albeit in adult animals (Molteni et al., 2010). Although Calabrese et al. (2013) found a reduction in total BDNF mRNA in homozygous adult rats when looking specifically at the ventral hippocampus, no heterozygous animals were included in their study and even these homozygous rats did not differ in total BDNF mRNA when the dorsal region of the hippocampus was investigated. Moreover, the significant reductions in total BDNF observed in the ventral hippocampus in homozygous adults were *not* found when investigating homozygous PND 7 rats (Calabrese et al., 2013). Thus, it is not surprising that EXP-X, investigating the entire hippocampus in the more moderate heterozygous rats at PND 7, did not find a genotype effect.

With respect to the frontal lobe, although previous work has found reduced total BDNF in the prefrontal cortex of PND 7 rats with a compromised SERT function, these investigations compared homozygous rats with wild-types and did not include the more moderate heterozygous genotype (Calabrese et al., 2013). In addition, research that did find reduced total BDNF in heterozygous animals observed so only in adult rats (Molteni et al., 2010). Finally, both these previous studies examined the 'prefrontal cortex,' which fundamentally differs from the less specific 'frontal lobe' region targeted in this thesis. In fact, data from Calabrese et al. (2010) support this point: these authors analysed total BDNF in both the 'prefrontal cortex' and the 'frontal cortex,' and found opposing effects of SERT genotype in each region. Specifically, total BDNF was downregulated in homozygous animals in the prefrontal cortex, but upregulated in homozygous animals in the frontal cortex. These findings emphasise the region-specific effects SERT genotype can have on total BDNF.

Although EXP-X found no effect of SERT genotype on total BDNF, an investigation of several prominent transcripts within the hippocampus revealed that SERT genotype did affect specific BDNF transcripts. For instance, in the hippocampus BDNF IV was upregulated in heterozygous animals relative to wild-types, whereas no genotype effect was found for BDNF VI. These findings support previous work suggesting that BDNF transcripts are differentially regulated (Aid et al., 2007; Pattabiraman et al., 2005).

The observation that heterozygous animals displayed upregulated BDNF IV, whilst having no significant upregulation of total BDNF is surprising. Previous research has found consistent patterns of direction between total BDNF and BDNF IV, suggesting BDNF IV is a primary driver of total BDNF. For example, both Molteni et al. (2010) and

Calabrese et al. (2013) found reductions in total BDNF and BDNF IV in homozygous adult animals. Moreover, where differences in total BDNF were *not* found in these previous studies, differences in BDNF IV were not found either. For instance, Molteni et al. (2010) found no difference in total BDNF or BDNF IV in adult heterozygous animals. In addition, Calabrese et al. (2013) found no difference in total BDNF or BDNF IV in PND 7 homozygous animals. In fact, consistent with the idea that BDNF IV may be a primary driver of total BDNF, Calabrese et al. (2013) only observed decreases in BDNF IV in homozygous animals from PND 21 onwards, which may explain why they found a decrease in total BDNF in adult animals but not PND 7 animals. Although the data in EXP-X seem to show a mismatch between total BDNF and BDNF IV within the hippocampus, a closer look at the statistical analyses reveals that heterozygous animals did have higher levels of total BDNF, but the difference was just short of significance ($p = .064$). Thus, the perceived mismatch between total BDNF and BDNF IV may be more related to a slightly larger variability in total BDNF levels in the heterozygous animals than a true inconsistency. Finally, the current finding of heterozygous animals displaying no difference in BDNF VI is in agreement with previous reports (Molteni et al., 2010).

Another key finding from EXP-X was that region-specific effects of VPA were found on total BDNF. Specifically, VPA significantly decreased total BDNF in the hippocampus, but significantly increased total BDNF in the frontal lobe. These findings are in accordance with previous research that has demonstrated VPA can induce both increases and decreases in total BDNF mRNA, depending on the region investigated (Almeida et al., 2014; Roullet et al., 2010). However, a vast range of experimental variables including the VPA treatment schedule, region of the brain investigated, and the age, strain, and species of the animal used, likely modulate the effects of prenatal VPA on BDNF gene expression and lead to differences amongst the literature. Taken together, the data from this chapter and previous papers suggest that prenatal exposure to VPA is able to produce dysregulation in BDNF gene expression in specific brain regions – reminiscent of the dysregulation seen in the blood of human patients.

Finally, EXP-X showed that VPA differentially impacted BDNF transcripts within the hippocampus. Specifically, BDNF IV was upregulated, but BDNF VI was unaffected by VPA. The observation that VPA can regulate BDNF expression in a transcript-specific manner is consistent with previous research. For instance, Almeida et al. (2014) found prenatal exposure to VPA increased specific BDNF transcripts (I, IV, VI) but not others, in foetal whole brains of mice. In addition, Yasuda et al. (2009) found VPA treatment

increased the level of BDNF IV (but not I, II, or VI) in rat cortical neuronal cultures. As discussed in chapter 1 of this thesis, VPA is an HDAC-I with the ability to regulate the expression of a wide number of genes (Lloyd, 2013). Literature investigating the effects of other HDAC-Is (such as TSA or sodium butyrate) on BDNF expression suggests that the mechanism by which VPA alters specific BDNF transcript expression is likely through the inhibition of HDAC. For instance, Yasuda et al. (2009) were able to find similar increases in BDNF IV using TSA and sodium butyrate that they found using VPA. In addition, Aid et al. (2007) found TSA increased the expression of specific BDNF transcripts (III, VII, IXA) in rat C6 glioma cells. Together, the findings here and elsewhere suggest that histone deacetylation may play a role in the regulation of BDNF expression.

Although EXP-X observed a VPA-induced increase in BDNF IV and no effect of VPA on BDNF VI, total BDNF in the hippocampus actually decreased. This finding was unexpected as typically the BDNF IV and VI transcripts have been seen as primary drivers of the level of total BDNF, particularly within the hippocampus (Calabrese et al., 2013). For instance, Almeida et al. (2014) found the VPA-induced increase in total BDNF to be associated with a 13 and 15 fold increase in the BDNF IV and VI transcripts, respectively. However, these authors analysed foetal whole brains in mice just 3 hr after being exposed to VPA and thus a number of experimental differences may account for the divergent effects of VPA on BDNF IV and VI. The data from EXP-X indicate that transcripts other than the IV and the VI are likely to account for the VPA-induced decrease in total BDNF mRNA in the hippocampus of PND 7 rats. Future research would benefit from an analysis of all BDNF transcripts.

EXP-XI aimed to measure IL-6 in serum. However, levels of IL-6 were too low (< 5 pg/ml) to be accurately detected in all experimental groups. The undetectably low amount of IL-6 reported here is in accordance with previous research finding undetectably low levels of IL-6 in control animals (Clark, Rinker, Lessov, Hazel, & Eckenstein, 1999; Givalois et al., 1994; Ulich, Guo, Remick, Del Castillo, & Yin, 1991). Thus, the moderate genetic and environmental manipulations used in this thesis do not seem to produce relevant (i.e., above background) increases in IL-6, relative to controls. More sensitive measures of IL-6 detection, such as a cytometric bead array, would be necessary for future research aiming to determine the precise levels of IL-6 in a prenatal exposure to VPA rat model. Nonetheless, based on the low levels of IL-6 reported here, prenatal

exposure to a moderate dose of VPA (400 mg/kg) does not produce physiologically relevant changes in IL-6 in either wild-type or heterozygous SERT rats, at least at PND 7.

Conclusion

Taken together, the data in this chapter show there is no interaction between SERT genotype and prenatal exposure to VPA on BDNF expression levels. However, SERT genotype and prenatal exposure to VPA demonstrated independent main effects on BDNF expression that were parameter-specific. Interestingly, prenatal exposure to VPA dysregulated BDNF expression in different ways, depending on the brain region investigated – once again highlighting the specificity of VPA's influence and its sensitivity to experimental variables. Finally, this *SERT reduction-prenatal exposure to VPA* model left levels of IL-6 undisturbed.

With the inclusion of the work in this chapter, the current thesis has provided an investigation of behavioural, biochemical, and immunological domains in the hopes of observing ASD-like symptomatology and determining the validity of the proposed gene-environment model for ASD.

CHAPTER 5: GENERAL DISCUSSION

ASD is a pervasive neurodevelopmental disorder characterised by social, communicative, and behavioural deficits. The apparent increase in prevalence over the past 20 years, the enormous burden to the patient and society, and the lack of effective pharmacological treatments have made ASD an increasing focus of research. Animal models play a major role in this research process, as they allow for the testing of specific hypotheses and the identification of novel therapies (Bauman et al., 2010). However, the recent rate of animal research translating into novel pharmacological therapies has been poor, particularly in the field of neuroscience (Kola & Landis, 2004). This low rate has prompted debate over the merit of animal research in general, and caused several major pharmaceutical companies to drastically reduce, or cut all together, animal research in areas with particularly low rates of success (Chandler, 2013; McGonigle & Ruggeri, 2014). Thus, there is an urgent need for improved animal models (Kola & Landis, 2004; McGonigle & Ruggeri, 2014; Seok et al., 2013).

It is now well understood that ASD develops as a result of an interaction between both genetic and environmental influences. However, most existing animal models for this disorder only take into account one of these aetiological contributors and have largely ignored looking at an interaction. There are a number of problems with this traditional modelling approach, but most prominent is the lack of construct validity involved in mimicking only one aspect of the aetiological process. For instance, one of the best models that currently exists for ASD is the prenatal exposure to VPA model that has been used in this thesis, yet this model only factors in one environmental influence and ignores the substantial contribution of genetic factors. In addition, VPA is likely only one of many environmental factors that interact with genotype in the development of ASD. Thus, the construct validity of even the best models are limited (Ranger & Ellenbroek, 2015). Given that construct validity may be the most important element of an animal model (van der Staay et al., 2009), steps are needed to improve the construct validity of animal models in this field. To improve the construct validity of ASD animal models one suggestion is to develop models that take a gene-environment interaction approach – an approach that is particularly lacking in preclinical ASD research.

The current thesis used two previously established environmental-based models for ASD and combined each of them with a genetic animal model that mimicked a genotype associated with ASD. The overall purpose was to develop a novel animal model for ASD

that demonstrated higher construct validity than traditional models due to its gene-environment approach. Specifically, in chapter 2 a maternal immune activation model (prenatal administration of LPS) was combined with a SERT knockout model and in chapters 3 and 4 a prenatal exposure to VPA model (prenatal administration of VPA) was combined with a SERT knockout model. These chapters tested the hypothesis that rats with a genetically compromised SERT function will be more vulnerable to the impacts of different environmental risk factors.

Collectively, the data from this thesis show that although both LPS and VPA interacted with SERT genotype on certain behavioural measures, only the prenatal exposure to VPA model interacted with SERT genotype on behavioural measures related to ASD. Specifically, the interaction between LPS and SERT genotype was only found on non-ASD type behaviours (locomotion), whereas prenatal exposure to VPA interacted with SERT on two of the three core dysfunctions in ASD: social behaviour and repetitive behaviour. Contrary to expectation, however, the data did not support the hypothesis that rats with a genetically compromised SERT function would be *more vulnerable* to the impacts of prenatal exposure to VPA. In fact, rats with a compromised SERT function appeared *more resilient* to the ASD-like effects of VPA. Extrapolating these findings to the human condition, the data presented in this thesis predict individuals with the S allele of the 5-HTTLPR may be more resilient to the ASD-related influence of prenatal VPA. Future research investigating the 5-HTTLPR genotype of ASD patients prenatally exposed to VPA would be required to test this prediction.

The reason rats with a compromised SERT genotype showed resilience, rather than vulnerability, to certain ASD-like effects of prenatal exposure to VPA is unclear. However, based on the considerable evidence demonstrating VPA's high sensitivity for experimental variables, a possible explanation for these unexpected findings is simply that the combination of experimental variables in this thesis were novel and therefore one could expect the unexpected. An alternative explanation might be that the timing of the VPA dose was wrong for detecting vulnerability. As outlined in chapter 1, *the timing of* VPA administration significantly influences the long term responses to this drug. Previous research demonstrated that a prenatal dose of VPA at GD 12 reliably produces ASD-like deficits in normal, wild-type rodents, and thus was an obvious choice for the current thesis. However, perhaps GD 12 is not the optimal day where the hypothesised enhanced vulnerability of heterozygous rats is exposed. In line with this idea are findings that prenatal VPA at GD 9 significantly impacts on the serotonergic system (Dufour-

Rainfray et al., 2010; Miyazaki et al., 2005; Narita et al., 2002). It is at least conceivable that VPA administration earlier than GD 12 may more strongly interact with the SERT genotype and expose the enhanced vulnerability hypothesised in this thesis. Future studies investigating the role of timing using a gene-environment approach would be needed to evaluate this suggestion. Although speculative at this point in time, should an enhanced vulnerability be found following an earlier dose of VPA, this would again emphasise the importance of tightly controlling the experimental variables when administering VPA.

Limitations

The overall goal of this thesis was to contribute to the development of animal models for ASD that possess higher construct validity and translatability to the human condition. To this aim, existing environmental animal models for ASD were crossed with a SERT knockout model. Thus, limitations in the typical methods of modelling maternal immune activation and prenatal exposure to VPA are present in the current thesis. Most notably, although strong face validity for the prenatal VPA model has been repeatedly observed, the typical treatment schedule used in this model does not quite mimic the human experience. Specifically, this thesis (and previous research) administered a single exposure of VPA during pregnancy, whereas in human pregnancies women typically take VPA for a prolonged period of time. More research investigating chronic VPA exposure in animals would be beneficial as it would increase the construct validity of the model by more accurately mimicking the human experience – this is especially important given the vital role that timing plays in response to VPA. A recent study investigating chronic exposure to low doses of VPA in rats reported disturbances in brain organisation equivalent to patients with ASD, thus supporting the suggestion for more work of this kind (Sabers et al., 2015).

Even though gene-environment approaches will likely improve the construct validity of animal models of ASD, the limitations of these types of models, particularly within this field, must be acknowledged. The aetiology of ASD is complex and diverse, making it uniquely difficult to model the causal components of ASD in an animal model. For instance, genetic linkage studies have had enormous difficulty finding specific genetic factors that are *consistently* found to be associated with ASD and failed replications of initial discoveries are common. Most single genetic effects are small and it is likely the combination of many genes that is responsible for the overall effect of an individual's genotype on their likelihood of developing ASD. Moreover, although certain

environmental agents such as VPA have been linked to the development of ASD, the majority of patients have not been exposed to VPA and thus a combination of environmental factors likely interacts with a susceptible genotype to produce the deficits observed in ASD. Given that gene-environment models will usually follow the approach in this thesis (i.e, the combining of a genetic risk factor and an environmental risk factor in the same model) it will likely be impossible in practical terms to model the number of risk factors necessary to mimic the causal process in humans. In short, it seems that inherent in the limitation of the gene-environment approach in the field of ASD, is the complexity of ASD itself. This raises the question of whether the enormous complexity of ASD will pose an insurmountable challenge towards developing truly predictive animal models for this disorder. In addition, if the answer to this question is yes, what is an alternative strategy to solve this problem?

Towards a better future

Despite the first reports of ASD occurring over 70 years ago, this disorder is still poorly understood. The heterogeneous clinical profile and truly complex aetiology of ASD have proven serious obstacles for researchers working in this field. At this stage, it is evident that in addition to experiencing their disorder differently, individuals with ASD likely developed their disorder in different ways. Thus, it is probable that different clusters of patients will require different pharmacological treatments. Indeed, it has been argued that it is time to abandon the search for a single cause or cure for ASD and instead investigate the specific core symptoms separately (Happé, Ronald, & Plomin, 2006). In support of this argument, population-based data indicate that the three core domains of ASD may not represent a coherent triad but may simply be three mostly independent domains of impairment. For instance, Ronald, Happe, Bolton, et al. (2006) found that the three core domains of ASD are genetically heterogeneous and although deficits in one domain increased the likelihood of a deficit in another, this increased risk was relatively low overall. In other words, rather than one underlying genetic deficit explaining the triad of behavioural symptoms in ASD, different genes are responsible for the different core dysfunctions present in this disorder. This finding likely accounts for why these deficits are often found in isolation within the general population (Ronald, Happe, Price, Baron-Cohen, & Plomin, 2006). If the different symptoms have different causes, it raises the question of whether ASD is even a unitary disorder at all. Although Ronald, Happe, Bolton, et al. (2006) found evidence for a small percentage of genes acting on multiple

parts of the triad, as Happé et al. (2006) argued, what is more interesting is the degree of separation. The degree of separation between the behavioural symptoms of ASD may explain why large scale genetic studies often provide inconsistent data when looking at ASD *as a whole*. For instance, although investigations into the role of the 5-HTTLPR in ASD provided remarkably heterogeneous findings overall (as described in chapter 1), when studies sub-grouped ASD by phenotype and genotype a clearer picture began to emerge (Brune et al., 2006; Kolevzon et al., 2014; Schauder et al., 2015; Tordjman et al., 2001). A strong focus on researching ASD as a unitary disorder may be a significant reason why so little is known about this disorder despite decades of research. Interestingly, a similar conclusion was drawn by Arnedo et al. (2015) with regards to schizophrenia. After providing evidence that schizophrenia is comprised of distinct clinical syndromes with heterogeneous genetic architecture, these authors concluded that much of schizophrenia's heritability has not been detected due to studies approaching schizophrenia as a whole, rather than using a more detailed analysis of patient phenotype (Arnedo et al., 2015).

Although Happé et al. (2006) called for a fractionation of ASD's triad of symptoms over 10 years ago, research has largely continued to approach this disorder as a whole (a disorder approach), in need of a singular explanation. In fact, the most recent edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) actually moved in the opposite direction from a fractionation and grouped previously distinguished developmental disorders together (Association, 2013). It is time to start taking the suggestion offered by Happé et al. (2006) seriously and acknowledge the possibility that ASD may be an illusory triad.

Approaching ASD as a unitary disorder (the disorder approach) should be abandoned and instead focus must move to the core symptoms as though they are independent impairments that simply co-exist in a number of people (a symptom approach). In this manner, the focus would shift from developing a model for ASD, towards developing different models for reduced sociability, reduced communication, and repetitive behaviour. In addition, effort should be made to model both genetic and environmental determinants of these symptoms. Under the traditional disorder approach, the heterogeneous aetiology and symptomatology of ASD pose insurmountable challenges to the development of good animal models for this disorder as it is impossible for a single animal model to mimic either all the causes or the range of symptoms. For instance, the prenatal exposure to VPA model cannot account for the majority of ASD cases that were

not induced by VPA. In addition, even though this model is credited with a very high degree of face validity, it cannot replicate the heterogeneity of symptoms observed in the clinical population. With the inability to create good, predictive animal models for ASD, it is not surprising that no effective pharmacological treatments have been developed.

Following a symptom approach into the future would be beneficial for a variety of reasons. First, it would lower the complex demands of a traditional ASD animal model and thus increase the likelihood of a predictive model in the specific domain of interest, such as reduced social behaviour. Second, if successful models of these core domains were developed it would lead to the development of treatments that may be used across multiple disorders. For instance, reduced social behaviour is also present in other psychopathological disorders such as schizophrenia. In addition, these treatments would be of use to individuals who experience difficulties in just one of the three domains and are currently undiagnosed for this reason. This point is of particular importance given that extreme deficits in these areas can occur in isolation within the general population (Ronald, Happe, Price, et al., 2006). Finally, even if ASD does represent a true triad of symptoms and the fractionation of these symptoms has been exaggerated, the non-success of ASD models up until now suggests a symptom approach may still be a more efficient method of modelling. In general, an efficient method of solving complex problems is to break down the larger problem into smaller, more easily solvable, problems.

In summary, the complexities of ASD's symptomatology and aetiology may be fundamental obstacles to modelling this disorder. In fact, the great irony in this field may be that the biggest factor holding back progress in ASD research is the very fact that scientists are focusing on ASD. The best way forward may be to transition from a disorder approach toward a symptom approach.

Conclusion

The aim of this thesis was to develop a novel animal model for ASD that demonstrated higher construct validity than traditional models by using a gene-environment approach. To this aim, this thesis investigated the hypothesis that rats with a genetically compromised SERT function would be more vulnerable to the impacts of different environmental risk factors. The data show that rats with a genetically compromised SERT function are *not* more vulnerable to the impact of either a maternal immune activation encountered during foetal development or prenatal exposure to VPA, at least with regards to the behavioural, biochemical, and immunological measures of ASD-like

symptomatology employed in this thesis. On the contrary, rats with a genetically compromised SERT function actually appeared more resilient to some ASD-like outcomes, at least following prenatal exposure to VPA. Future attempts to improve animal models in this field should continue modelling both genetic and environmental risk factors, but would benefit from taking a symptom-based approach rather than the more traditional disorder-based approach.

Appendix

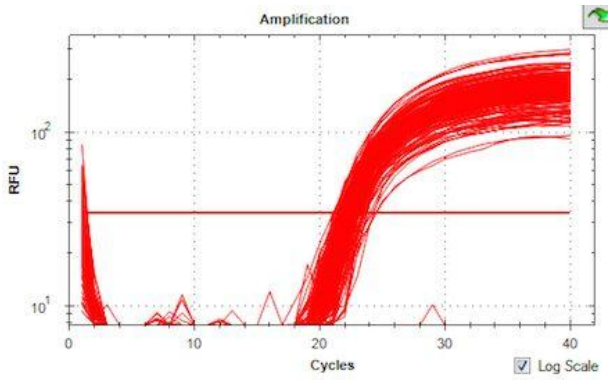

Figure 30. Amplification graph for total BDNF in the hippocampus of PND 7 rats.

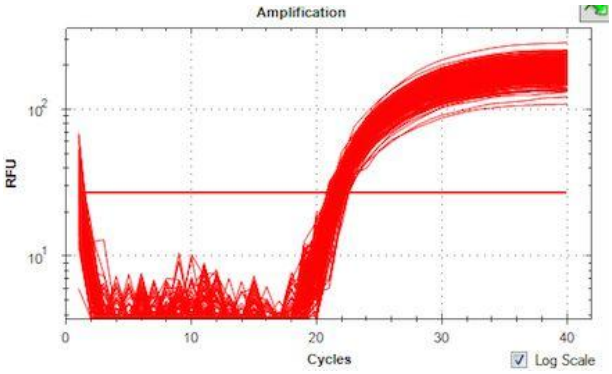

Figure 31. Amplification graph for total BDNF in the frontal lobe of PND 7 rats.

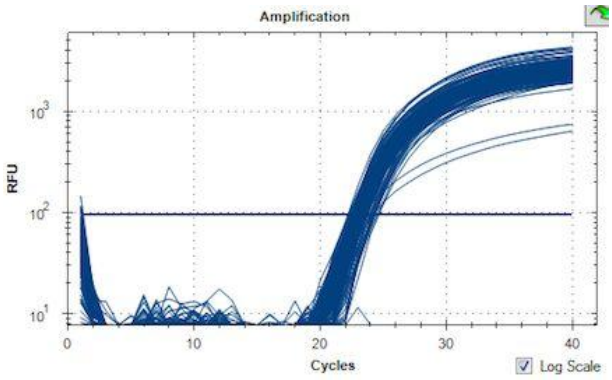

Figure 32. Amplification graph for the housekeeping gene (36B4) in the hippocampus of PND 7 rats.

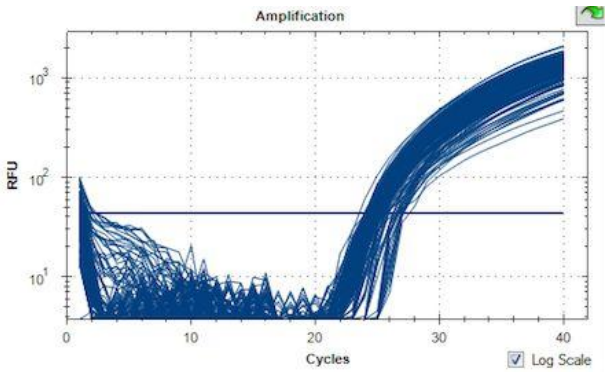

Figure 33. Amplification graph for the housekeeping gene (36B4) in the frontal lobe of PND 7 rats.

Table 6. Absorbance (nm) values of the serum samples (minus the absorbance value of the 0 pg/ml standard, or 'the blank'). Each column represents an experimental group. Each cell represents a different sample.

Wild-type Saline	**Wild-type VPA**	**Heterozygous Saline**	**Heterozygous VPA**
-0.0155	-0.0205	-0.0215	-0.031
-0.006	-0.007	-0.021	-0.0175
-0.0145	-0.016	-0.0235	-0.0235
-0.0285	-0.0125	-0.005	-0.0235
-0.0105	-0.0215	-0.0215	-0.02
-0.008		-0.009	-0.014
-0.0135		-0.023	-0.028
-0.024		-0.0165	-0.0265
-0.018			-0.026

References

Abdallah, M., Mortensen, E., Greaves-Lord, K., Larsen, N., Bonefeld-Jørgensen, E. C., Nørgaard-Pedersen, B., . . . Grove, J. (2013). Neonatal levels of neurotrophic factors and risk of autism spectrum disorders. *Acta Psychiatrica Scandinavica, 128*(1), 61-69.

Adab, N., Kini, U., Vinten, J., Ayres, J., Baker, G., Clayton-Smith, J., . . . Gregg, J. (2004). The longer term outcome of children born to mothers with epilepsy. *Journal of Neurology, Neurosurgery & Psychiatry, 75*(11), 1575-1583.

Adams, D. B. (1976). The relation of scent-marking, olfactory investigation, and specific postures in the isolation-induced fighting of rats. *Behaviour, 56*(3), 286-297.

Aid, T., Kazantseva, A., Piirsoo, M., Palm, K., & Timmusk, T. (2007). Mouse and rat BDNF gene structure and expression revisited. *Journal of neuroscience research, 85*(3), 525-535.

Al-Amin, M. M., Rahman, M. M., Khan, F. R., Zaman, F., & Reza, H. M. (2015). Astaxanthin improves behavioral disorder and oxidative stress in prenatal valproic acid-induced mice model of autism. *Behavioural brain research, 286*, 112-121.

Al-Ayadhi, L. Y. (2012). Relationship between Sonic hedgehog protein, brain-derived neurotrophic factor and oxidative stress in autism spectrum disorders. *Neurochemical research, 37*(2), 394-400.

Al Deeb, S., Al Moutaery, K., Arshaduddin, M., & Tariq, M. (2000). Vitamin E decreases valproic acid induced neural tube defects in mice. *Neuroscience Letters, 292*(3), 179-182.

Allen, M., Bird, C., Feng, W., Liu, G., Li, W., Perrone-Bizzozero, N. I., & Feng, Y. (2013). HuD promotes BDNF expression in brain neurons via selective stabilization of the BDNF long 3'UTR mRNA. *PloS one, 8*(1), e55718.

Allin, J. T., & Banks, E. M. (1972). Functional aspects of ultrasound production by infant albino rats (Rattus norvegicus). *Animal Behaviour, 20*(1), 175-185.

Almeida, L. E., Roby, C. D., & Krueger, B. K. (2014). Increased BDNF expression in fetal brain in the valproic acid model of autism. *Molecular and Cellular Neuroscience, 59*, 57-62.

Andersen, J. K. (2004). Oxidative stress in neurodegeneration: cause or consequence?

Arakawa, H., Blanchard, D. C., Arakawa, K., Dunlap, C., & Blanchard, R. J. (2008). Scent marking behavior as an odorant communication in mice. *Neuroscience & Biobehavioral Reviews, 32*(7), 1236-1248.

Arieff, Z., Kaur, M., Gameeldien, H., Van der Merwe, L., & Bajic, V. B. (2010). 5-HTTLPR polymorphism: Analysis in South African autistic individuals. *Human biology, 82*(3), 291-300.

Arnedo, J., Svrakic, D. M., del Val, C., Romero-Zaliz, R., Hernández-Cuervo, H., Fanous, A. H., . . . Cloninger, C. R. (2015). Uncovering the hidden risk architecture of the schizophrenias: confirmation in three independent genome-wide association studies. *American Journal of Psychiatry*.

Ashdown, H., Dumont, Y., Ng, M., Poole, S., Boksa, P., & Luheshi, G. (2005). The role of cytokines in mediating effects of prenatal infection on the fetus: implications for schizophrenia. *Molecular psychiatry, 11*(1), 47-55.

Ashwood, P., Krakowiak, P., Hertz-Picciotto, I., Hansen, R., Pessah, I., & Van de Water, J. (2011). Elevated plasma cytokines in autism spectrum disorders provide evidence of immune dysfunction and are associated with impaired behavioral outcome. *Brain, behavior, and immunity, 25*(1), 40-45.

Association, A. P. (2013). *Diagnostic and statistical manual of mental disorders (DSM-5®)*: American Psychiatric Pub.

Atladóttir, H., Thorsen, P., Østergaard, L., Schendel, D., Lemcke, S., Abdallah, M., & Parner, E. (2010). Maternal Infection Requiring Hospitalization During Pregnancy and Autism Spectrum Disorders. *Journal of autism and developmental disorders, 40*(12), 1423-1430. doi:10.1007/s10803-010-1006-y

Avallone, A., Piccirillo, M. C., Delrio, P., Pecori, B., Di Gennaro, E., Aloj, L., . . . Cavalcanti, E. (2014). Phase 1/2 study of valproic acid and short-course radiotherapy plus capecitabine as preoperative treatment in low-moderate risk rectal cancer-V-shoRT-R3 (Valproic acid-short RadioTherapy-rectum 3rd trial). *BMC cancer, 14*(1), 875.

Azmitia, E. C., Singh, J. S., & Whitaker-Azmitia, P. M. (2011). Increased serotonin axons (immunoreactive to 5-HT transporter) in postmortem brains from young autism donors. *Neuropharmacology, 60*(7), 1347-1354.

Baharnoori, M., Bhardwaj, S. K., & Srivastava, L. K. (2012). Neonatal behavioral changes in rats with gestational exposure to lipopolysaccharide: a prenatal infection model for developmental neuropsychiatric disorders. *Schizophrenia bulletin, 38*(3), 444-456.

Bailey, A., Le Couteur, A., Gottesman, I., Bolton, P., Simonoff, E., Yuzda, E., & Rutter, M. (1995). Autism as a strongly genetic disorder: evidence from a British twin study. *Psychological medicine, 25*(01), 63-77.

Bambini-Junior, V., Rodrigues, L., Behr, G. A., Moreira, J. C. F., Riesgo, R., & Gottfried, C. (2011). Animal model of autism induced by prenatal exposure to valproate: behavioral changes and liver parameters. *Brain Research, 1408*, 8-16.

Banji, D., Banji, O. J., Abbagoni, S., Hayath, M. S., Kambam, S., & Chiluka, V. L. (2011). Amelioration of behavioral aberrations and oxidative markers by green tea extract in valproate induced autism in animals. *Brain research, 1410*, 141-151.

Banks, W. A., Kastin, A. J., & Gutierrez, E. G. (1994). Penetration of interleukin-6 across the murine blood-brain barrier. *Neuroscience Letters, 179*(1–2), 53-56. doi:http://dx.doi.org/10.1016/0304-3940(94)90933-4

Barfield, R. J., Auerbach, P., Geyer, L. A., & McIntosh, T. K. (1979). Ultrasonic vocalizations in rat sexual behavior. *American Zoologist, 19*(2), 469-480.

Barker, E. T., Hartley, S. L., Seltzer, M. M., Floyd, F. J., Greenberg, J. S., & Orsmond, G. I. (2011). Trajectories of emotional well-being in mothers of adolescents and adults with autism. *Developmental psychology, 47*(2), 551.

Basta-Kaim, A., Szczęsny, E., Leśkiewicz, M., Głombik, K., Ślusarczyk, J., Budziszewska, B., . . . Wędzony, K. (2012). Maternal immune activation leads to age-related behavioral and immunological changes in male rat offspring-the effect of antipsychotic drugs. *Pharmacological Reports, 64*(6), 1400-1410.

Bauman, M. D., Crawley, J. N., & Berman, R. F. (2010). Autism: animal models. *eLS*.

Bauman, M. D., Iosif, A.-M., Smith, S. E., Bregere, C., Amaral, D. G., & Patterson, P. H. (2014). Activation of the maternal immune system during pregnancy alters behavioral development of rhesus monkey offspring. *Biological psychiatry, 75*(4), 332-341.

Beck, G., & Habicht, G. S. (1996). Immunity and the invertebrates. *Scientific American, 275*(5), 60-66.

Belzung, C., Leman, S., Vourc'h, P., & Andres, C. (2005). Rodent models for autism: a critical review. *Drug discovery today: Disease models, 2*(2), 93-101.

Betancur, C. (2011). Etiological heterogeneity in autism spectrum disorders: more than 100 genetic and genomic disorders and still counting. *Brain research, 1380*, 42-77.

Betancur, C., Corbex, M., Spielewoy, C., Philippe, A., Laplanche, J.-L., Launay, J.-M., . . . Giros, B. (2002). Serotonin transporter gene polymorphisms and hyperserotonemia in autistic disorder. *Molecular psychiatry, 7*(1), 67.

Binder, D. K., & Scharfman, H. E. (2004). Mini review. *Growth factors, 22*(3), 123-131.

Bittigau, P., Sifringer, M., Genz, K., Reith, E., Pospischil, D., Govindarajalu, S., . . . Dikranian, K. (2002). Antiepileptic drugs and apoptotic neurodegeneration in the developing brain. *Proceedings of the National Academy of Sciences, 99*(23), 15089-15094.

Blanchard, R. J., Blanchard, D. C., Agullana, R., & Weiss, S. M. (1991). Twenty-two kHz alarm cries to presentation of a predator, by laboratory rats living in visible burrow systems. *Physiology & behavior, 50*(5), 967-972.

Blasi, F., Bacchelli, E., Carone, S., Toma, C., Monaco, A. P., Bailey, A. J., & Maestrini, E. (2005). SLC25A12 and CMYA3 gene variants are not associated with autism in the IMGSAC multiplex family sample. *European Journal of Human Genetics, 14*(1), 123-126.

Bollino, D., Balan, I., & Aurelian, L. (2015). Valproic acid induces neuronal cell death through a novel calpain-dependent necroptosis pathway. *Journal of neurochemistry*.

Bonora, E., Beyer, K., Lamb, J., Parr, J., Klauck, S., Benner, A., . . . Poustka, A. (2003). Analysis of reelin as a candidate gene for autism. *Molecular psychiatry, 8*(10), 885-892.

Borrell, J., Vela, J. M., Arévalo-Martin, A., Molina-Holgado, E., & Guaza, C. (2002). Prenatal immune challenge disrupts sensorimotor gating in adult rats: implications for the etiopathogenesis of schizophrenia.

Boukhris, T., Sheehy, O., Mottron, L., & Bérard, A. (2015). ANtidepressant use during pregnancy and the risk of autism spectrum disorder in children. *JAMA Pediatrics*, 1-8. doi:10.1001/jamapediatrics.2015.3356

Brodie, S. A., & Brandes, J. C. (2014). Could valproic acid be an effective anticancer agent? The evidence so far. *Expert review of anticancer therapy, 14*(10), 1097-1100.

Brudzynski, S. M. (2013). Ethotransmission: communication of emotional states through ultrasonic vocalization in rats. *Current opinion in neurobiology*.

Brudzynski, S. M., Kehoe, P., & Callahan, M. (1999). Sonographic structure of isolation-induced ultrasonic calls of rat pups. *Developmental psychobiology, 34*(3), 195-204.

Brugha, T. S., McManus, S., Bankart, J., Scott, F., Purdon, S., Smith, J., . . . Meltzer, H. (2011). Epidemiology of autism spectrum disorders in adults in the community in England. *Archives of general psychiatry, 68*(5), 459-465.

Brune, C., Kim, S.-J., Salt, J., Leventhal, B., Lord, C., & Cook, E. (2006). 5-HTTLPR genotype-specific phenotype in children and adolescents with autism. *American Journal of Psychiatry, 163*(12), 2148-2156.

Brunelli, S. A., Shair, H. N., & Hofer, M. A. (1994). Hypothermic vocalizations of rat pups (Rattus norvegicus) and direct maternal search behavior. *Journal of Comparative Psychology, 108*(3), 298.

Brunner, D., Buhot, M., Hen, R., & Hofer, M. (1999). Anxiety, motor activation, and maternal–infant interactions in 5HT 1B knockout mice. *Behavioral neuroscience, 113*(3), 587.

Bryn, V., Halvorsen, B., Ueland, T., Isaksen, J., Kolkova, K., Ravn, K., & Skjeldal, O. (2015). Brain derived neurotrophic factor (BDNF) and autism spectrum disorders (ASD) in childhood. *European Journal of Paediatric Neurology*.

Buescher, A. V., Cidav, Z., Knapp, M., & Mandell, D. S. (2014). Costs of autism spectrum disorders in the United Kingdom and the United States. *JAMA pediatrics, 168*(8), 721-728.

Burgdorf, J., Kroes, R. A., Moskal, J. R., Pfaus, J. G., Brudzynski, S. M., & Panksepp, J. (2008). Ultrasonic vocalizations of rats (Rattus norvegicus) during mating, play, and aggression: Behavioral concomitants, relationship to reward, and self-administration of playback. *Journal of comparative psychology, 122*(4), 357.

Burmeister, M., McInnis, M. G., & Zöllner, S. (2008). Psychiatric genetics: progress amid controversy. *Nature Reviews Genetics, 9*(7), 527-540.

Cadman, T., Eklund, H., Howley, D., Hayward, H., Clarke, H., Findon, J., . . . Glaser, K. (2012). Caregiver burden as people with autism spectrum disorder and attention-deficit/hyperactivity disorder transition into adolescence and adulthood in the United Kingdom. *Journal of the American Academy of Child & Adolescent Psychiatry, 51*(9), 879-888.

Calabrese, F., Guidotti, G., Middelman, A., Racagni, G., Homberg, J., & Riva, M. A. (2013). Lack of serotonin transporter alters BDNF expression in the rat brain during early postnatal development. *Molecular neurobiology, 48*(1), 244-256.

Calabrese, F., Molteni, R., Cattaneo, A., Macchi, F., Racagni, G., Gennarelli, M., . . . Riva, M. A. (2010). Long-Term duloxetine treatment normalizes altered brain-derived neurotrophic factor expression in serotonin transporter knockout rats through the modulation of specific neurotrophin isoforms. *Molecular pharmacology, 77*(5), 846-853.

Calcagnetti, D. J., & Schechter, M. D. (1992). Place conditioning reveals the rewarding aspect of social interaction in juvenile rats. *Physiology & behavior, 51*(4), 667-672.

Caspi, A., & Moffitt, T. E. (2006). Gene–environment interactions in psychiatry: joining forces with neuroscience. *Nature Reviews Neuroscience, 7*(7), 583-590.

Caspi, A., Moffitt, T. E., Cannon, M., McClay, J., Murray, R., Harrington, H., . . . Braithwaite, A. (2005). Moderation of the effect of adolescent-onset cannabis use on adult psychosis by a functional polymorphism in the catechol-O-methyltransferase gene: longitudinal evidence of a gene X environment interaction. *Biological psychiatry, 57*(10), 1117-1127.

Caspi, A., Sugden, K., Moffitt, T. E., Taylor, A., Craig, I. W., Harrington, H., . . . Braithwaite, A. (2003). Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science Signaling, 301*(5631), 386.

Chandler, D. J. (2013). Something's got to give: psychiatric disease on the rise and novel drug development on the decline. *Drug Discovery Today, 18*(3–4), 202-206. doi:http://dx.doi.org/10.1016/j.drudis.2012.08.003

Chaste, P., & Leboyer, M. (2012). Autism risk factors: genes, environment, and gene-environment interactions. *Dialogues in clinical neuroscience, 14*(3), 281.

Chez, M. G., Dowling, T., Patel, P. B., Khanna, P., & Kominsky, M. (2007). Elevation of tumor necrosis factor-alpha in cerebrospinal fluid of autistic children. *Pediatric neurology, 36*(6), 361-365.

Cho, I. H., Yoo, H. J., Park, M., Lee, Y. S., & Kim, S. (2007). Family-based association study of 5-HTTLPR and the 5-HT2A receptor gene polymorphisms with autism spectrum disorder in Korean trios. *Brain research, 1139*, 34-41.

Choi, C. S., Hong, M., Kim, K. C., Kim, J.-W., Yang, S. M., Seung, H., . . . Shin, C. Y. (2014). Effects of Atomoxetine on Hyper-Locomotive Activity of the Prenatally Valproate-Exposed Rat Offspring. *Biomolecules & therapeutics, 22*(5), 406.

Chomczynski, P., & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical biochemistry, 162*(1), 156-159.

Christensen, J., Grønborg, T. K., Sørensen, M. J., Schendel, D., Parner, E. T., Pedersen, L. H., & Vestergaard, M. (2013). Prenatal Valproate Exposure and Risk of Autism Spectrum Disorders and Childhood AutismPrenatal Valproate and Autism. *Jama, 309*(16), 1696-1703.

Christianson, A. L., Chester, N., & Kromberg, J. G. (1994). Fetal Valproate Syndrome: Clinical and Neuro-developmental Features in Two Sibling Pairs. *Developmental Medicine & Child Neurology, 36*(4), 361-369.

Ciaranello, A. L., & Ciaranello, R. D. (1995). The neurobiology of infantile autism. *The Neuroscientist, 1*(6), 361-367.

Cidav, Z., Marcus, S. C., & Mandell, D. S. (2012). Implications of childhood autism for parental employment and earnings. *Pediatrics, 129*(4), 617-623.

Clark, W. M., Rinker, L. G., Lessov, N. S., Hazel, K., & Eckenstein, F. (1999). Time course of IL-6 expression in experimental CNS ischemia. *Neurological research, 21*(3), 287-292.

Cohen, O. S., Varlinskaya, E. I., Wilson, C. A., Glatt, S. J., & Mooney, S. M. (2013). Acute prenatal exposure to a moderate dose of valproic acid increases social behavior and alters gene expression in rats. *International Journal of Developmental Neuroscience, 31*(8), 740-750.

Colvert, E., Tick, B., McEwen, F., Stewart, C., Curran, S. R., Woodhouse, E., . . . Garnett, T. (2015). Heritability of Autism Spectrum Disorder in a UK Population-Based Twin Sample. *JAMA psychiatry*.

Connolly, A. M., Chez, M., Streif, E. M., Keeling, R. M., Golumbek, P. T., Kwon, J. M., . . . Deuel, R. M. K. (2006). Brain-derived neurotrophic factor and autoantibodies to neural antigens in sera of children with autistic spectrum disorders, Landau-Kleffner syndrome, and epilepsy. *Biological psychiatry, 59*(4), 354-363.

Conroy, J., Meally, E., Kearney, G., Fitzgerald, M., Gill, M., & Gallagher, L. (2004). Serotonin transporter gene and autism: a haplotype analysis in an Irish autistic population. *Molecular psychiatry, 9*(6), 587-593.

Cook, E., Courchesne, R., Lord, C., Cox, N. J., Yan, S., Lincoln, A., . . . Leventhal, B. L. (1997). Evidence of linkage between the serotonin transporter and autistic disorder. *Molecular psychiatry, 2*, 247-250.

Cook Jr, E. H., & Leventhal, B. L. (1996). The serotonin system in autism. *Current opinion in pediatrics, 8*(4), 348-354.

Correia, C., Coutinho, A., Sequeira, A., Sousa, I., Lourenco Venda, L., Almeida, J., . . . Conroy, J. (2010). Increased BDNF levels and NTRK2 gene association suggest a disruption of BDNF/TrkB signaling in autism. *Genes, Brain and behavior, 9*(7), 841-848.

Courchesne, E., Carper, R., & Akshoomoff, N. (2003). Evidence of brain overgrowth in the first year of life in autism. *Jama, 290*(3), 337-344.

Courchesne, E., Karns, C., Davis, H., Ziccardi, R., Carper, R., Tigue, Z., . . . Lord, C. (2001). Unusual brain growth patterns in early life in patients with autistic disorder an MRI study. *Neurology, 57*(2), 245-254.

Crawley, J. N. (2007). Mouse Behavioral Assays Relevant to the Symptoms of Autism*. *Brain Pathology, 17*(4), 448-459.

Crawley, J. N., Chen, T., Puri, A., Washburn, R., Sullivan, T. L., Hill, J. M., . . . Young, L. J. (2007). Social approach behaviors in oxytocin knockout mice: comparison of two independent lines tested in different laboratory environments. *Neuropeptides, 41*(3), 145-163.

Davies, K. J. (1995). *Oxidative stress: the paradox of aerobic life.* Paper presented at the Biochemical Society Symposia.

Davies, K. J. (2000). Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. *IUBMB life, 50*(4-5), 279-289.

De Theije, C. G., Koelink, P. J., Korte-Bouws, G. A., da Silva, S. L., Korte, S. M., Olivier, B., . . . Kraneveld, A. D. (2014). Intestinal inflammation in a murine model of autism spectrum disorders. *Brain, behavior, and immunity, 37*, 240-247.

Dean, J., Hailey, H., Moore, S., Lloyd, D., Turnpenny, P., & Little, J. (2002). Long term health and neurodevelopment in children exposed to antiepileptic drugs before birth. *Journal of medical genetics, 39*(4), 251-259.

Defoort, E. N., Kim, P. M., & Winn, L. M. (2006). Valproic acid increases conservative homologous recombination frequency and reactive oxygen species formation: a potential mechanism for valproic acid-induced neural tube defects. *Molecular pharmacology, 69*(4), 1304-1310.

Devlin, B., Bennett, P., Dawson, G., Figlewicz, D. A., Grigorenko, E. L., McMahon, W., . . . Spence, M. A. (2004). Alleles of a reelin CGG repeat do not convey liability to autism in a sample from the CPEA network. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics, 126*(1), 46-50.

Devlin, B., Cook, E., Coon, H., Dawson, G., Grigorenko, E., McMahon, W., . . . Spence, M. (2005). Autism and the serotonin transporter: the long and short of it. *Molecular psychiatry, 10*(12), 1110-1116.

Di Forti, M. (2008). Genes and environment in psychiatry. *Medicine, 36*(9), 493-495.

Diav-Citrin, O., Shechtman, S., Bar-Oz, B., Cantrell, D., Arnon, J., & Ornoy, A. (2008). Pregnancy Outcome after In Utero Exposure to Valproate. *CNS drugs, 22*(4), 325-334.

Dinarello, C. A. (2000). Proinflammatory cytokines. *CHEST Journal, 118*(2), 503-508.

Douma, T., Millan, M., Verdouw, P., Oosting, R., Olivier, B., & Groenink, L. (2014). Valproate improves prepulse inhibition deficits induced by corticotropin-releasing factor independent of GABA A and GABA B receptor activation. *Neuropharmacology, 79*, 66-74.

Dufour-Rainfray, D., Vourc'h, P., Le Guisquet, A.-M., Garreau, L., Ternant, D., Bodard, S., . . . Andres, C. R. (2010). Behavior and serotonergic disorders in rats exposed prenatally to valproate: a model for autism. *Neuroscience letters, 470*(1), 55-59.

Dufour-Rainfray, D., Vourc'h, P., Tourlet, S., Guilloteau, D., Chalon, S., & Andres, C. R. (2011). Fetal exposure to teratogens: evidence of genes involved in autism. *Neuroscience & Biobehavioral Reviews, 35*(5), 1254-1265.

Eikel, D., Lampen, A., & Nau, H. (2006). Teratogenic effects mediated by inhibition of histone deacetylases: evidence from quantitative structure activity relationships of 20 valproic acid derivatives. *Chemical research in toxicology, 19*(2), 272-278.

Einat, H., Yuan, P., Gould, T. D., Li, J., Du, J., Zhang, L., . . . Chen, G. (2003). The role of the extracellular signal-regulated kinase signaling pathway in mood modulation. *The Journal of neuroscience, 23*(19), 7311-7316.

El-Khodor, B. F., Dimmler, M. H., Amara, D. A., Hofer, M., Hen, R., & Brunner, D. (2004). Juvenile 5HT 1B receptor knockout mice exhibit reduced pharmacological sensitivity to 5HT 1A receptor activation. *International Journal of Developmental Neuroscience, 22*(5), 405-413.

Enstrom, A. M., Onore, C. E., Van de Water, J. A., & Ashwood, P. (2010). Differential monocyte responses to TLR ligands in children with autism spectrum disorders. *Brain, behavior, and immunity, 24*(1), 64-71.

Farrell, W. J., & Alberts, J. R. (2002). Stimulus control of maternal responsiveness to Norway rat (Rattus norvegicus) pup ultrasonic vocalizations. *Journal of Comparative Psychology, 116*(3), 297.

Fatemi, S. H., Reutiman, T. J., Folsom, T. D., Huang, H., Oishi, K., Mori, S., . . . Sohr, R. (2008). Maternal infection leads to abnormal gene regulation and brain atrophy in mouse offspring: implications for genesis of neurodevelopmental disorders. *Schizophrenia research, 99*(1), 56-70.

Fathe, K., Palacios, A., & Finnell, R. H. (2014). Brief report novel mechanism for valproate-induced teratogenicity. *Birth Defects Research Part A: Clinical and Molecular Teratology, 100*(8), 592-597.

Ferretti, C. J., & Hollander, E. (2015). The Role of Inflammation in Autism Spectrum Disorder *Immunology and Psychiatry* (pp. 275-312): Springer.

Fortier, M.-E., Luheshi, G. N., & Boksa, P. (2007). Effects of prenatal infection on prepulse inhibition in the rat depend on the nature of the infectious agent and the stage of pregnancy. *Behavioural Brain Research, 181*(2), 270-277. doi:http://dx.doi.org/10.1016/j.bbr.2007.04.016

Freitag, C. M. (2006). The genetics of autistic disorders and its clinical relevance: a review of the literature. *Molecular psychiatry, 12*(1), 2-22.

Fujiki, R., Sato, A., Fujitani, M., & Yamashita, T. (2013). A proapoptotic effect of valproic acid on progenitors of embryonic stem cell-derived glutamatergic neurons. *Cell death & disease, 4*(6), e677.

Fukuchi, M., Nii, T., Ishimaru, N., Minamino, A., Hara, D., Takasaki, I., . . . Tsuda, M. (2009). Valproic acid induces up-or down-regulation of gene expression responsible for the neuronal excitation and inhibition in rat cortical neurons through its epigenetic actions. *Neuroscience Research, 65*(1), 35-43.

Fumagalli, F., Calabrese, F., Luoni, A., Bolis, F., Racagni, G., & Riva, M. A. (2012). Modulation of BDNF expression by repeated treatment with the novel antipsychotic lurasidone under basal condition and in response to acute stress. *The International Journal of Neuropsychopharmacology, 15*(02), 235-246.

Gandal, M. J., Edgar, J. C., Ehrlichman, R. S., Mehta, M., Roberts, T. P., & Siegel, S. J. (2010). Validating γ oscillations and delayed auditory responses as translational biomarkers of autism. *Biological psychiatry, 68*(12), 1100-1106.

Gayle, D. A., Beloosesky, R., Desai, M., Amidi, F., Nunez, S. E., & Ross, M. G. (2004). Maternal LPS induces cytokines in the amniotic fluid and corticotropin releasing hormone in the fetal rat brain. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 286*(6), R1024-R1029.

Geier, D. A., Kern, J. K., & Geier, M. R. (2010). The biological basis of autism spectrum disorders: Understanding causation and treatment by clinical geneticists. *Acta Neurobiol Exp (Wars), 70*(2), 209-226.

Gilmore, J. H., & Fredrik Jarskog, L. (1997). Exposure to infection and brain development: cytokines in the pathogenesis of schizophrenia. *Schizophrenia research, 24*(3), 365-367.

Givalois, L., Dornand, J., Mekaouche, M., Solier, M., Bristow, A., Ixart, G., . . . Barbanel, G. (1994). Temporal cascade of plasma level surges in ACTH, corticosterone, and cytokines in endotoxin-challenged rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 267*(1), R164-R170.

Grant, E. (1963). An analysis of the social behaviour of the male laboratory rat. *Behaviour, 21*(3), 260-281.
Grishina, O., Schmoor, C., Döhner, K., Hackanson, B., Lubrich, B., May, A. M., . . . Lübbert, M. (2015). DECIDER: prospective randomized multicenter phase II trial of low-dose decitabine (DAC) administered alone or in combination with the histone deacetylase inhibitor valproic acid (VPA) and all-trans retinoic acid (ATRA) in patients> 60 years with acute myeloid leukemia who are ineligible for induction chemotherapy. *BMC cancer, 15*(1), 430.
Guenther, S., Bauer, S., Hagge, M., Knake, S., Olmes, D. G., Tackenberg, B., . . . Hamer, H. M. (2014). Chronic valproate or levetiracetam treatment does not influence cytokine levels in humans. *Seizure, 23*(8), 666-669.
Guhathakurta, S., Ghosh, S., Sinha, S., Chatterjee, A., Ahmed, S., Chowdhury, S. R., . . . Usha, R. (2006). Serotonin transporter promoter variants: Analysis in Indian autistic and control population. *Brain research, 1092*(1), 28-35.
Gurvich, N., Berman, M. G., Wittner, B. S., Gentleman, R. C., Klein, P. S., & Green, J. B. (2005). Association of valproate-induced teratogenesis with histone deacetylase inhibition in vivo. *The FASEB journal, 19*(9), 1166-1168.
Haber, J. E. (1999). DNA recombination: the replication connection. *Trends in Biochemical Sciences, 24*(7), 271-275. doi:http://dx.doi.org/10.1016/S0968-0004(99)01413-9
Hallmayer, J., Cleveland, S., Torres, A., Phillips, J., Cohen, B., Torigoe, T., . . . Smith, K. (2011). Genetic heritability and shared environmental factors among twin pairs with autism. *Archives of general psychiatry, 68*(11), 1095-1102.
Hansson, S., Mezey, E., & Hoffman, B. (1998). Serotonin transporter messenger RNA in the developing rat brain: early expression in serotonergic neurons and transient expression in non-serotonergic neurons. *Neuroscience, 83*(4), 1185-1201.
Happé, F., Ronald, A., & Plomin, R. (2006). Time to give up on a single explanation for autism. *Nature neuroscience, 9*(10), 1218-1220.
Hartley, S., Sikora, D., & McCoy, R. (2008). Prevalence and risk factors of maladaptive behaviour in young children with autistic disorder. *Journal of Intellectual Disability Research, 52*(10), 819-829.
Harvey, L., & Boksa, P. (2014). Do prenatal immune activation and maternal iron deficiency interact to affect neurodevelopment and early behavior in rat offspring? *Brain, behavior, and immunity, 35*, 144-154.
Hodgson, R. A., Guthrie, D. H., & Varty, G. B. (2008). Duration of ultrasonic vocalizations in the isolated rat pup as a behavioral measure: sensitivity to anxiolytic and antidepressant drugs. *Pharmacology Biochemistry and Behavior, 88*(3), 341-348.
Holmes, P. V. (2003). Rodent models of depression: reexamining validity without anthropomorphic inference. *Critical Reviews™ in Neurobiology, 15*(2).
Homberg, J. R., Molteni, R., Calabrese, F., & Riva, M. A. (2014). The serotonin–BDNF duo: Developmental implications for the vulnerability to psychopathology. *Neuroscience & Biobehavioral Reviews, 43*, 35-47.
Homberg, J. R., Olivier, J. D. A., Smits, B. M. G., Mul, J. D., Mudde, J., Verheul, M., . . . Cuppen, E. (2007). Characterization of the serotonin transporter knockout rat: A selective change in the functioning of the serotonergic system. *Neuroscience, 146*(4), 1662-1676. doi:http://dx.doi.org/10.1016/j.neuroscience.2007.03.030
Homberg, J. R., Schiepers, O. J., Schoffelmeer, A. N., Cuppen, E., & Vanderschuren, L. J. (2007). Acute and constitutive increases in central serotonin levels reduce social play behaviour in peri-adolescent rats. *Psychopharmacology, 195*(2), 175-182.

Hrupka, B., & Langhans, W. (2001). A role for serotonin in lipopolysaccharide-induced anorexia in rats. *Pharmacology Biochemistry and Behavior, 68*(2), 355-362.
Hsia, Y., Wong, A. Y., Murphy, D. G., Simonoff, E., Buitelaar, J. K., & Wong, I. C. (2014). Psychopharmacological prescriptions for people with autism spectrum disorder (ASD): a multinational study. *Psychopharmacology, 231*(6), 999-1009.
Hsieh, C.-L., Wang, H.-E., Tsai, W.-J., Peng, C.-C., & Peng, R. Y. (2012). Multiple point action mechanism of valproic acid-teratogenicity alleviated by folic acid, vitamin C, And N-acetylcysteine in chicken embryo model. *Toxicology, 291*(1–3), 32-42. doi:http://dx.doi.org/10.1016/j.tox.2011.10.015
Hsieh, C. L., Chen, K. C., Lin, P. X., Peng, C. C., & Peng, R. Y. (2014). Resveratrol and vitamin E rescue valproic acid-induced teratogenicity: The mechanism of action. *Clinical and Experimental Pharmacology and Physiology, 41*(3), 210-219.
Hu, J.-P., Xie, J.-W., Wang, C.-Y., Wang, T., Wang, X., Wang, S.-L., . . . Wang, Z.-Y. (2011). Valproate reduces tau phosphorylation via cyclin-dependent kinase 5 and glycogen synthase kinase 3 signaling pathways. *Brain research bulletin, 85*(3), 194-200.
Hu, X.-Z., Lipsky, R. H., Zhu, G., Akhtar, L. A., Taubman, J., Greenberg, B. D., . . . Kennedy, J. L. (2006). Serotonin transporter promoter gain-of-function genotypes are linked to obsessive-compulsive disorder. *The American Journal of Human Genetics, 78*(5), 815-826.
Huang, C. H., & Santangelo, S. L. (2008). Autism and serotonin transporter gene polymorphisms: A systematic review and meta-analysis. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics, 147*(6), 903-913.
Ichiyama, T., Okada, K., Lipton, J. M., Matsubara, T., Hayashi, T., & Furukawa, S. (2000). Sodium valproate inhibits production of TNF-α and IL-6 and activation of NF-κB. *Brain research, 857*(1–2), 246-251. doi:http://dx.doi.org/10.1016/S0006-8993(99)02439-7
Ingram, J. L., Peckham, S. M., Tisdale, B., & Rodier, P. M. (2000). Prenatal exposure of rats to valproic acid reproduces the cerebellar anomalies associated with autism. *Neurotoxicology and Teratology, 22*(3), 319-324.
Insel, T. R., Hill, J. L., & Mayor, R. B. (1986). Rat pup ultrasonic isolation calls: possible mediation by the benzodiazepine receptor complex. *Pharmacology Biochemistry and Behavior, 24*(5), 1263-1267.
Ivanov, M., Barragan, I., & Ingelman-Sundberg, M. (2014). Epigenetic mechanisms of importance for drug treatment. *Trends in pharmacological sciences, 35*(8), 384-396.
Jacob, J., Ribes, V., Moore, S., Constable, S. C., Sasai, N., Gerety, S. S., . . . Briscoe, J. (2014). Valproic acid silencing of ascl1b/Ascl1 results in the failure of serotonergic differentiation in a zebrafish model of fetal valproate syndrome. *Disease models & mechanisms, 7*(1), 107-117.
Jamain, S., Quach, H., Betancur, C., Råstam, M., Colineaux, C., Gillberg, I. C., . . . Gillberg, C. (2003). Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. *Nature genetics, 34*(1), 27-29.
James, E. J., Gu, J., Ramirez-Vizcarrondo, C. M., Hasan, M., Truszkowski, T. L., Tan, Y., . . . Aizenman, C. D. (2015). Valproate-Induced Neurodevelopmental Deficits in Xenopus laevis Tadpoles. *The Journal of Neuroscience, 35*(7), 3218-3229.
Järbrink, K. (2007). The economic consequences of autistic spectrum disorder among children in a Swedish municipality. *Autism, 11*(5), 453-463.

Jeon, G. S., Park, S.-H., Lee, K.-J., Lee, M.-S., Chun, B.-G., & Shin, K.-H. (2006). Valproate prevents MK801-induced changes in brain-derived neurotrophic factor mRNA in the rat brain. *European Journal of Pharmacology, 545*(2), 142-146.

Jeong, M. R., Hashimoto, R., Senatorov, V. V., Fujimaki, K., Ren, M., Lee, M. S., & Chuang, D.-M. (2003). Valproic acid, a mood stabilizer and anticonvulsant, protects rat cerebral cortical neurons from spontaneous cell death: a role of histone deacetylase inhibition. *FEBS letters, 542*(1), 74-78.

Jiang, Y.-h., & Ehlers, M. D. (2013). Modeling autism by SHANK gene mutations in mice. *Neuron, 78*(1), 8-27.

Johannessen, C. U., & Johannessen, S. I. (2003). Valproate: past, present, and future. *CNS drug reviews, 9*(2), 199-216.

Jones, K. L., Smith, R. M., Edwards, K. S., Givens, B., Tilley, M. R., & Beversdorf, D. Q. (2010). Combined effect of maternal serotonin transporter genotype and prenatal stress in modulating offspring social interaction in mice. *International Journal of Developmental Neuroscience, 28*(6), 529-536.

Jyonouchi, H., Sun, S., & Le, H. (2001). Proinflammatory and regulatory cytokine production associated with innate and adaptive immune responses in children with autism spectrum disorders and developmental regression. *Journal of Neuroimmunology, 120*(1), 170-179.

Kalueff, A., Fox, M., Gallagher, P., & Murphy, D. (2007). Hypolocomotion, anxiety and serotonin syndrome-like behavior contribute to the complex phenotype of serotonin transporter knockout mice. *Genes, Brain and behavior, 6*(4), 389-400.

Kalueff, A. V., Ren-Patterson, R. F., & Murphy, D. L. (2007). The developing use of heterozygous mutant mouse models in brain monoamine transporter research. *Trends in pharmacological sciences, 28*(3), 122-127.

Kane, M. J., Angoa-Peréz, M., Briggs, D. I., Sykes, C. E., Francescutti, D. M., Rosenberg, D. R., & Kuhn, D. M. (2012). Mice genetically depleted of brain serotonin display social impairments, communication deficits and repetitive behaviors: possible relevance to autism. *PloS one, 7*(11), e48975.

Kataoka, S., Takuma, K., Hara, Y., Maeda, Y., Ago, Y., & Matsuda, T. (2013). Autism-like behaviours with transient histone hyperacetylation in mice treated prenatally with valproic acid. *The International Journal of Neuropsychopharmacology, 16*(01), 91-103.

Kawai, Y., & Arinze, I. J. (2006). Valproic Acid–Induced Gene Expression through Production of Reactive Oxygen Species. *Cancer research, 66*(13), 6563-6569.

Kim, H.-G., Kishikawa, S., Higgins, A. W., Seong, I.-S., Donovan, D. J., Shen, Y., . . . Kutsche, K. (2008). Disruption of neurexin 1 associated with autism spectrum disorder. *The American Journal of Human Genetics, 82*(1), 199-207.

Kim, J.-W., Choi, C. S., Kim, K. C., Park, J. H., Seung, H., Joo, S. H., . . . Park, S. H. (2013). Gastrointestinal Tract Abnormalities Induced by Prenatal Valproic Acid Exposure in Rat Offspring. *Toxicological research, 29*(3), 173.

Kim, K. C., Kim, P., Go, H. S., Choi, C. S., Park, J. H., Kim, H. J., . . . Ryu, J. H. (2013). 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, K. C., Kim, P., Go, H. S., Choi, C. S., Yang, S.-I., Cheong, J. H., . . . Ko, K. H. (2011). The critical period of valproate exposure to induce autistic symptoms in Sprague–Dawley rats. *Toxicology letters, 201*(2), 137-142.

Kinney, D. K., Munir, K. M., Crowley, D. J., & Miller, A. M. (2008). Prenatal stress and risk for autism. *Neuroscience & Biobehavioral Reviews, 32*(8), 1519-1532. doi:http://dx.doi.org/10.1016/j.neubiorev.2008.06.004

Kirsten, T. B., Chaves-Kirsten, G. P., Chaible, L. M., Silva, A. C., Martins, D. O., Britto, L. R., . . . Bernardi, M. M. (2012). Hypoactivity of the central dopaminergic system and autistic-like behavior induced by a single early prenatal exposure to lipopolysaccharide. *Journal of neuroscience research, 90*(10), 1903-1912.

Kirsten, T. B., Taricano, M., Flório, J. C., Palermo-Neto, J., & Bernardi, M. M. (2010). Prenatal lipopolysaccharide reduces motor activity after an immune challenge in adult male offspring. *Behavioural brain research, 211*(1), 77-82. doi:http://dx.doi.org/10.1016/j.bbr.2010.03.009

Kirsten, T. B., Taricano, M., Maiorka, P. C., Palermo-Neto, J., & Bernardi, M. M. (2010). Prenatal lipopolysaccharide reduces social behavior in male offspring. *Neuroimmunomodulation, 17*(4), 240-251.

Kiser, D., SteemerS, B., Branchi, I., & Homberg, J. R. (2012). The reciprocal interaction between serotonin and social behaviour. *Neuroscience & Biobehavioral Reviews, 36*(2), 786-798.

Klauck, S. M., Poustka, F., Benner, A., Lesch, K.-P., & Poustka, A. (1997). Serotonin transporter (5-HTT) gene variants associated with autism? *Human Molecular Genetics, 6*(13), 2233-2238.

Knutson, B., Burgdorf, J., & Panksepp, J. (1998). Anticipation of play elicits high-frequency ultrasonic vocalizations in young rats. *Journal of comparative psychology, 112*(1), 65.

Koishi, S., Yamamoto, K., Matsumoto, H., Koishi, S., Enseki, Y., Oya, A., . . . Iga, T. (2006). Serotonin transporter gene promoter polymorphism and autism: a family-based genetic association study in Japanese population. *Brain and Development, 28*(4), 257-260.

Kola, I., & Landis, J. (2004). Can the pharmaceutical industry reduce attrition rates? *Nature reviews Drug discovery, 3*(8), 711-716.

Kolevzon, A., Lim, T., Schmeidler, J., Martello, T., Cook Jr, E. H., & Silverman, J. M. (2014). Self-injury in autism spectrum disorder: an effect of serotonin transporter gene promoter variants. *Psychiatry research, 220*(3), 987-990.

Koren, G., Nava-Ocampo, A. A., Moretti, M. E., Sussman, R., & Nulman, I. (2006). Major malformations with valproic acid. *Canadian family physician, 52*(4), 441-442.

Kornberg, R. D. (1977). Structure of chromatin. *Annual review of biochemistry, 46*(1), 931-954.

Kwan, P., Sills, G. J., & Brodie, M. J. (2001). The mechanisms of action of commonly used antiepileptic drugs. *Pharmacology & therapeutics, 90*(1), 21-34.

Kyzar, E. J., Pham, M., Roth, A., Cachat, J., Green, J., Gaikwad, S., & Kalueff, A. V. (2012). Alterations in grooming activity and syntax in heterozygous SERT and BDNF knockout mice: the utility of behavior-recognition tools to characterize mutant mouse phenotypes. *Brain research bulletin, 89*(5), 168-176.

Lam, K. S., Aman, M. G., & Arnold, L. E. (2006). Neurochemical correlates of autistic disorder: a review of the literature. *Research in Developmental Disabilities, 27*(3), 254-289.

Laskar, S., Bhattacharya, U., & Basak, B. (1991). Modified ninhydrin spray reagent for the identification of amino acids on thin-layer chromatography plates. *Analyst, 116*(6), 625-626.

Leblond, C. S., Nava, C., Polge, A., Gauthier, J., Huguet, G., Lumbroso, S., . . . Mouzat, K. (2014). Meta-analysis of SHANK mutations in autism spectrum disorders: a gradient of severity in cognitive impairments.

Lee, E.-J., Choi, S. Y., & Kim, E. (2015). NMDA receptor dysfunction in autism spectrum disorders. *Current opinion in pharmacology, 20*, 8-13.

Lehman, M. N., & Adams, D. B. (1977). A Statistical and Motivational Analysis of the Social Behaviors of the Male Laboratory Rat. *Behaviour, 61*(3/4), 238-275. Retrieved from http://www.jstor.org/stable/4533820

Lehrman, G., Hogue, I. B., Palmer, S., Jennings, C., Spina, C. A., Wiegand, A., . . . Mellors, J. W. (2005). Depletion of latent HIV-1 infection in vivo: a proof-of-concept study. *The Lancet, 366*(9485), 549-555.

Lesch, K.-P., Bengel, D., Heils, A., Sabol, S. Z., Greenberg, B. D., Petri, S., . . . Murphy, D. L. (1996). Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. *Science, 274*(5292), 1527-1531.

Levy, S. E., Mandell, D. S., & Schultz, R. T. (2009). Autism. *Lancet, 374*, 1627-1638.

Li, G., & Reinberg, D. (2011). Chromatin higher-order structures and gene regulation. *Current opinion in genetics & development, 21*(2), 175-186.

Li, J., Nguyen, L., Gleason, C., Lotspeich, L., Spiker, D., Risch, N., & Myers, R. M. (2004). Lack of evidence for an association between WNT2 and RELN polymorphisms and autism. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics, 126*(1), 51-57.

Li, X., Chauhan, A., Sheikh, A. M., Patil, S., Chauhan, V., Li, X.-M., . . . Malik, M. (2009). Elevated immune response in the brain of autistic patients. *Journal of Neuroimmunology, 207*(1), 111-116.

Libbey, J. E., Sweeten, T. L., McMahon, W. M., & Fujinami, R. S. (2005). Autistic disorder and viral infections. *Journal of neurovirology, 11*(1), 1-10.

Lin, Y.-L., Lin, S.-Y., & Wang, S. (2012). Prenatal lipopolysaccharide exposure increases anxiety-like behaviors and enhances stress-induced corticosterone responses in adult rats. *Brain, behavior, and immunity, 26*(3), 459-468.

Liu, X., & Osawa, T. (2009). Astaxanthin protects neuronal cells against oxidative damage and is a potent candidate for brain food.

Lloyd, K. A. (2013). A scientific review: mechanisms of valproate-mediated teratogenesis. *Bioscience Horizons, 6*, hzt003.

Loohuis, N. O., Kole, K., Glennon, J., Karel, P., Van der Borg, G., Van Gemert, Y., . . . Shahabipour, F. (2015). Elevated microRNA-181c and microRNA-30d levels in the enlarged amygdala of the valproic acid rat model of autism. *Neurobiology of disease, 80*, 42-53.

Łopuch, S., & Popik, P. (2011). Cooperative behavior of laboratory rats (Rattus norvegicus) in an instrumental task. *Journal of comparative psychology, 125*(2), 250.

Macchi, F., Homberg, J. R., Calabrese, F., Zecchillo, C., Racagni, G., Riva, M. A., & Molteni, R. (2013). Altered inflammatory responsiveness in serotonin transporter mutant rats. *Journal of neuroinflammation, 10*(1), 1-10.

Maestrini, E., Lai, C., Marlow, A., Matthews, N., Wallace, S., Bailey, A., . . . Monaco, A. (1999). Serotonin transporter (5-HTT) and-aminobutyric acid receptor subunit 3 (GABRB3) gene polymorphisms are not associated with autism in the IMGSA families. The International Molecular Genetic Study of Autism Consortium. *Am J Med Genet, 88*(5), 492-496.

Makkonen, I., Riikonen, R., Kokki, H., Airaksinen, M. M., & Kuikka, J. T. (2008). Serotonin and dopamine transporter binding in children with autism determined by SPECT. *Developmental Medicine & Child Neurology, 50*(8), 593-597.
Malik, M., Sheikh, A. M., Wen, G., Spivack, W., Brown, W. T., & Li, X. (2011). Expression of inflammatory cytokines, Bcl2 and cathepsin D are altered in lymphoblasts of autistic subjects. *Immunobiology, 216*(1), 80-85.
Malkova, N. V., Yu, C. Z., Hsiao, E. Y., Moore, M. J., & Patterson, P. H. (2012). Maternal immune activation yields offspring displaying mouse versions of the three core symptoms of autism. *Brain, behavior, and immunity, 26*(4), 607-616.
Mansour, M., Mohamed, A., Azam, H., & Henedy, M. (2010). Brain derived neurotrophic factor in autism. *Current Psychiatry, 17*(1), 23-29.
Martinowich, K., & Lu, B. (2008). Interaction between BDNF and serotonin: role in mood disorders. *Neuropsychopharmacology, 33*(1), 73-83.
McCauley, J., Olson, L., Dowd, M., Amin, T., Steele, A., Blakely, R., . . . Sutcliffe, J. (2004). Linkage and association analysis at the serotonin transporter (SLC6A4) locus in a rigid-compulsive subset of autism. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics, 127*(1), 104-112.
McDougle, C. J., Naylor, S. T., Cohen, D. J., Aghajanian, G. K., Heninger, G. R., & Price, L. H. (1996). Effects of tryptophan depletion in drug-free adults with autistic disorder. *Archives of general psychiatry, 53*(11), 993.
McDougle, C. J., Naylor, S. T., Cohen, D. J., Volkmar, F. R., Heninger, G. R., & Price, L. H. (1996). A double-blind, placebo-controlled study of fluvoxamine in adults with autistic disorder. *Archives of general psychiatry, 53*(11), 1001.
McFarlane, H., Kusek, G., Yang, M., Phoenix, J., Bolivar, V., & Crawley, J. (2008). Autism-like behavioral phenotypes in BTBR T+ tf/J mice. *Genes, Brain and behavior, 7*(2), 152-163.
McGonigle, P., & Ruggeri, B. (2014). Animal models of human disease: Challenges in enabling translation. *Biochemical Pharmacology, 87*(1), 162-171. doi:http://dx.doi.org/10.1016/j.bcp.2013.08.006
Meador, K., Reynolds, M. W., Crean, S., Fahrbach, K., & Probst, C. (2008). Pregnancy outcomes in women with epilepsy: a systematic review and meta-analysis of published pregnancy registries and cohorts. *Epilepsy research, 81*(1), 1-13.
Meaney, M. J., & Stewart, J. (1981). A descriptive study of social development in the rat (Rattus norvegicus). *Animal Behaviour, 29*(1), 34-45.
Mehta, M. V., Gandal, M. J., & Siegel, S. J. (2011). mGluR5-antagonist mediated reversal of elevated stereotyped, repetitive behaviors in the VPA model of autism. *PloS one, 6*(10), e26077.
Menegola, E., Di Renzo, F., Broccia, M. L., Prudenziati, M., Minucci, S., Massa, V., & Giavini, E. (2005). Inhibition of histone deacetylase activity on specific embryonic tissues as a new mechanism for teratogenicity. *Birth Defects Research Part B: Developmental and Reproductive Toxicology, 74*(5), 392-398.
Meyer, U., Feldon, J., & Dammann, O. (2011). Schizophrenia and autism: both shared and disorder-specific pathogenesis via perinatal inflammation? *Pediatric research, 69*, 26R-33R.
Meyer, U., Feldon, J., & Fatemi, S. H. (2009). In-vivo rodent models for the experimental investigation of prenatal immune activation effects in neurodevelopmental brain disorders. *Neuroscience & Biobehavioral Reviews, 33*(7), 1061-1079.
Miyazaki, K., Narita, N., & 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), 287-297.
Miyazaki, K., Narita, N., Sakuta, R., Miyahara, T., Naruse, H., Okado, N., & Narita, M. (2004). Serum neurotrophin concentrations in autism and mental retardation: a pilot study. *Brain and Development, 26*(5), 292-295.
MohanKumar, S. M., MohanKumar, P., & Quadri, S. (1999). Lipopolysaccharide-induced changes in monoamines in specific areas of the brain: blockade by interleukin-1 receptor antagonist. *Brain research, 824*(2), 232-237.
Moldrich, R. X., Leanage, G., She, D., Dolan-Evans, E., Nelson, M., Reza, N., & Reutens, D. C. (2013). Inhibition of histone deacetylase in utero causes sociability deficits in postnatal mice. *Behavioural Brain Research, 257*, 253-264.
Molteni, R., Cattaneo, A., Calabrese, F., Macchi, F., Olivier, J. D., Racagni, G., . . . Riva, M. A. (2010). Reduced function of the serotonin transporter is associated with decreased expression of BDNF in rodents as well as in humans. *Neurobiology of Disease, 37*(3), 747-755.
Moore, S., Turnpenny, P., Quinn, A., Glover, S., Lloyd, D., Montgomery, T., & Dean, J. (2000). A clinical study of 57 children with fetal anticonvulsant syndromes. *Journal of Medical Genetics, 37*(7), 489-497.
Morrow, J., Russell, A., Guthrie, E., Parsons, L., Robertson, I., Waddell, R., . . . Craig, J. (2006). Malformation risks of antiepileptic drugs in pregnancy: a prospective study from the UK Epilepsy and Pregnancy Register. *Journal of Neurology, Neurosurgery & Psychiatry, 77*(2), 193-198.
Mosienko, V., Beis, D., Alenina, N., & Wöhr, M. (2015). Reduced isolation-induced pup ultrasonic communication in mouse pups lacking brain serotonin. *Molecular Autism, 6*(1), 13.
Mosienko, V., Bert, B., Beis, D., Matthes, S., Fink, H., Bader, M., & Alenina, N. (2012). Exaggerated aggression and decreased anxiety in mice deficient in brain serotonin. *Translational psychiatry, 2*(5), e122.
Mowery, T. M., Wilson, S. M., Kostylev, P. V., Dina, B., Buchholz, J. B., Prieto, A. L., & Garraghty, P. E. (2015). Embryological exposure to valproic acid disrupts morphology of the deep cerebellar nuclei in a sexually dimorphic way. *International Journal of Developmental Neuroscience, 40*, 15-23.
Moy, S., Nadler, J., Perez, A., Barbaro, R., Johns, J., Magnuson, T., . . . Crawley, J. (2004). Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. *Genes, Brain and behavior, 3*(5), 287-302.
Moy, S. S., Nadler, J. J., Young, N. B., Nonneman, R. J., Grossman, A. W., Murphy, D. L., . . . Lauder, J. M. (2009). Social approach in genetically engineered mouse lines relevant to autism. *Genes, Brain and Behavior, 8*(2), 129-142.
Moy, S. S., Nadler, J. J., Young, N. B., Perez, A., Holloway, L. P., Barbaro, R. P., . . . Lauder, J. M. (2007). Mouse behavioral tasks relevant to autism: phenotypes of 10 inbred strains. *Behavioural brain research, 176*(1), 4-20.
Mulleners, W. M., McCrory, D. C., & Linde, M. (2014). Antiepileptics in migraine prophylaxis: An updated Cochrane review. *Cephalalgia*, 0333102414534325.
Muller, C., Anacker, A., & Veenstra-VanderWeele, J. (2015). The serotonin system in autism spectrum disorder: From biomarker to animal models. *Neuroscience*.
Murray, M. L., Hsia, Y., Glaser, K., Simonoff, E., Murphy, D. G., Asherson, P. J., . . . Wong, I. C. (2014). Pharmacological treatments prescribed to people with autism spectrum disorder (ASD) in primary health care. *Psychopharmacology, 231*(6), 1011-1021.

Na, L., Wartenberg, M., Nau, H., Hescheler, J., & Sauer, H. (2003). Anticonvulsant valproic acid inhibits cardiomyocyte differentiation of embryonic stem cells by increasing intracellular levels of reactive oxygen species. *Birth Defects Research Part A: Clinical and Molecular Teratology, 67*(3), 174-180.

Nadler, J., Moy, S., Dold, G., Simmons, N., Perez, A., Young, N., . . . Crawley, J. (2004). Automated apparatus for quantitation of social approach behaviors in mice. *Genes, Brain and behavior, 3*(5), 303-314.

Nakamura, M., Ueno, S., Sano, A., & Tanabe, H. (2000). The human serotonin transporter gene linked polymorphism (5-HTTLPR) shows ten novel allelic variants. *Molecular psychiatry, 5*(1), 32-38.

Narita, N., Kato, M., Tazoe, M., Miyazaki, K., Narita, M., & Okado, N. (2002). Increased monoamine concentration in the brain and blood of fetal thalidomide-and valproic acid–exposed rat: putative animal models for autism. *Pediatric Research, 52*(4), 576-579.

Nelson, K. B., Grether, J. K., Croen, L. A., Dambrosia, J. M., Dickens, B. F., Jelliffe, L. L., . . . Phillips, T. M. (2001). Neuropeptides and neurotrophins in neonatal blood of children with autism or mental retardation. *Annals of neurology, 49*(5), 597-606.

Nestler, E. J., & Hyman, S. E. (2010). Animal models of neuropsychiatric disorders. *Nature neuroscience, 13*(10), 1161-1169.

Newschaffer, C. J., Croen, L. A., Daniels, J., Giarelli, E., Grether, J. K., Levy, S. E., . . . Reaven, J. (2007). The epidemiology of autism spectrum disorders*. *Annu. Rev. Public Health, 28*, 235-258.

Nickl-Jockschat, T., & Michel, T. (2011). The role of neurotrophic factors in autism. *Molecular psychiatry, 16*(5), 478-490.

Nicolini, C., Ahn, Y., Michalski, B., Rho, J. M., & Fahnestock, M. (2015). Decreased mTOR signaling pathway in human idiopathic autism and in rats exposed to valproic acid. *Acta neuropathologica communications, 3*(1), 3.

Niesink, R. J., & Van Ree, J. M. (1982). Short-term isolation increases social interactions of male rats: a parametric analysis. *Physiology & behavior, 29*(5), 819-825.

Nijmeijer, J. S., Hartman, C. A., Rommelse, N. N., Altink, M. E., Buschgens, C. J., Fliers, E. A., . . . Sergeant, J. A. (2010). Perinatal risk factors interacting with catechol O-methyltransferase and the serotonin transporter gene predict ASD symptoms in children with ADHD. *Journal of Child Psychology and Psychiatry, 51*(11), 1242-1250.

O'Roak, B. J., & State, M. W. (2008). Autism genetics: strategies, challenges, and opportunities. *Autism Research, 1*(1), 4-17.

O'Roak, B. J., Vives, L., Girirajan, S., Karakoc, E., Krumm, N., Coe, B. P., . . . Smith, J. D. (2012). Sporadic autism exomes reveal a highly interconnected protein network of de novo mutations. *Nature, 485*(7397), 246-250.

Ohkawara, T., Katsuyama, T., Ida-Eto, M., Narita, N., & Narita, M. (2014). Maternal viral infection during pregnancy impairs development of fetal serotonergic neurons. *Brain and Development*.

Olivier, J., Jans, L., Korte-Bouws, G., Korte, S., Deen, P., Cools, A., . . . Blokland, A. (2008). Acute tryptophan depletion dose dependently impairs object memory in serotonin transporter knockout rats. *Psychopharmacology, 200*(2), 243-254.

Ornoy, A. (2009). Valproic acid in pregnancy: how much are we endangering the embryo and fetus? *Reproductive Toxicology, 28*(1), 1-10.

Pattabiraman, P. P., Tropea, D., Chiaruttini, C., Tongiorgi, E., Cattaneo, A., & Domenici, L. (2005). Neuronal activity regulates the developmental expression and

subcellular localization of cortical BDNF mRNA isoforms in vivo. *Molecular and Cellular Neuroscience, 28*(3), 556-570.
Patterson, P. H. (2002). Maternal infection: window on neuroimmune interactions in fetal brain development and mental illness. *Current opinion in neurobiology, 12*(1), 115-118.
Patterson, P. H. (2009). Immune involvement in schizophrenia and autism: etiology, pathology and animal models. *Behavioural brain research, 204*(2), 313-321.
Patterson, P. H. (2011). Modeling autistic features in animals. *Pediatric Research, 69*, 34R-40R.
Peartree, N. A., Hood, L. E., Thiel, K. J., Sanabria, F., Pentkowski, N. S., Chandler, K. N., & Neisewander, J. L. (2012). Limited physical contact through a mesh barrier is sufficient for social reward-conditioned place preference in adolescent male rats. *Physiology & behavior, 105*(3), 749-756.
Penner, J. D., & Brown, A. S. (2007). Prenatal infectious and nutritional factors and risk of adult schizophrenia. *Expert review of neurotherapeutics, 7*(7), 797-805.
Perry, W., Minassian, A., Lopez, B., Maron, L., & Lincoln, A. (2007). Sensorimotor Gating Deficits in Adults with Autism. *Biological Psychiatry, 61*(4), 482-486. doi:http://dx.doi.org/10.1016/j.biopsych.2005.09.025
Persico, A. M., Militerni, R., Bravaccio, C., Schneider, C., Melmed, R., Conciatori, M., . . . Keller, F. (2000). Lack of association between serotonin transporter gene promoter variants and autistic disorder in two ethnically distinct samples. *American journal of medical genetics, 96*(1), 123-127.
Phiel, C. J., Zhang, F., Huang, E. Y., Guenther, M. G., Lazar, M. A., & Klein, P. S. (2001). Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *Journal of Biological Chemistry, 276*(39), 36734-36741.
Politte, L. C., & McDougle, C. J. (2014). Atypical antipsychotics in the treatment of children and adolescents with pervasive developmental disorders. *Psychopharmacology, 231*(6), 1023-1036.
Pruunsild, P., Kazantseva, A., Aid, T., Palm, K., & Timmusk, T. (2007). Dissecting the human BDNF locus: bidirectional transcription, complex splicing, and multiple promoters. *Genomics, 90*(3), 397-406.
Qing, H., He, G., Ly, P. T., Fox, C. J., Staufenbiel, M., Cai, F., . . . Chen, C.-H. (2008). Valproic acid inhibits Aβ production, neuritic plaque formation, and behavioral deficits in Alzheimer's disease mouse models. *The Journal of experimental medicine, 205*(12), 2781-2789.
Rabionet, R., McCauley, J., Jaworski, J., Ashley-Koch, A., Martin, E., Sutcliffe, J., . . . Wright, H. (2006). Lack of association between autism and SLC25A12. *American Journal of Psychiatry, 163*(5), 929-931.
Ramoz, N., Reichert, J. G., Corwin, T. E., Smith, C. J., Silverman, J. M., Hollander, E., & Buxbaum, J. D. (2006). Lack of evidence for association of the serotonin transporter gene SLC6A4 with autism. *Biological psychiatry, 60*(2), 186-191.
Ramoz, N., Reichert, J. G., Smith, C. J., Silverman, J. M., Bespalova, I. N., Davis, K. L., & Buxbaum, J. D. (2004). Linkage and association of the mitochondrial aspartate/glutamate carrier SLC25A12 gene with autism. *American Journal of Psychiatry, 161*(4), 662-669.
Ranger, P., & Ellenbroek, B. A. (2015). Perinatal Influences of Valproate on Brain and Behaviour: An Animal Model for Autism (pp. 1-24): Springer Berlin Heidelberg.

Rasalam, A., Hailey, H., Williams, J., Moore, S., Turnpenny, P., Lloyd, D., & Dean, J. (2005). Characteristics of fetal anticonvulsant syndrome associated autistic disorder. *Developmental Medicine & Child Neurology, 47*(08), 551-555.

Ricci, S., Businaro, R., Ippoliti, F., Vasco, V. L., Massoni, F., Onofri, E., . . . Ricciardi, M. R. (2013). Altered cytokine and BDNF levels in autism spectrum disorder. *Neurotoxicity research, 24*(4), 491-501.

Rodier, P. M., & Hyman, S. L. (1998). Early environmental factors in autism. *Mental Retardation and Developmental Disabilities Research Reviews, 4*(2), 121-128.

Rodier, P. M., Ingram, J. L., Tisdale, B., & Croog, V. J. (1997). Linking etiologies in humans and animal models: studies of autism. *Reproductive Toxicology, 11*(2), 417-422.

Rogers, S. J., Hepburn, S., & Wehner, E. (2003). Parent reports of sensory symptoms in toddlers with autism and those with other developmental disorders. *Journal of autism and developmental disorders, 33*(6), 631-642.

Ronald, A., Happe, F., Bolton, P., Butcher, L. M., Price, T. S., Wheelwright, S., . . . Plomin, R. (2006). Genetic heterogeneity between the three components of the autism spectrum: a twin study. *Journal of the American Academy of Child & Adolescent Psychiatry, 45*(6), 691-699.

Ronald, A., Happe, F., Price, T. S., Baron-Cohen, S., & Plomin, R. (2006). Phenotypic and genetic overlap between autistic traits at the extremes of the general population. *Journal of the American Academy of Child & Adolescent Psychiatry, 45*(10), 1206-1214.

Ronald, A., & Hoekstra, R. A. (2011). Autism spectrum disorders and autistic traits: a decade of new twin studies. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics, 156*(3), 255-274.

Roullet, F., Wollaston, L., Decatanzaro, D., & Foster, J. (2010). Behavioral and molecular changes in the mouse in response to prenatal exposure to the anti-epileptic drug valproic acid. *Neuroscience, 170*(2), 514-522.

Roullet, F. I., Lai, J. K., & Foster, J. A. (2013). In utero exposure to valproic acid and autism–a current review of clinical and animal studies. *Neurotoxicology and Teratology*.

Ryan, B. C., Young, N. B., Crawley, J. N., Bodfish, J. W., & Moy, S. S. (2010). Social deficits, stereotypy and early emergence of repetitive behavior in the C58/J inbred mouse strain. *Behavioural Brain Research, 208*(1), 178-188.

Sabers, A., Bertelsen, F. C., Scheel-Krüger, J., Nyengaard, J. R., & Møller, A. (2015). Corrigendum to "Long-term valproic acid exposure increases the number of neocortical neurons in the developing rat brain"[Neurosci. Lett. 580 (2014) 12–16]: A possible new animal model of autism. *Neuroscience letters*.

Sandin, S., Lichtenstein, P., Kuja-Halkola, R., Larsson, H., Hultman, C. M., & Reichenberg, A. (2014). The familial risk of autism. *Jama, 311*(17), 1770-1777.

Sara, S. J., Dyon-Laurent, C., & Hervé, A. (1995). Novelty seeking behavior in the rat is dependent upon the integrity of the noradrenergic system. *Cognitive Brain Research, 2*(3), 181-187. doi:http://dx.doi.org/10.1016/0926-6410(95)90007-1

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

Schauder, K. B., Muller, C. L., Veenstra-VanderWeele, J., & Cascio, C. J. (2015). Genetic variation in serotonin transporter modulates tactile hyperresponsiveness in ASD. *Research in autism spectrum disorders, 10*, 93-100.

Schletter, J., Heine, H., Ulmer, A. J., & Rietschel, E. T. (1995). Molecular mechanisms of endotoxin activity. *Archives of microbiology, 164*(6), 383-389.
Schneider, T., & Przewlocki, R. (2005). Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. *Neuropsychopharmacology, 30*(1), 80-89.
Schneider, T., Roman, A., Basta-Kaim, A., Kubera, M., Budziszewska, B., Schneider, K., & Przewłocki, R. (2008). Gender-specific behavioral and immunological alterations in an animal model of autism induced by prenatal exposure to valproic acid. *Psychoneuroendocrinology, 33*(6), 728-740.
Schumacher, J., Jamra, R. A., Becker, T., Ohlraun, S., Klopp, N., Binder, E. B., . . . Höfels, S. (2005). Evidence for a relationship between genetic variants at the brain-derived neurotrophic factor (BDNF) locus and major depression. *Biological psychiatry, 58*(4), 307-314.
Schwartzer, J., Careaga, M., Onore, C., Rushakoff, J., Berman, R., & Ashwood, P. (2013). Maternal immune activation and strain specific interactions in the development of autism-like behaviors in mice. *Translational psychiatry, 3*(3), e240.
Segurado, R., Conroy, J., Meally, E., Fitzgerald, M., Gill, M., & Gallagher, L. (2005). Confirmation of association between autism and the mitochondrial aspartate/glutamate carrier SLC25A12 gene on chromosome 2q31. *American Journal of Psychiatry, 162*(11), 2182-2184.
Seok, J., Warren, H. S., Cuenca, A. G., Mindrinos, M. N., Baker, H. V., Xu, W., . . . Hennessy, L. (2013). Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proceedings of the National Academy of Sciences, 110*(9), 3507-3512.
Shair, H. N., Rupert, D. D., Rosko, L. M., Hofer, M. A., Myers, M. M., & Welch, M. G. (2015). Effects of maternal deprivation and the duration of reunion time on rat pup ultrasonic vocalization responses to isolation: Possible implications for human infant studies. *Developmental psychobiology, 57*(1), 63-72.
Shi, L., Fatemi, S. H., Sidwell, R. W., & Patterson, P. H. (2003). Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. *The Journal of Neuroscience, 23*(1), 297-302.
Shi, L., Smith, S. E., Malkova, N., Tse, D., Su, Y., & Patterson, P. H. (2009). Activation of the maternal immune system alters cerebellar development in the offspring. *Brain, behavior, and immunity, 23*(1), 116-123.
Shiah, I.-S., Yatham, L. N., Yeh, C.-B., & Ravindran, A. V. (2005). Effect of valproate on plasma levels of interleukin-6 in healthy male humans. *International clinical psychopharmacology, 20*(6), 295-298.
Short, S. J., Lubach, G. R., Karasin, A. I., Olsen, C. W., Styner, M., Knickmeyer, R. C., . . . Coe, C. L. (2010). Maternal influenza infection during pregnancy impacts postnatal brain development in the rhesus monkey. *Biological psychiatry, 67*(10), 965-973.
Sies, H. (1997). Oxidative stress: oxidants and antioxidants. *Experimental physiology, 82*(2), 291-295.
Silverman, J. L., Yang, M., Lord, C., & Crawley, J. N. (2010). Behavioural phenotyping assays for mouse models of autism. *Nature Reviews Neuroscience, 11*(7), 490-502. doi:http://dx.doi.org/10.1038/nrn2851
Simonoff, E., Pickles, A., Charman, T., Chandler, S., Loucas, T., & Baird, G. (2008). Psychiatric disorders in children with autism spectrum disorders: prevalence,

comorbidity, and associated factors in a population-derived sample. *Journal of the American Academy of Child & Adolescent Psychiatry, 47*(8), 921-929.
Sinn, D.-I., Kim, S.-J., Chu, K., Jung, K.-H., Lee, S.-T., Song, E.-C., . . . Roh, J.-K. (2007). Valproic acid-mediated neuroprotection in intracerebral hemorrhage via histone deacetylase inhibition and transcriptional activation. *Neurobiology of Disease, 26*(2), 464-472. doi:http://dx.doi.org/10.1016/j.nbd.2007.02.006
Smith, C. J., Wilkins, K. B., Mogavero, J. N., & Veenema, A. H. (2015). Social Novelty Investigation in the Juvenile Rat: Modulation by the μ-Opioid System. *Journal of neuroendocrinology, 27*(10), 752-764.
Smith, S., Li, J., Garbett, K., Mirnics, K., & Patterson, P. H. (2007). Maternal immune activation alters fetal brain development through interleukin-6. *The Journal of Neuroscience, 27*(40), 10695-10702.
Smith, V., & Brown, N. (2014). Prenatal valproate exposure and risk of autism spectrum disorders and childhood autism. *Archives of disease in childhood-Education & practice edition*, edpract-2013-305636.
Smits, B. M., Mudde, J. B., van de Belt, J., Verheul, M., Olivier, J., Homberg, J., . . . Plasterk, R. H. (2006). Generation of gene knockouts and mutant models in the laboratory rat by ENU-driven target-selected mutagenesis. *Pharmacogenetics and genomics, 16*(3), 159-169.
Smotherman, W. P., Bell, R. W., Starzec, J., Elias, J., & Zachman, T. A. (1974). Maternal responses to infant vocalizations and olfactory cues in rats and mice. *Behavioral biology, 12*(1), 55-66.
Sperner-Unterweger, B. (2005). Immunological aetiology of major psychiatric disorders. *Drugs, 65*(11), 1493-1520.
Steckler, T., Spooren, W., & Murphy, D. (2014). Autism spectrum disorders-an emerging area in psychopharmacology. *Psychopharmacology, 231*(6), 977-978.
Štefánik, P., Olexová, L., & Kršková, L. (2015). Increased sociability and gene expression of oxytocin and its receptor in the brains of rats affected prenatally by valproic acid. *Pharmacology Biochemistry and Behavior, 131*, 42-50.
Steffenburg, S., Gillberg, C., Hellgren, L., Andersson, L., Gillberg, I. C., Jakobsson, G., & Bohman, M. (1989). A twin study of autism in Denmark, Finland, Iceland, Norway and Sweden. *Journal of Child Psychology and Psychiatry, 30*(3), 405-416.
Steinborn, B., Żarowski, M., Winczewska-Wiktor, A., Wójcicka, M., Młodzikowska-Albrecht, J., & Losy, J. (2014). Concentration of Il-1β, Il-2, Il-6, TNFα in the blood serum in children with generalized epilepsy treated by valproate. *Pharmacological Reports, 66*(6), 972-975.
Stigger, F., Lovatel, G., Marques, M., Bertoldi, K., Moysés, F., Elsner, V., . . . Marcuzzo, S. (2013). Inflammatory response and oxidative stress in developing rat brain and its consequences on motor behavior following maternal administration of LPS and perinatal anoxia. *International Journal of Developmental Neuroscience, 31*(8), 820-827. doi:http://dx.doi.org/10.1016/j.ijdevneu.2013.10.003
Stuart, M., & McGrew, J. H. (2009). Caregiver burden after receiving a diagnosis of an autism spectrum disorder. *Research in autism spectrum disorders, 3*(1), 86-97.
Südhof, T. C. (2008). Neuroligins and neurexins link synaptic function to cognitive disease. *Nature, 455*(7215), 903-911.
Sykes, N. H., & Lamb, J. A. (2007). Autism: the quest for the genes. *Expert Rev Mol Med, 9*(24), 1-15.
Takuma, K., Hara, Y., Kataoka, S., Kawanai, T., Maeda, Y., Watanabe, R., . . . Ago, Y. (2014). Chronic treatment with valproic acid or sodium butyrate attenuates novel

object recognition deficits and hippocampal dendritic spine loss in a mouse model of autism. *Pharmacology Biochemistry and Behavior, 126*, 43-49.
Taurines, R., Segura, M., Schecklmann, M., Albantakis, L., Grünblatt, E., Walitza, S., . . . Theisen, F. M. (2014). Altered peripheral BDNF mRNA expression and BDNF protein concentrations in blood of children and adolescents with autism spectrum disorder. *Journal of Neural Transmission, 121*(9), 1117-1128.
Thiel, K. J., Okun, A. C., & Neisewander, J. L. (2008). Social reward-conditioned place preference: a model revealing an interaction between cocaine and social context rewards in rats. *Drug and alcohol dependence, 96*(3), 202-212.
Timmusk, T., Palm, K., Metsis, M., Reintam, T., Paalme, V., Saarma, M., & Persson, H. (1993). Multiple promoters direct tissue-specific expression of the rat BDNF gene. *Neuron, 10*(3), 475-489.
Tordjman, S., Gutknecht, L., Carlier, M., Spitz, E., Antoine, C., Slama, F., . . . Roubertoux, P. (2001). Role of the serotonin transporter gene in the behavioral expression of autism. *Molecular psychiatry, 6*(4), 434-439.
Tordjman, S., Somogyi, E., Coulon, N., Kermarrec, S., Cohen, D., Bronsard, G., . . . Lauth, B. (2014). Gene× environment interactions in autism spectrum disorders: role of epigenetic mechanisms. *Frontiers in psychiatry, 5*.
Trezza, V., Damsteegt, R., & Vanderschuren, L. J. (2009). Conditioned place preference induced by social play behavior: parametrics, extinction, reinstatement and disruption by methylphenidate. *European Neuropsychopharmacology, 19*(9), 659-669.
Trikalinos, T. A., Ntzani, E. E., Contopoulos-Ioannidis, D. G., & Ioannidis, J. P. (2004). Establishment of genetic associations for complex diseases is independent of early study findings. *European Journal of Human Genetics, 12*(9), 762-769.
Trinka, E., Höfler, J., Zerbs, A., & Brigo, F. (2014). Efficacy and safety of intravenous valproate for status epilepticus: a systematic review. *CNS drugs, 28*(7), 623-639.
Tsai, S.-J. (2005). Is autism caused by early hyperactivity of brain-derived neurotrophic factor? *Medical hypotheses, 65*(1), 79-82.
Tuchman, R., & Rapin, I. (2002). Epilepsy in autism. *The Lancet Neurology, 1*(6), 352-358.
Tung, E. W., & Winn, L. M. (2010). Epigenetic modifications in valproic acid-induced teratogenesis. *Toxicology and applied pharmacology, 248*(3), 201-209.
Tung, E. W., & Winn, L. M. (2011). Valproic acid increases formation of reactive oxygen species and induces apoptosis in postimplantation embryos: a role for oxidative stress in valproic acid-induced neural tube defects. *Molecular pharmacology, 80*(6), 979-987.
Ulich, T. R., Guo, K., Remick, D., Del Castillo, J., & Yin, S. (1991). Endotoxin-induced cytokine gene expression in vivo. III. IL-6 mRNA and serum protein expression and the in vivo hematologic effects of IL-6. *The Journal of Immunology, 146*(7), 2316-2323.
Van den Berg, C. L., Pijlman, F. T., Koning, H. A., Diergaarde, L., Van Ree, J. M., & Spruijt, B. M. (1999). Isolation changes the incentive value of sucrose and social behaviour in juvenile and adult rats. *Behavioural brain research, 106*(1), 133-142.
van der Poll, T., Keogh, C. V., Guirao, X., Buurman, W. A., Kopf, M., & Lowry, S. F. (1997). Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia. *Journal of Infectious Diseases, 176*(2), 439-444.
van der Staay, F. J., Arndt, S. S., & Nordquist, R. E. (2009). Evaluation of animal models of neurobehavioral disorders. *Behavioral and Brain Functions, 5*(1), 11.

van Ijzendoorn, M., Belsky, J., & Bakermans-Kranenburg, M. (2012). Serotonin transporter genotype 5HTTLPR as a marker of differential susceptibility? A meta-analysis of child and adolescent gene-by-environment studies. *Translational psychiatry, 2*(8), e147.

Vargas, D. L., Nascimbene, C., Krishnan, C., Zimmerman, A. W., & Pardo, C. A. (2005). Neuroglial activation and neuroinflammation in the brain of patients with autism. *Annals of neurology, 57*(1), 67-81.

Verrotti, A., Basciani, F., Trotta, D., Greco, R., Morgese, G., & Chiarelli, F. (2001). Effect of anticonvulsant drugs on interleukins-1,-2 and-6 and monocyte chemoattractant protein-1. *Clinical and Experimental Medicine, 1*(3), 133-136.

Vicario, A., Colliva, A., Ratti, A., Davidovic, L., Baj, G., Gricman, Ł., . . . Bardoni, B. (2015). Dendritic targeting of short and long 3′ UTR BDNF mRNA is regulated by BDNF or NT-3 and distinct sets of RNA-binding proteins. *Frontiers in molecular neuroscience, 8*.

Viinikainen, K., Eriksson, K., Mönkkönen, A., Äikiä, M., Nieminen, P., Heinonen, S., & Kälviäinen, R. (2006). The effects of valproate exposure in utero on behavior and the need for educational support in school-aged children. *Epilepsy & Behavior, 9*(4), 636-640.

Vorstman, J. A., Spooren, W., Persico, A. M., Collier, D. A., Aigner, S., Jagasia, R., . . . Buitelaar, J. K. (2014). Using genetic findings in autism for the development of new pharmaceutical compounds. *Psychopharmacology, 231*(6), 1063-1078.

Wang, C., Luan, Z., Yang, Y., Wang, Z., Cui, Y., & Gu, G. (2011). Valproic acid induces apoptosis in differentiating hippocampal neurons by the release of tumor necrosis factor-α from activated astrocytes. *Neuroscience letters, 497*(2), 122-127.

Watson, K. K., Ghodasra, J. H., & Platt, M. L. (2009). Serotonin transporter genotype modulates social reward and punishment in rhesus macaques. *PloS one, 4*(1), e4156.

Wei, H., Alberts, I., & Li, X. (2013). Brain IL-6 and autism. *Neuroscience, 252*, 320-325.

Wei, H., Chadman, K. K., McCloskey, D. P., Sheikh, A. M., Malik, M., Brown, W. T., & Li, X. (2012). Brain IL-6 elevation causes neuronal circuitry imbalances and mediates autism-like behaviors. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1822*(6), 831-842.

Wei, H., Zou, H., Sheikh, A. M., Malik, M., Dobkin, C., Brown, W. T., & Li, X. (2011). IL-6 is increased in the cerebellum of autistic brain and alters neural cell adhesion, migration and synaptic formation. *J Neuroinflammation, 8*(52.10), 1186.

Weintraub, K. (2011). Autism counts. *Nature, 479*(7371), 22-24.

Weller, A., Leguisamo, A. C., Towns, L., Ramboz, S., Bagiella, E., Hofer, M., . . . Brunner, D. (2003). Maternal effects in infant and adult phenotypes of 5HT1A and 5HT1B receptor knockout mice. *Developmental psychobiology, 42*(2), 194-205.

Wells, P. G., Kim, P. M., Laposa, R. R., Nicol, C. J., Parmana, T., & Winn, L. M. (1997). Oxidative damage in chemical teratogenesis. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 396*(1), 65-78.

Wells, P. G., Lee, C. J., McCallum, G. P., Perstin, J., & Harper, P. A. (2010). Receptor- and reactive intermediate-mediated mechanisms of teratogenesis *Adverse Drug Reactions* (pp. 131-162): Springer.

Wells, P. G., McCallum, G. P., Chen, C. S., Henderson, J. T., Lee, C. J., Perstin, J., . . . Wong, A. W. (2009). Oxidative stress in developmental origins of disease: teratogenesis, neurodevelopmental deficits, and cancer. *Toxicological sciences, 108*(1), 4-18.

Williams, P. G., & Hersh, J. H. (1997). A male with fetal valproate syndrome and autism. *Developmental Medicine & Child Neurology, 39*(9), 632-634.
Willner, P. (1984). The validity of animal models of depression. *Psychopharmacology, 83*(1), 1-16.
Wingate, M., Kirby, R. S., Pettygrove, S., Cunniff, C., Schulz, E., Ghosh, T., . . . Constantino, J. (2014). Prevalence of autism spectrum disorder among children aged 8 years-autism and developmental disabilities monitoring network, 11 sites, United States, 2010. *MMWR Surveillance Summaries, 63*(2).
Winn, L. M. (2003). Homologous recombination initiated by benzene metabolites: a potential role of oxidative stress. *Toxicological sciences, 72*(1), 143-149.
Winn, L. M., & Wells, P. G. (1997). Evidence for embryonic prostaglandin H synthase-catalyzed bioactivation and reactive oxygen species-mediated oxidation of cellular macromolecules in phenytoin and benzo [a] pyrene teratogenesis. *Free Radical Biology and Medicine, 22*(4), 607-621.
Winn, L. M., & Wells, P. G. (1999). Maternal administration of superoxide dismutase and catalase in phenytoin teratogenicity 1. *Free Radical Biology and Medicine, 26*(3), 266-274.
Winslow, J. T., & Insel, T. R. (1990). Serotonergic and catecholaminergic reuptake inhibitors have opposite effects on the ultrasonic isolation calls of rat pups. *Neuropsychopharmacology*.
Wischhof, L., Irrsack, E., Osorio, C., & Koch, M. (2015). Prenatal LPS-exposure–a neurodevelopmental rat model of schizophrenia–differentially affects cognitive functions, myelination and parvalbumin expression in male and female offspring. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 57*, 17-30.
Wöhr, M., Roullet, F. I., & Crawley, J. N. (2011). Reduced scent marking and ultrasonic vocalizations in the BTBR T+ tf/J mouse model of autism. *Genes, Brain and behavior, 10*(1), 35-43.
Wöhr, M., & Scattoni, M. L. (2013). Models of Autism Spectrum Disorders: Current Standards and New Developments. *Behavioural brain research*.
Wöhr, M., & Schwarting, R. K. (2007). Ultrasonic communication in rats: can playback of 50-kHz calls induce approach behavior. *PloS one, 2*(12), e1365.
Wöhr, M., van Gaalen, M. M., & Schwarting, R. K. (2015). Affective communication in rodents: serotonin and its modulating role in ultrasonic vocalizations. *Behavioural pharmacology, 26*(6), 506-521.
Wu, E. Q., Birnbaum, H. G., Shi, L., Ball, D. E., Kessler, R. C., Moulis, M., & Aggarwal, J. (2005). The economic burden of schizophrenia in the United States in 2002. *Journal of Clinical Psychiatry*.
Wyszynski, D., Nambisan, M., Surve, T., Alsdorf, R., Smith, C., & Holmes, L. (2005). Increased rate of major malformations in offspring exposed to valproate during pregnancy. *Neurology, 64*(6), 961-965.
Xuan, I. C., & Hampson, D. R. (2014). Gender-dependent effects of maternal immune activation on the behavior of mouse offspring.
Yan, J., Noltner, K., Feng, J., Li, W., Schroer, R., Skinner, C., . . . Sommer, S. S. (2008). Neurexin 1α structural variants associated with autism. *Neuroscience letters, 438*(3), 368-370.
Yang, C.-J., Liu, C.-L., Sang, B., Zhu, X.-M., & Du, Y.-J. (2015). The combined role of serotonin and interleukin-6 as biomarker for autism. *Neuroscience, 284*, 290-296.
Yasuda, S., Liang, M., Marinova, Z., Yahyavi, A., & Chuang, D. (2009). The mood stabilizers lithium and valproate selectively activate the promoter IV of brain-derived neurotrophic factor in neurons. *Molecular psychiatry, 14*(1), 51-59.

Yirmiya, N., Pilowsky, T., Nemanov, L., Arbelle, S., Feinsilver, T., Fried, I., & Ebstein, R. P. (2001). Evidence for an association with the serotonin transporter promoter region polymorphism and autism. *American journal of medical genetics, 105*(4), 381-386.

Zaken, V., Kohen, R., & Ornoy, A. (2000). The development of antioxidant defense mechanism in young rat embryos in vivo and in vitro. *Early pregnancy (Online), 4*(2), 110-123.

Zanettini, C., Carola, V., Lo Iacono, L., Moles, A., Gross, C., & d'Amato, F. (2010). Postnatal handling reverses social anxiety in serotonin receptor 1A knockout mice. *Genes, Brain and Behavior, 9*(1), 26-32.

Zhang, B., Wang, X., & Nazarali, A. (2010). Ascorbic acid reverses valproic acid-induced inhibition of hoxa2 and maintains glutathione homeostasis in mouse embryos in culture. *Cellular and molecular neurobiology, 30*(1), 137-148.

Zhang, Q.-b., Jiang, L.-f., & Lu, Y.-J. (2014). Serum brain-derived neurotrophic factor levels in Chinese children with autism spectrum disorders: a pilot study. *International Journal of Developmental Neuroscience, 37*, 65-68.

Zhong, N., Ye, L., Ju, W., Tsiouris, J., Cohen, I., & Brown, W. T. (1999). 5-HTTLPR variants not associated with autistic spectrum disorders. *Neurogenetics, 2*(2), 129-131.

Zhou, F. C., Sari, Y., & Zhang, J. K. (2000). Expression of serotonin transporter protein in developing rat brain. *Developmental Brain Research, 119*(1), 33-45.

Zhuang, X., Gross, C., Santarelli, L., Compan, V., Trillat, A.-C., & Hen, R. (1999). Altered emotional states in knockout mice lacking 5-HT1A or 5-HT1B receptors. *Neuropsychopharmacology, 21*, 52S-60S.

Zimmerberg, B., & Germeyan, S. C. (2015). Effects of neonatal fluoxetine exposure on behavior across development in rats selectively bred for an infantile affective trait. *Developmental psychobiology, 57*(2), 141-152.

Zoghbi, H. Y., & Bear, M. F. (2012). Synaptic dysfunction in neurodevelopmental disorders associated with autism and intellectual disabilities. *Cold Spring Harbor perspectives in biology, 4*(3), a009886.

Zöllner, S., & Pritchard, J. K. (2007). Overcoming the winner's curse: estimating penetrance parameters from case-control data. *The American Journal of Human Genetics, 80*(4), 605-615.

Zuccato, C., & Cattaneo, E. (2009). Brain-derived neurotrophic factor in neurodegenerative diseases. *Nature Reviews Neurology, 5*(6), 311-322.

Zuckerman, L., Rehavi, M., Nachman, R., & Weiner, I. (2003). Immune activation during pregnancy in rats leads to a postpubertal emergence of disrupted latent inhibition, dopaminergic hyperfunction, and altered limbic morphology in the offspring: a novel neurodevelopmental model of schizophrenia. *Neuropsychopharmacology*.

Zuckerman, L., & Weiner, I. (2005). Maternal immune activation leads to behavioral and pharmacological changes in the adult offspring. *Journal of psychiatric research, 39*(3), 311-323.