The Effect of Prenatal Valproate Exposure in Serotonin Transporter Knockout Rats On Anxiety and Cognition: A Gene*Environment Interaction Model of Autism Spectrum Disorder

By

Caren L. August

A thesis

submitted to the Victoria University of Wellington

in fulfilment of the requirements for the degree of

Master of Science

Victoria University of Wellington

2015

Abstract

Autism Spectrum Disorder is a complex neurodevelopmental disorder which is often associated with increased anxiety and deficits in cognitive ability. The present research investigated a potential gene*environment interaction between two factors previously implicated in ASD in a rat model; prenatal exposure to valproate (VPA) and genetic reduction of the serotonin transporter (SERT). Wildtype and heterozygous SERT knockout rats prenatally exposed to VPA or saline on gestational day12.5 (G12.5) were assessed on measures of anxiety: elevated plus-maze and novelty suppressed-feeding and cognitive ability: prepulse inhibition and latent inhibition. A significant main effect was found for VPA exposure in all paradigms, showing increased anxiety-typical behaviour and abnormal cognitive ability. However, no significant effect of genotype or interaction was observed. Results from the present study do not confirm gene*environment interaction between prenatal VPA and heterozygous SERT knockout but this may be due to several factors that are discussed within the thesis. In any case, this study represents a starting point for further studies investigating other combinations of genetic and environmental factors as models of ASD pathogenesis.

Keywords: Valproate, Serotonin Transporter knockout, Autism Spectrum Disorder, Gene*Environment.

The Effect of Prenatal Valproate Exposure in Serotonin Transporter Knockout Rats On Anxiety and Cognition: A Gene*Environment Interaction Model of Autism Spectrum Disorder

Autism Spectrum Disorder (ASD) is a complex neurodevelopmental disorder arising from the interaction of an as yet unknown number of genetic and environmental components (Miyazaki, Narita, & Narita, 2005; Persico & Bourgeron, 2006; Volkmar & Pauls, 2003). It is characterized in the Diagnostic and Statistical Manual of Mental Disorders, 5th edition, by a dyad of impairment: social interaction/communication and repetitive/repetitive behaviour (American Psychiatric Asociation, 2013), with cognitive rigidity and increased anxiety also often present. A recent survey found rates of ASD to have now reached one in 91 children being affected (Kogan et al., 2009) with the prevalence being four times higher in males (Fombonne, 2003). As yet, there is no 'cure', and current therapies and pharmacological treatments have only limited success. When factoring in increasing prevalence rates (Herbert, 2010a) and increasing health care costs (Leslie & Martin, 2007) of ASD it becomes even more apparent that investigating underlying causes is hugely important.

The effect of ASD on the family environment as well as for society in general is substantial (DiCicco-Bloom et al., 2006). Definitive financial impacts of ASD have proven difficult to ascertain, but it has been estimated on a broad level that health care costs for individuals with ASD exceeds $35 billion US dollars annually in America (Ganz, 2007). British estimates put the health costs of children with ASD at £2.7 billion pounds and adult health care exceeding £25 billion pounds per annum (Knapp, Romeo, & Beecham, 2009). High financial impacts have also been shown at the family level, with studies showing increased financial burden and time costs associated with rearing a child diagnosed with ASD (Jarbrink & Knapp, 2001). In addition to the substantial economic impact, there is a significant escalation in caregiver burden. In one study looking at family stress in recently diagnosed children with ASD it was shown that increased symptom severity lead to increased levels of individual and family burden (Stuart & McGrew, 2009). Taken together, these growing social and financial costs highlight the increasing importance of studying ASD.

Confounding the progress in etiological investigation and the efficacy of psychosocial rehabilitation is the high comorbidity burden within those diagnosed with ASD (Matson & Nebel-Schwalm, 2007). In a recent study, it was found that 70% of young people with ASD

also met the criteria for at least one comorbid disorder and over 40% had two or more (Simonoff et al., 2008). Of these, the most prevalent co-occurring disorders were social anxiety disorder (29.2%) and attentional deficit/hyperactivity disorder (28.2%) (Simonoff et al., 2008). Furthermore, in one of the largest exploratory retrospective prevalence studies to date, Kohane et al (2012) examined the recorded comorbidity rates within 14,000 individuals diagnosed with ASD. It was found that there were remarkably higher rates of cranial anomalies (12.45% cf. 1.19%), epilepsy (19.44% cf. 2.19%), Schizophrenia (2.43% cf. 0.24%) and bowl disorders (11.74% cf. 4.5%) in patients diagnosed with ASD compared to matched samples without ASD (Kohane et al., 2012).

The serotonin (5-HT) system has long been implicated in ASD because, along with its role as a neurotransmitter, 5-HT has a major role in brain development (Whitaker-Azmitia & Azmitia, 1986). Serotonin is crucial in temporally sensitive synaptogenesis, migration and apoptosis (programmed cell death) with research suggesting this system is significantly altered in ASD (Whitaker-Azmitia, 2001). Studies have reliably shown that the serotonergic system is altered in patients with ASD with asymmetrical synthesis of 5-HT observed in the frontal cortex, thalamus and dentate nucleus compared to healthy controls (Chugani et al., 1997). This is of particular note as these three areas are integral to verbal communication and sensory integration; both of which are domains altered in ASD. Hyperserotonemia (50% increase of 5-HT in blood cf. normal) is also common in ASD and is widely recognized as one of the first biomarkers of ASD identified (Veenstra-VanderWeele et al., 2012).Based on these findings, altered 5-HT homeostasis is often implicated as a potential mechanism of ASD (Veenstra-VanderWeele et al., 2012).

In further support of the involvement of 5-HT in ASD, is the early development and its widespread nature within the brain, and its interactions with other neurotransmitter and hormone pathways (Whitaker-Azmitia, 2005). Serotonin neurons project throughout the brain; innervated to such an extent that they play a role in the many key functions, both behavioural and physiological. This diverse area of effect, including a wide range of functions outside of the central nervous system, is proposed to explain at least part of the variability and pleiotropic nature of behaviours stemming from alterations to serotonergic development (Berger, Gray, & Roth, 2009). Furthermore, 5-HT is known to have direct effects on behaviour through' regulation of mood and basic functions, such as hunger and body temperature. Through impacts on hormone systems such as oxytocin, 5-HT is linked

with sexual behaviours, arousal and a host of other functions (Lam, Aman, & Arnold, 2006). Based on the countless brain and behaviour systems impacted by 5-HT, coupled with the broad pattern of impairment observed in ASD, alterations to the serotonergic system present a sound target for research into the pathogenesis of ASD.

Due to the critical role of 5-HT during brain development, any prolonged alterations to its availability early in life is likely to lead to disturbed neural development through interference with temporally and concentration sensitive pathways (Veenstra-VanderWeele et al., 2012). It has been found that exposure to prolonged higher, or lower, than normal levels of 5-HT during early development can increase risk of ASD and anxiety (Veenstra-VanderWeele et al., 2012; Yang, Tan, & Du, 2014). Extracellular levels of 5-HT are largely controlled by the 5-HT transporter (SERT), but it can also (to a much lesser extent) bind with some other neurotransmitter transporters, such as Norepinephrine (Daws, 2009). Serotonin transporter binding has been shown to be altered in specific brain regions in people with ASD, with indications this is underpinned by lower numbers of serotonergic nerve terminals (Makkonen, Riikonen, Kokki, Airaksinen, & Kuikka, 2008).

Using single-photon emission computed tomography; Makkonen et al (2008) found that in patients with ASD, SERT binding is decreased in the Medial Frontal Cortex (Makkonen et al., 2008). This finding is of particular interest as the Medial Frontal Cortex is known to have a role in recognizing and understanding the intentions of others; a trait noticeably impaired in ASD (Frith & Frith, 1999). This reduction in binding belies either a decrease in 5-HT synapses, reduced overall amounts of SERT proteins, or a combination thereof (Makkonen et al., 2008). In addition, SERT is the site of action of Selective Serotonin Reuptake Inhibitors which have been shown to lessen some behavioural symptoms of ASD, with studies showing that 5-HT depletion exacerbates symptoms (McDougle et al., 1996). Thus, disruptions to the 5-HT system have been strongly implicated in the pathology of ASD with specific focus on the SERT.

The focus to-date on the serotonin system has stimulated much research into proposed mechanisms underlying the HSN coupled with low brain 5-HT often present in instances of ASD (McNamara, Borella, Bialowas, & Whitaker-Azmitia, 2008). The serotonergic system possesses the ability to autoregulate during development; a trait which is often implicated as a mechanism of ASD. During neural development high levels of serotonin elicits negative

feedback causing a decline in the number of serotonin terminals and therefore SERT present later in life (Whitaker-Azmitia & Azmitia, 1986). Evidence for autoregulation causing long-term changes is accumulating; experiments have demonstrated that rats exposed to serotonin agonists *in utero* (Whitaker-Azmitia, 2001), as well as ASD children with HSN (Croen, Grether, Yoshida, Odouli, & Hendrick, 2011), develop lower numbers of serotonin neurons. Much research has implicated the permeability of the blood-brain barrier of the developing foetus, ambient prenatal serotonin levels and the ability of 5-HT to autoregulate as contributing factors for ASD (Kahne et al., 2002; McNamara et al., 2008; Whitaker-Azmitia, 2005).

Early on in pregnancy the blood-brain barrier of the foetus is still permeable to 5-HT, such that 5-HT present in the blood can enter into the brain which is not possible later on in development (Hadjikhani, 2010; Whitaker-Azmitia, 2005). During the earliest stages, placental serotonin provided by the mother has a robust impact on brain development. Bonnin et al (2011) were able to show that 5-HT synthesized by the placenta accounts for the level of 5-HT present in the developing forebrain during early embryogenesis before the endogenous serotonergic system innervates; thus, suggesting a viable pathway for prenatal exposure to teratogens to cause systemic alterations (Bonnin et al., 2011; Veenstra-VanderWeele & Blakely, 2012). This dependence shifts later in development to serotonin produced by the foetus (Bonnin & Levitt, 2011). In this manner, impacts on environmental 5-HT from the mother and endogenous 5-HT from the developing brain can influence pathophysiology. It is thus suggested that higher than normal levels of blood 5-HT during this critical period in pregnant mothers can cause the loss of 5-HT terminals in the foetus through autoregulation and negative feedback, thereby playing a significant role in the disruption of normal serotonergic growth (Kahne et al., 2002; Whitaker-Azmitia, 2005).

Prenatal exposure to drugs which increase levels of 5-HT in blood, such as cocaine (Davis et al., 1992), alcohol (Aronson, Hagberg, & Gillberg, 1997), SSRIs (Croen et al., 2011), and VPA (Kinast, Peeters, Kolk, Schubert, & Homberg, 2013), is associated with significantly increased risk of ASD; contributing to Developmental Hyperserotonemia (DHS) as a proposed mechanism of ASD. Likewise, genetic alterations in SERT are likely to lead to increased levels of 5-HT. The corresponding asymmetric decrease of serotonergic neurons throughout parts of the brain is suggested as an explanation for the wide variety of behavioural and cognitive aberrations seen in ASD and is known as the DHS model of ASD

(Hadjikhani, 2010; McNamara et al., 2008).The DHS model accounts for the intuitively dissonant characteristics commonly associated with ASD; high blood serotonin coupled with decreased serotonin in specific brain regions (McNamara et al., 2008).

One way in which Developmental Hyperserotonemia has been posited to detrimentally affect emotional regulation and social behaviour is through its implication in the disruption of normal functioning in the hypothalamo-pituatary axis, amygdala and oxytocin regulation (Whitaker-Azmitia, 2005). Oxytocin is a pro-social hormone which is involved in social recognition and alongside 5-HT is often associated with ASD (Whitaker-Azmitia, 2005). Developmental Hyperserotonemia has been shown as a causative factors in decreasing levels of oxytocin present in the paraventricular nucleus of the hypothalamus as well as an increased concentration of calcitonin gene-related peptide in the amygdala, specifically the central nucleus; both of which are linked with social interactions and shown to be disturbed in ASD (Yang et al., 2014).

Of all neuropsychiatric disorders, ASD is known to have the strongest genetic component (Levy, Mandell, & Schultz, 2009) with a 92% concordance rate between monozygotic twins and 10% between dizygotic twins for a broad spectrum of ASD phenotypes (Le Couteur et al., 1996). However, because the concordance rate is less than 100%, it suggests that environmental factors play a role in the development of ASD and may alter penetrance of phenotypic expression (Bill & Geschwind, 2009). As it stands, less than 10% of cases can be directly attributed to a specific cause, genetic or medical (Fombonne, 1999).

The hunt for underlying contributing factors has led to multiple types of genetic variation being investigated for their possible role in ASD development; variable number tandem repeats (VNTRs), single nucleotide polymorphisms (SNPs), as well as haplotypes which are sets of genes often inherited together (Levy et al., 2009). Thus far, very few definitive genes of high impact have been identified. More often, genes are implicated as contributing risk factors of only moderate effect. One estimate has placed the number of genes which are in involved in the development of ASD between 5 and 20 (Stokstad, 2001). Thus, many researchers have turned towards a model of genetic complexity with gene-gene, gene networks and gene-environment models now under investigations. Furthermore, many genes with systemic effects are now being investigated (Herbert et al., 2006).

Numerous diverse regions of the genome, specific gene variants and protein processes are currently under investigation to assess their viability as candidates contributing to ASD (Persico & Napolioni, 2013). Genome-Wide Linkage studies (GWLS) identify areas of genetic risk by comparing gene sequences of people with ASD and their family members without ASD. Genome-Wide Association studies (GWAS) compare genetic regions and variants across a single population and are proposed to have a higher efficacy when compared to similar sized linkage studies (Yang & Gill, 2007). In a literature review of ASD risk regions identified by GWLS 16 different chromosomal regions were specified as harbouring susceptibility genes by at least three separate studies. A total of 18 loci spanning 14 chromosomal regions were identified by a GWAS, and of these seven regions converged with those highlighted by GWLS.

Special emphasis has been put on the investigation into common polymorphisms identified as susceptibility genes which are known to effect transmission pathways and cortical development, as they present a potential neurodevelopmental pathway to ASD (Persico & Napolioni, 2013). So far, genes implicated in ASD pathogenesis are involved in a varied array of systems; including hormonal pathways, neurotransmitter regulation and cell adhesion molecules (Persico & Napolioni, 2013). *OXTR* which encodes the receptor for the hormone Oxytocin has also been consistently replicated in previous GWAS as containing common polymorphisms associated with ASD. Cell adhesion proteins have been targeted in ASD investigation, for example *ITGB3,* which encodes Integrin beta 3.Another example, the gene *RELN* responsible for encoding Reelin; a secreted extracellular matrix protein important for neuronal migration as part of brain development, has been investigated and has been found to harbour common polymorphisms linked with susceptibility to ASD (Persico & Napolioni, 2013). However, gene expression studies and candidate gene studies have also highlighted the genomic region containing *SLC6A4*, which encodes SERT, as a particularly likely susceptibility region and worthy of further investigation (Yang & Gill, 2007).

The serotonin system, in particular SERT, represents a point where findings from genetic research, neurochemistry and pathology intersect (Veenstra-VanderWeele et al., 2012; Yang & Gill, 2007) and therefore is an ideal candidate for further investigation if we are to understand the etiology of ASD. Much of the research into the physiological underpinnings of ASD has focused on functional changes in the 5-HT pathway, with the strong evidence pointing towards differences in the SERT in specific (Levy et al.,

2009).Given the above mentioned crucial role of 5-HT and the SERT, many researchers have focused on the SERT gene, *SLC6A4* genomic location in cytogenetic band 17q11.2, which is associated with ASD and extracellular concentrations of 5-HT in the brain and the blood (Veenstra-VanderWeele et al., 2012). Commonplace polymorphisms located in the upstream promoter region of this gene (*5-HTTLPR*); caused by a 44-base pair insertion/deletion, alter the overall number of SERT proteins by moderating transcription efficiency (Holmes, Murphy, & Crawley, 2003). The shorter variant of this polymorphism, the "s" allele, demonstrably leads to a reduction of the SERT protein and has been recognized as a risk factor for developing depression, anxiety and ASD (Olivier et al., 2008; Veenstra-VanderWeele et al., 2012). There are at least nine other known SNPs located on *SLC6A4*, along with the *5-HTTLPR* (Ramoz et al., 2006). However, because SERT polymorphisms are only a risk-factor, it suggests that environmental factors play a role in ASD development (Nijmeijer et al., 2010).

Environmental factors are now being accredited as at least partially responsible for the increasing prevalence of ASD (Herbert, 2010b). A wealth of research is now implicating not only gene-gene, but also gene-environment interactions and environmentally vulnerable genes in the development of ASD (Herbert, 2010a). One of the most interesting environmental risk factors is Valproic Acid (VPA; also referred to as Valproate), an antiepileptic and mood stabilizing drug which has been repeatedly linked to increasing risk of developing ASD if prenatal exposure occurs (Rasalam et al., 2005). Prenatal exposure, especially in the first trimester of pregnancy during a critical period of organogenesis, increases risk of ASD (Williams et al., 2001) with symptom severity occurring in a dose dependent manner (Mawhinney et al., 2012). In one of the original investigations into the correlation between VPA and ASD, Moore et al (2000) found that 11% of children born from women taking VPA during pregnancy developed ASD, which is significantly higher than the rate in the general population (Moore et al., 2000). In line with this, high doses of prenatal VPA in rats creates gender specific changes, with males showing more signs of ASD (Schneider et al., 2008) such as increased repetitive behaviour (Markram, Rinaldi, La Mendola, Sandi, & Markram, 2008), increased anxiety and decreased exploration in novel environments (Schneider & Przewlocki, 2005), HSN, abnormal 5-HT neuronal placement and migration (Narita et al., 2002), and impaired cognitive function (Narita et al., 2010). However, because not all children prenatally exposed to VPA develop ASD, it suggests that it interacts with a pre-existing vulnerability.

Relatively large doses of VPA are required in order to produce therapeutic effect and it is known to exert effects on intracellular signaling pathways, extracellular neurotransmitters and epigenetic changes (Lagace, O'Brien, Gurvich, Nachtigal, & Klein, 2004). It has been shown that VPA moves into and out of the central nervous system via both the cerebrospinal fluid barrier and the blood-brain barrier. One of the mechanisms by which VPA causes long term changes and possibly alters development is by inhibiting histone deacetylases (HDACs) which are enzymes that suppress gene transcription long term (Grunstein, 1997). Therefore, VPA inhibits natural repression of gene expression which has long lasting behavioural effects (Kataoka et al., 2013).

In a mouse model investigation into the effects of VPA and Valpromide, an analog of VPA lacking HDAC inhibitory activity, it was found that behavioural changes in response to anxiety were only displayed by animals prenatally exposed to VPA (Kataoka et al., 2013). This provides compelling evidence that *in utero* VPA exposure leads to long lasting behavioural changes due to its' epigenetic effects. VPA inhibition of HDACs has been noted in both humans and rodents allowing for comparative research across species in this regard (Göttlicher, 2003; Kim et al., 2011). Interestingly, VPA has also been associated with changes in methylation states on genes, potentially leading to transcription of previously inactive genes (as cited in (Lagace et al., 2004). Furthermore, the glutaminergic modulation of excitatory neurotransmission is also affected by VPA along with increased in gamma-aminobutyric acid (GABA, an inhibitory neurotransmitter) (Lagace et al., 2004).The enkephalinergic system, an endogenous opioid peptide system involved in anxiety and emotional response, is also altered in the VPA rat model as shown by decreased proenkephalin mRNA expression in specific brain regions (Schneider, Ziolkowska, Gieryk, Tyminska, & Przewlocki, 2007). Taken together, these long last alterations in brain due to the varied effects of VPA can be associated with numerous behavioural changes noted in ASD.

Timing is a determining factor in the degree of penetrance of ASD-like behaviour caused by prenatal VPA exposure. Much research into VPA and development in ASD points to a vulnerable period during early development when the nervous system and developing foetus are most susceptible to environmental insults (Rice & Barone, 2000). By correlating ASD with comorbid conditions with known temporal origins of errors, systemic alterations and neural developmental changes, it was shown that ASD arises in a stage of early development called organogenesis (Ploeger, Raijmakers, van der Maas, & Galis, 2010).

Furthermore, Rasalam et al (2005) found that aberrations in the migration and 5-HT neuronal placement were more severe in rats if VPA was administered on day 11 of gestation (G11) as opposed to day 9. Gestational day 11 also happens to coincide with the time of neural tube closure in rat fetuses (Rasalam et al., 2005). Interestingly, 5-HT neurons are first detectable in rats around 12 days after conception (McNamara et al., 2008), which coincides perfectly with the time period for prenatal VPA exposure linked with the most severe expression of ASD-like behavioural and physiological changes. At the macro level, prenatal VPA exposure on G12.5, which is around the time when 5-HT neurons are first detectable, produces abnormalities in multiple brain regions such as the brain stem and cerebellar (Ingram, Peckham, Tisdale, & Rodier, 2000) which are in line with findings from brain imaging studies and autopsy results of humans with ASD (Bauman & Kemper, 2005).

There has been much research has been conducted to investigate the behavioural, immunological and neurodevelopmental alterations present in the VPA rat model of ASD. In line with the sexual disparity in prevalence, effect of sex in the pervasiveness of these behavioural and neurological alterations, with males being disproportionately impaired as in humans; pointing to divergent reactions to VPA in early neurodevelopment (Schneider et al., 2008). Behavioural changes documented in male VPA rats include decreased exploratory activity and an increase in behaviours associated with anxiety, such as investigating new environments (Schneider et al., 2008). Consistent with aberrations seen in human patients with ASD, prenatal VPA exposure during early neurodevelopment (gestational day 9-12) alters development of the serotonergic system via changes to neuronal differentiation and migration and postnatally induces HSN. It is also known to increase basal levels of 5-HT in the hippocampus and Frontal Cortex of rats (Dufour-Rainfray et al., 2010). Further support comes from findings of neuroendocrine and immunological changes in male VPA rats, consistent with findings in humans with ASD (Schneider et al., 2008).

"A gene-environment interaction occurs when the effect of exposure to an environmental pathogen on a person's health is conditional on his/her genotype" (Caspi & Moffitt, 2006). Building off research implicating both heritable genetic and environmental factors in the etiology of ASD, Pessah and Lein (2008) suggest that these factors interact through converging to interfere with the same developmental pathway (Pessah & Lein, 2008). Such gene-environment (G*E) interactions have already been reliably identified in other neurodevelopmental disorders such as Schizophrenia (Clarke, Tanskanen, Huttunen, Murray,

& Cannon, 2009). A heritable aberration in interconnectivity between neurons in foetuses at risk of ASD would then be particularly responsive to environmental insults, such as VPA, which act on the same neurotransmitter networks culminating in disrupted neuronal development and therefore ASD. Thereby, one of more genetic factors with minimal individual effect can amplify of be exacerbated by exposure to an environmental insult during crucial periods of development such as organogenesis (Pessah et al., 2008). This theory accounts for the varying severity of ASD and range of symptoms displayed by patients (Veenstra-VanderWeele & Blakely, 2012); allowing for combinations of heritable ASD risk factors and varied environmental insults which interact uniquely but affect the same system (Pessah et al., 2008). Sex hormones are also a mitigating factor in this model as ASD and potentially mediate the degree of expressivity through epigenetics modifications (Kaminsky, Wang, & Petronis, 2006).

The existence of G*E interactions is not a recent discovery, but the field is currently burgeoning. Furthermore, it is not confined to the realms of neuropsychiatric disorders; much research has also gone into investigating G*E interactions in the physiological world. Moffitt et al (2006) mention several relatively recent studies implicating environmental factors whose impact on a person is contingent on their genetic make-up, and vice versa (Moffitt, Caspi, & Rutter, 2005). They make mention of research implicating gene-reliant effects of environmental events, such as the increased likelihood of developing psychotic symptoms following cannabis use based on the polymorphism of the catechol *O*-methyltransferase gene a person posessed (Caspi et al., 2005). Likewise, they recount research indicating a link between genotpye and the likelihood of a smoker developing a cronary heart disease (Humphries et al., 2001).

There is much research to be done in this burgeoning field of gene-environment interactions as a causative factor for the development of ASD. With both VPA and SERT strongly linked with ASD their interaction is of particular interest. The current study will look at responsiveness to VPA as an environmental factor conferred by varied numbers of SERT present due to genotype and assess the phenotypic penetrance on cognitive function and anxiety using a relevant animal model.

The rat has long been used as a species to model psychiatric, behavioural and medical conditions in humans. Through the use of *N*-ethyl-*N*-nitrosourea (ENU) targeted mutagenesis

a premature stop codon was produced in *SLC6A4* which renders the SERT protein nonfunctional and signals the resulting mRNA for nonsense mediated decay, thereby creating a strain of SERT knockout (KO) rats (Homberg et al., 2007; Smits et al., 2006). Through careful measurement of citalopram binding Homberg et al (2007) concluded that heterozygous animals (HZ/SERT$^{+/-}$) showed a gene-dose effect regarding SERT prevalence providing subsequent researchers with a means to explore the effect of the *5-HTTLPR* as a genetic predisposition in the aetiology of autism (Homberg et al., 2007; Wohr & Scattoni, 2013).

The utility of the rat animal models of comes from the ability to create paradigms designed to quantify and assess aspects and behaviours associated with neuropsychiatric disorders. Rat animal models are interesting in that they allow for assessment of anxiety and cognitive behaviours (Wohr & Scattoni, 2013) while maintaining control of independent variables; holding conditions constant which would be impossible to ethically and scientifically attempt in humans such as genotype and environmental conditions. In essence, rat models allow us to draw conclusions based on causality rather than simply correlation.

Previous research conducted by Olivier et al (2008) into the behavioural phenotype of the SERT KO rat has already indicated its potential as an animal model for investigating human genetic susceptibility to anxiety and depression (Olivier et al., 2008). It was found that on numerous measures of depression-like- and anxious behaviour homozygous SERT KO rats displayed significantly different patterns of behaviour than their wildtype (WT) counterparts. In the Novelty Suppressed Feeding paradigm (NSF), male homozygous SERT KO rats showed increased latency to begin eating; indicative of increased level of anxiety. Furthermore, they spent significantly more time in the closed arms of the Elevated Plus-Maze (EPM), again indicating higher levels of anxiety. With regards to depression-typical behaviour homozygous SERT KO rats displayed decreased preference for sucrose, signifying anhedonia (Olivier et al., 2008).

Perturbations in anxiety have long been associated with ASD, since the first characterization of the pervasive developmental disorder (Kanner, 1943; White, Oswald, Ollendick, & Scahill, 2009). While it is not recognized as a core deficit, it is often present and is thought to exacerbate social impairment (White et al., 2009). Levels of anxiety have been found to be significantly higher in people with ASD compared to controls subjects, a

phenomenon which has been replicated and reliably found in studies looking specifically at anxiety and comorbidity in ASD (White et al., 2009). This pattern of increased anxiety is echoed in animal models such as the prenatal VPA rat model of ASD which has consistently shown behaviours indicative of increased anxiety (Schneider & Przewlocki, 2005; Schneider, Turczak, & Przewlocki, 2005; Schneider et al., 2007). It has been posited that the behaviours observed in VPA rats point to a disruption in the systems responsible for regulating reaction to stressful stimuli (Schneider et al., 2007). Behaviour robustly associated with increased levels of anxiety has been shown by VPA rats on the elevated plus-maze (Schneider et al., 2007). The present study will therefore assess anxiety behaviour through use of the elevated plus-maze, and the novelty suppressed feeding paradigm, which elicit natural avoidance of open spaces; both of which have been successfully used to this effect in the past.

Cognitive impairment is often referred to in the diagnosis of ASD (American Psychiatric Association , 2013). Precise facets of cognitive ability reported perturbed in ASD include: executive function which incorporates aspects of memory and inhibition (Hill, 2004), learning (McLaren & Mackintosh, 2000) and sensorimotor gating (Perry, Minassian, Lopez, Maron, & Lincoln, 2007). Latent inhibition refers to the subsequent stunting of association-based learning due to pre-exposure to a stimulus (McLaren & Mackintosh, 2000). Associative learning has been demonstrated to be impaired in ASD (Preissler, 2008), and LI will be assessed using a conditioned taste-aversion paradigm. Conditioned taste aversion usually develops rapidly after only one pairing with LiCl following exposure to a novel stimulus; resulting from the negative physiological state induced by the LiCl (Yamamoto, Shimura, Sako, Yasoshima, & Sakai, 1994). Sensorimotor gating will be analysed through assessment of acoustic prepulse inhibition (PPI), habituation and startle response. Prepulse inhibition occurs when exposure to a weak stimulus immediately antecedent to a strong startle stimulus, usually of the same type, attenuates subsequent response. A particular strength of PPI measurement is that it easily transferrable across species with similar patterns of performance in both human and rat models of ASD (McAlonan et al., 2002).

The aim of this research is two-fold; to investigate the interaction between genetically determined SERT prevalence and prenatal exposure to VPA in rats, and the utility of the construct as an animal model of cognitive function and anxiety-liked behaviour typical of ASD. The SERT-/- phenotype is an extreme one, and a human case is yet to be reported to the best of this author’s knowledge. Therefore the present study is going to assess interaction

between VPA and SERT genotype through investigation of HZ SERT KOs. While past experiments have shown that HZ SERT genotype does not present with a severe phenotype in general they most closely translate to the genetic susceptibility identified in the *5-HTTLPR* in people with ASD. Furthermore, this research will focus predominantly on anxiety and cognition as both of these aspects are strongly implicated in ASD and are affected by VPA, SERT or hypothetically both.

The hypotheses of this research are threefold. Firstly, it is hypothesized that there will be significant differences noted overall between prenatal treatment groups; with VPA treated animals displaying reduced cognitive ability and increased portrayal of anxiety-related behaviour compared with saline-treated groups. Secondly, it is expected that with genetically reduced levels of SERT, the $SERT^{+/-}$ rats will display significantly poorer performance on measures of anxiety and cognitive ability compared to genetic WTs ($SERT^{+/+}$) However, as $SERT^{+/-}$ still retain 50% of the SERT proteins and the central nervous system has a strong capacity to compensate, it is possible that the behavioural phenotype of $SERT^{+/-}$ is very mild. Finally, it is hypothesized that there will be a gene-environment interaction noted in the VPA treated HZ animals, such that they will perform significantly worse on the measures of cognitive function and also display behaviour typical of increased anxiety compared to the saline treated $SERT^{+/-}$ cohort and the VPA $SERT^{+/+}$.

Method

Animals

$SERT^{+/-}$ and $SERT^{+/+}$ Wistar males were mated with $SERT^{+/-}$ females to produce $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ offspring. Pairs were mated overnight and the presence of a vaginal plug was taken as a sign of successful conception and counted as Gestational Day 1 (GD1). On GD12.5 half the pregnant females were subcutaneously injected with 400mg/kg of VPA with the remaining half injected with saline 1mg/kg. As a result, six groups were produced, of which four were used; VPA/ $SERT^{+/-}$, VPA/ $SERT^{+/+}$, SAL/ $SERT^{+/-}$ and SAL/ $SERT^{+/+}$. Only male offspring were utilized in experiments. The rats were kept on 12hr light/dark cycles with free access to food and water aside from during specific cognitive experiments which required food restriction (85-90% of free-feeding weight).

Elevated Plus-Maze

A robust and well used method for measuring anxiety in rats, the elevated plus-maze (EPM) consists of four arms of equal length (50cm X 10cm), with two opposing arms enclosed by opaque walls (closed arms) and the remaining arms having only a shallow raised transparent lip (open arms). The entire apparatus sits one meter off the ground. Thirty six saline prenatally treated rats were used (14 $SERT^{+/+}$ and 22 $SERT^{+/-}$) along with thirty one VPA treated (12 $SERT^{+/+}$ and 19 $SERT^{+/-}$) were used in this paradigm. The rats were handled for the two days prior to the experiment to habituate them to pre-test stressors (home-cage movement and removal from home cage) (Hogg, 1996). Immediately before placement into the EPM the rats were placed in an open field for five minutes to encourage exploratory behaviour (Pellow, Chopin, File, & Briley, 1985).

At the onset of the experiment the rat was placed in one of the closed arms and its movements were recorded by Ethovision XT9® from Noldus for five minutes. The recording is then analysed to assess the latency to first emerge from the entry arm, frequency and durations of entry into each subsequent arm, as well as overall percentage of time spent in either open or closed arms. Total distance moved and velocity was also noted. Entry into an arm was defined as all four paws crossing into the arm. Comparison of results shows level of anxiety across groups (with faster latency to emerge from the entry arm, increased time spent and entries into open arms, showing lower anxiety).

Novelty Suppressed Feeding

Using the conflict between hunger and trepidation of the unknown to measure anxiety, the Novelty Suppressed feeding paradigm entailed depriving the rats of food for 24 hours prior to commencing the experiment (with water still freely available), to increase food seeking behaviour. Nineteen saline prenatally treated rats were used (9 $SERT^{+/+}$ and 10 $SERT^{+/-}$) along with twenty three VPA treated (11$SERT^{+/+}$ and 12 $SERT^{+/-}$) were used in this paradigm. The experiment begun as the rat was placed near the wall in a well-lit circular open-field (50cm radius) and its movements were digitally recorded. A food pellet was placed in the center of the test arena on a 3cm radius circle of filter paper and the latency until the rat began eating was recorded as an indication of the level of anxiety as the rat must leave their preferred area of the wall and venture into the center of the open field (Lira et al., 2003).

Latent Inhibition

A Conditioned Taste Aversion paradigm aimed to investigate an aspect of cognitive performance, especially the ability to filter out irrelevant information. It consisted of three stages spanning five days; pre-exposure (3 days), conditioning (1 day) and testing (1 day). Six groups were used in this analysis; prenatal treatment group (VPA or Saline), Genotype (WT or HZ) and finally pre-exposure group (non- or pre-exposed to sucrose solution). The water bottles were removed from all cages 24 hours before the beginning of the experiment in order to increase drinking behaviour during experimentation. Pre-exposure lasted for three days, with the individual animals given 30 minutes of free access to either a 5% sucrose solution (pre-exposure group) or water (non-pre-exposure group). During conditioning, all the subjects were given free access to the sucrose solution for 30 minutes immediately followed by an intraperitoneal injection of 75mg/kg Lithium Chloride (10mg/ml). On the testing day animals were individually given free access to both the sucrose solution and water for 30 minutes. The bottles were weighed immediately before and immediately after the 30 minute time period to ascertain the amount of each fluid consumed.

Prepulse Inhibition

The startle response and PPI was measured using standard startle equipment (SR Lab, San Diego Instruments). The rats were placed in transparent cylinders which sit atop a piezo-electrical element measuring movement of the rat as an indication of startle response. The sounds attenuating chamber emits a background of white noise (70dB) and rats were subject to a five minute habituation period to adjust. After this time brief increases in sound intensity

(120dB, 20ms) served as the startle stimuli. The experiment consisted of three trial blocks. The first comprises of five presentations of the startle stimuli (120dB). The second and largest block contains 61 data points; startle stimuli, no stimuli or various prepulse bursts, randomly delivered with 10-20 second inter-trial intervals. On select trials, prepulse bursts preceded the startle stimuli by 100ms at varying levels (72, 74, 76, and 82dB, 20ms). The final block again consists only of startle stimuli.

Average displacement during the first block consisted of five 120dB startle stimuli was calculated to represent basal startle response. Percentage startle habituation denotes the change in average from the first and second blocks of 120dB startle stimuli. Finally, accumulated PPI intensities were averaged and divided by the mean of the median startle trial block to give an overall PPI value.

Statistics

Data collected from all paradigms was submitted to factorial Analyses of Variance to determine statistically significant differences. All statistical tests were completed using IBM SPSS Statistics 20. A p-value $\leq .05$ was considered to be significant.

Results

Elevated Plus-Maze

Valproate prenatally treated animals spent significantly less time in the open arms of the EPM compared to saline animals ($F(1, 61)=12.97$, $p=.001$, see fig 1), but no difference was found in the number of entries made ($p= .227$). No other significant results were found. The main effect of genotype was non-significant ($p=.519$) as was the treatment*genotype interaction ($p=.343$). The other key indicator of anxiety, number of entries made into the open arms, showed no statistically significant differences between the cohorts: prenatal treatment ($p= .227$), genotype ($p= .471$) or an interaction ($p= .501$).

Latency to first emerge from the closed arm initially placed in was nearing significance for prenatal treatment, $F(1, 61)= 3.78$, $p=.056$, while genotype and the interaction were not ($p= .945$, $p= .911$, respectively). There was no significant differences in the total distance moved for prenatal treatment ($p=.950$), genotype ($p=.390$) or the interaction ($p=.119$). No statistically significant differences were found in cumulative distance moved or velocity (data not shown).

Please refer to table 1 for average percentage of entries made into open arms and latency to first emerge from initial arm.

Novelty Suppressed Feeding

Animals prenatally treated with VPA had an increased latency to begin eating in the NSF paradigm (see fig 2). A statistically significant main effect was again seen for prenatal treatment in the latency to begin eating, $F(1, 40)= 5.22$, $p= .028$, with a longer average time observed for the VPA prenatally treated rats compared to those treated with saline. The main effect for Genotype and the interaction were again non-significant ($p=.678$ and $p= .124$, respectively).

Latent Inhibition

The test of latent inhibition showed the anticipated statistically significant main effect for pre-exposure ($F(1, 53)= 16.50$, $p< .001$). As expected, animals pre-exposed to sucrose solution consumed a higher proportion of sucrose on the test day compared to those who were not previously exposed (see fig 3). Furthermore, prenatal treatment also showed a statistically significant main effect, $F(1, 53)= 7.64$, $p= .008$, with those exposed to VPA consuming higher percentage of sucrose solution than those prenatally exposed to saline. However, there was no prenatal treatment * pre-exposure interaction, suggesting that prenatal

valproate decreased sucrose intake both in the pre-exposed and non-pre-exposed animals. Genotype did not show a statistically significant main effect (p= .139) and nor was any significant interaction between genotype & prenatal treatment group evident (p= .853). No other interactions between any of the three independent variables (genotype, prenatal treatment or sucrose pre-exposure group) were significant. However, the interaction between genotype and sucrose pre-exposure group was trending towards significance (p= .115, see figure 3).

Prepulse Inhibition

Since there were no interaction effects of either prenatal treatment or genotype and prepulse intensity, the prepulse data were collapsed into one average prepulse value (see fig 4). A significant main effect was found in percentage of prepulse inhibition (PPI) for prenatal treatment, $F(1,48)=6.49$, p= .014, see figure 4. No main effect for genotype (p= .283) or any interaction (p= .631) was observed in PPI. Basal startle response and percentage of habituation yielded no significant main effects for prenatal exposure (p=.087 and p= .379) or genotype (p=.589 and p= .670), nor any interaction (p=.887 and p=.711, startle response and percent habituation respectively). Please refer to table 3 for group average startle response and percent habituation, standard deviations and sample sizes.

Discussion

The results from the present study corroborate previous literature that prenatal exposure to a single dose of VPA (400mg/kg) exposure at G12.5 leads to statistically significant long-term alterations in anxiety-related behaviour and cognitive performance on a range of paradigms; supporting the first hypothesis of this research. The hypothesis that genotype would have an impact on performance, specifically that the HZ animals would perform worse than the WT cohort was not supported by the results obtained as there was no statistically significant difference between groups. Furthermore, there were no significant results obtained which could support the presence of a G*E interaction; contrary to the third hypothesis of this research that HZ animals prenatally exposed to VPA would perform significantly worse than any other group.

In line with previous research, animals prenatally exposed to VPA spent a significantly smaller percentage of time in the open arms of the EPM compared to their saline counterparts. Interestingly, Schneider et al (2005) reported that the average percentage of time spent in the open arms for adult male rats prenatally exposed to VPA was ~5%, (cf. ~30% for saline treated animals) which is significantly lower than the averages found in the present study (see fig 1). This finding was echoed in other similar studies on the effects of prenatal VPA exposure (Schneider & Przewlocki, 2005). However, in their paradigm experimental animals were exposed to 600mg/kg of VPA which is a substantially larger dose than that administered in the current research (400mg/kg). It is possible that the single larger dose of VPA was sufficient to produce a stronger display of anxiety in their animals than that demonstrated presently. This may explain why the pattern of behaviour is the same but to a less dramatic degree and could be taken as a potential indication of a dose sensitive effect during critical period of development. Indeed this dose-response relationship has been identified in clinical studies showing increased risk and behavioural phenotype following higher daily doses of VPA administration during pregnancy (Roullet, Lai, & Foster, 2013).

Different protocols for the same paradigm may also explain the differences in averages reported by the present research and that of previous studies. For example, Schneider et al (2007) used a different protocol which spanned two days when running EPM. In the 2007 study, time spent in open arms of the EPM for animals prenatally treated with VPA did not breach 15% for day 1 or 10% for day 2 compared to roughly 25% and 19% for saline treated animals. In addition it is well known that (even subtle) differences in lighting in

the room have a very strong effect of the percentage of time exploring the open arms of a plus maze. It is thus difficult to compare absolute levels of anxiety-like behavior across laboratories.

Currently the novelty suppressed feeding paradigm appears absent from the VPA rat model literature, a gap which has been addressed by the present research. To further corroborate the anxiety-like profile of VPA rats, we found that in the NSF paradigm, VPA rats showed an increased latency to start feeding in a novel environment. Like the EPM, the NSF paradigm is in essence a conflict paradigm; in this case between the drive to eat and fear for a novel environment.

Longer latency to begin eating food placed in the center of a brightly lit open field, as in the NSF paradigm, is reliably linked to increased anxiety in rats (Lira et al., 2003). In previous research, it was found that rats with a complete genetic deletion of the SERT also showed an increase latency to start eating (Olivier et al., 2008). In line with performance on the extensively documented paradigm in VPA rat model literature, the EPM (Schneider & Przewlocki, 2005; Schneider, Turczak, & Przewlocki, 2005); rats prenatally exposed to VPA had a significantly longer latency to begin feeding than the saline cohort in the present study. These results are also congruent with studies linking ASD with increased anxiety (White et al., 2009). In the NSF paradigm, there was a small non-significant effect observed in the HZ SERT KO rats prenatally treated with saline, which was also seen in the EPM. While not significant it is indicative of a slightly stronger anxiety-like response and given that a complete deletion of the SERT leads to significant increase in anxiety (Olivier et al., 2008). This trend suggests that 50% reduction is not strong enough to produce the anxious phenotype.

While not ideal due to inherent differences across species, behaviour of the HZ genotype when not exacerbated by other developmental stressors, has been shown to be intermediary between that of homozygous SERT KO and WT in mouse models (Joeyen-Waldorf, Edgar, & Sibille, 2009). In the EPM, no statistical differences on most measures between HZ and both other genotypes were seen (Kalueff, Fox, Gallagher, & Murphy, 2007). While a significant difference was seen for total time spent in the closed arms of the EPM between homozygous and WT SERT KO genotypes, the HZ showed an intermediate score but did not reach significance (Kalueff et al., 2007). However, in another study HZ SERT KO mice performed similarly to WTs in the open field test, another measure of anxiety, but more

closely related to the homozygous KO genotype in the EPM (Joeyen-Waldorf et al., 2009). These mixed results suggest that HZ produces a transitional behaviour pattern between homozygous and WT but also that this display is contingent on the paradigm used to investigate anxiety. This pattern of behaviour in the SERT KO mouse model suggests that while HZ is functionally the closest genotype to that in humans, HZ alone is not enough to consistently produce the anxiety-related phenotype displayed in ASD.

A conditioned taste-aversion paradigm was used to investigate the potential effect of prenatal VPA and SERT genotype on the latent inhibition phenomenon. Conditioned taste-aversion (CTA) refers to the cognitive association which forms following presentation of a neutral or appetitive stimulus with exposure to an aversive stimulus (Yamamoto et al., 1994). A type of associative learning, LI is thought to represent the ability to filter out irrelevant information after pre-exposure to stimuli (McLaren & Mackintosh, 2000). Because no interaction was observed between prenatal VPA treatment or genotype and sucrose pre-exposure it does not appear to be impaired in this study. Under usual circumstances, when previously exposed to the novel stimulus/solution, the association between LiCl and sucrose solution is lessened (Yamamoto et al., 1994). Results from the present experiment displayed the typical pattern of animals pre-exposed to sucrose consuming proportionately more during testing than those not previously exposed (see figure 3).

Rather than an effect on latent inhibition per se, it appears that VPA prenatal treatment at G12.5 has a general effect on sucrose consumption; indicative of a lesser association with a negative stimulus (LiCl) which could signify impairment in associative learning or a decreased susceptibility to the LiCl conditioned taste aversion paradigm. These data are in line with previous research on associative learning and VPA in rat models (Schneider et al., 2007). In a conditioned place aversion paradigm, VPA prenatally treated rats demonstrated a significantly reduced naloxone induced place aversion compared to control animals when injected with 0.2 or 0.5mg/kg. However, no significant difference was found between the groups when exposed to 1mg/kg of naloxone, meaning that VPA animals displayed decreased sensitivity to lower doses/ needed higher doses to lead to the same degree of conditioned place aversion as their control counterparts (Schneider et al., 2007). No corresponding deficit was found in measures of learning and memory included in their study of spontaneous non-matching to sample, so it was concluded that this was not due to any overarching impairment in learning (Schneider et al., 2007).

No significant effect of genotype was observed in the CTA test of LI however there was a trend towards lower sucrose consumption in all groups, except HZ SERT KO rats prenatally treated with VPA and not previously exposed to the sucrose solution. While not disparate enough to be statistically significant, this may indicate a trend towards increased CTA compared to the genetically WT rats. The behavioural pattern of the HZ SERT KO rats in the present study echo findings in children with low functioning autism; where associative learning is found to be significantly altered compared to healthy controls (Preissler, 2008). However, because the CTA paradigm has not previously been researched using SERT KO rats, it remains unclear whether this is a reliable trend which could become significant using the homozygous genotype.

Prepulse Inhibition of acoustic startle response allows quantification of sensorimotor gating and processing (Swerdlow, Geyer, & Braff, 2001). It has been repeatedly demonstrated to be deficient in both ASD patients and VPA animal models of ASD and performance is regulated by the FC, limbic system and other key structures known to be perturbed during development by VPA exposure (Swerdlow et al., 2001). In line with VPA rat models previously reported following exposure on G12.5, the present findings show significant reductions in overall PPI, indicating a reduced ability to dampen startle response to an intense acoustic stimuli following immediate pre-exposure to a weaker stimuli (prepulse)(Markram et al., 2008; Schneider & Przewlocki, 2005; Schneider et al., 2005). The lack of significant differences produced for startle response and habituation in the present study also echoes previous research cited. However, not all studies have produced the same pattern (Dendrinos, Hemelt, & Keller, 2011). This pattern of impairment demonstrated by rats prenatally exposed to VPA corresponds with what has often, but not always, been observed in people with ASD; with decreased PPI but no corresponding significant difference in base startle response or overall habituation (Kohl et al., 2014).

While not all research teams have been able to replicate these findings in people with ASD it is suggested that this lack of consistent association may be dependent on differences between phenotypically separate groups- high vs. low function ASD (Orekhova et al., 2008). In a study investigating the sensorimotor pattern of impairment in adults with Autistic Disorder using an acoustic paradigm, a significant difference in PPI but not startle response was found (Perry et al., 2007). In a study investigating startle response in patients with Asperger's Syndrome it was found that in trials with high intensity prepulses, a reduction in inhibition was found compared to control subjects with no indication of significant

differences in basal startle response (McAlonan et al., 2002). However, other studies have found the opposite pattern in adults with high functioning ASD (Kohl et al., 2014) or no significant difference in either measure in children with ASD (Oranje, Lahuis, van Engeland, van der Gaag, & Kemner, 2013). Age has been suggested as a confounding factor and may be applicable in future rat model studies too (Oranje et al., 2013). Indeed, in a longitudinal VPA rat study abnormal startle response was found in juvenile rats but not adults prenatally treated with either one or two administrations of VPA; along with no significant impairment in PPI for any group except single dose VPA juveniles rats (Dendrinos et al., 2011).

Overall, the results of our present study are largely in agreement with previous research showing that prenatal VPA at GD 12.5 leads to significant alterations reminiscent of ASD, consistent in most of the paradigms used. However, our central hypothesis, namely that rats with genetically compromised SERT functioning would be more sensitive to the disruptive effect of VPA was not confirmed in the present experiments. There are several reasons why this might be the case.

When treating patients, maintenance of VPA in therapeutically relevant doses requires daily administration (Birnbaum et al., 2004), and therefore the premise of using only one moderate dose exposure bares further consideration. Although in the present study the often used single administration paradigm was utilized, and indeed significantly altered behaviour in the offspring was found, repeated exposure to lower doses of VPA during the critical developmental period could lead to an interaction not observable in the current research. In a study designed to evaluate the impact of a single vs. multiple administrations of VPA in an animal model significant differences in behavioural markers was observed (Dendrinos et al., 2011). Two moderate (200mg/kg) doses of VPA on G11 and G13 were compared with a single administration of 400mg/kg or 500mg/kg on G12.5 to investigate potential differences between single and multiple doses. Although the authors did not find any evidence for an accumulation of the two 200mg/kg doses (Dendrinos et al., 2011), this is not surprising given the 2.5 hour half-life of VPA in rats (cf. with 9-18hours in humans) (Loscher, 1999). However their administration schedule was not daily as in patients being treated for epilepsy or mood stabilization and also missed the day G12.5 which has previously identified as the critical window for developmental disruption in ASD (Rice & Barone, 2000).

In order to adhere to more realistic administrations routines, Sabers et al (2014) chronically injected pregnant rats with different doses of VPA, 20mg/kg and 100mg/kg for

the last 9-12 days of pregnancy and continue to inject the dams throughout rearing until PND23, when neuronal growth was analysed (Sabers, Bertelsen, Scheel-Kruger, Nyengaard, & Moller, 2014). A significant increase in neocortical neurons was found after chronic VPA exposure compared to non-exposed pups. A dose related effect was found in frontal cortical thickness, with 100mg/kg leading to increase compared to 20mg/kg and controls signifying an overgrowth of neurons in the frontal lobes (Sabers et al., 2014). As yet, effects on cognition and other core areas of impairment in ASD (including possible alterations in 5-HT) have yet to be investigated in this chronic VPA exposure model. Based on the findings of significant neurodevelopmental aberrations due to chronic prenatal VPA administration (Sabers et al., 2014), future research would benefit from investigating the long term behavioural effects of chronic VPA administration throughout development. It is possible that prolonged exposure to comparatively low doses may allow observation of an interaction with SERT genotype otherwise masked by high doses.

The timing of exposure to prenatal VPA may also be important for potential interactions with SERT genotype due to different critical windows based on temporally sensitive divergences between behaviour and neuronal development. For instance, a single exposure to VPA on G9 led to significant alterations to synthesis, maturation and distribution of serotonergic neurons in rats (Miyazaki, Narita, & Narita, 2005), whereas exposure on G12.5 has been repeatedly used in behavioural studies (Bambini-Junior et al., 2011; Markram et al., 2008; Schneider & Przewlocki, 2005; Schneider et al., 2005; Schneider et al., 2007). Attempts to identify behavioural changes due to a single VPA exposure earlier than G12.5 have met with mixed success. Kim et al (2011) found gradual decreases in social behaviour from time of prenatal VPA exposure G9 to peak at G12.5 (Kim, Kim, Jeon, Han, & Shin, 2011). Inhibitory function was significantly decreased in rats administered VPA on G12.5 compared to any other exposure time as identified by electroshock seizure threshold. This reduced threshold has been likened to the decreased inhibitory ability seen in patients with ASD (Kim et al., 2011).

Alterations in the social behaviour of rats have been inconsistent across studies, with significant differences found compared to control found in some research (600mg/kg at G9), (Dufour-Rainfray et al., 2010) and not in others (800mg/kg at G9) (Narita et al., 2010). Furthermore, changes to reflexes and motor development have also produced contradictory results after VPA exposure on G9 (Dufour-Rainfray et al., 2010; Narita et al., 2010). Because of this apparent discrepancy between morphological changes in the serotonergic

system (seen especially after with GD9 administration) and behavioural changes (seen especially with GD12.5 administration), it would be interesting to see if VPA exposure in the earlier temporal window would show interaction with SERT genotype. Likewise, early exposure coupled with a more chronic administration of VPA (which would be more in line with the clinical situation) might lead to G*E interactive effects.

Olivier et al (2008) found significant differences between performance of homozygous SERT KO rats and their WT counterparts on both the Elevated Plus-Maze and Novelty Suppressed Feeding paradigms; with homozygous SERT KO animals showing increased latency to begin eating on NSF, and decreased percentage of entries and time spent in open arms of the EPM compared to the WT rats (Olivier et al., 2008). Results from the present study replicates patterns of behaviour found on the EPM and NSF, but without the difference reaching statistical significance (see table 1 & 2). In the present set of experiments HZ animals were specifically chosen, to avoid a potential ceiling effect. However, it is possible that if homozygous rats had been used significant differences or an interaction with VPA exposure could have been found.

So far, much of the research has focused on a comparison between homozygous SERT KO rats, which represents an extreme phenotype/genotype not found in humans, and control WT rats, neglecting the HZ genotype. Studies that have included HZ SERT KO genotype have done so in mice and have generally found a mild intermediate phenotype in measures of anxiety, rarely distinguishable in behaviour from WT animals (Adamec, Burton, Blundell, Murphy, & Holmes, 2006; Kalueff et al., 2007). The present study addresses the present gap in the rat literature by utilizing HZ genotype specifically as it represents the most translatable to humans. VPA and SERT showed no significant interaction but was trending that way in multiple experiments; in two specific behaviours on the EPM and also in NSF (p= 0.1), so potentially there is too mild of a genetic effect to produce a strong enough pattern of behavior without a substantially larger sample size.

It is possible that due to system redundancy even with only 50% of SERT available in HZ animals it is enough to compensate for any deficits and explain why no genetic effect was observed. The DHS theory of ASD pathogenesis (see introduction) focusses on the neurotrophic role of 5-HT early in development. As previously mentioned, 5-HT concentration is originally determined by maternal factors due to placental permeability and it is only later that fetuses' genetics play more of a significant role (Bonnin & Levitt, 2011). All

rats used in the present study were conceived and reared by HZ dams which display relatively normal levels of 5-HT (Homberg et al., 2007). Given the role of maternal 5-HT concentration in early brain development it would be interesting to see whether the long-term behavioural changes in HZ pups depend on the maternal environment, i.e. whether pups are reared by dams of different genotypes (homozygous KO, HZ and WT) show exaggerated phenotypes. Assuming the behavioural deficits are larger in pups reared by homozygous SERT KO mothers, a potential G*E interaction with VPA might be uncovered.

Conversely, genotype alone may not be the reason for the lack of interaction found between SERT and prenatal VPA. Previous research has found statistically significant associations between both the VNTR polymorphism in the promoter region *5-HTTLPR*, and SNPs on *SLC6A4*, and ASD (Yang & Gill, 2007). However, genetic screening of the *SLC6A4* gene has yet to find any single mutation which reliably associates with ASD (Kim et al., 2002). While it has been suggested that small sample sizes may be to blame for inconsistent association findings, Ramoz et al (2006) posit that defining specific phenotypic subsets may increase the likelihood of finding homogenous results. Sometimes called endophenotypes, specific traits such as age of first word and restricted repetitive behaviours can be used to increase statistical power (Bill & Geschwind, 2009).

By isolating age of first word as an explicit endophenotype Alarcon et al (2008) identified *CNTNAP2* as an Autism Susceptibility Candidate Gene (ASCG) through linkage, association and gene expression analysis (Alarcon et al., 2008). This is supported by the work of Buxbaum et al (2001) who found that using Phrase Speech Delay as a phenotypic subset led to the identification of chromosome 2q31 as a candidate gene region (Buxbaum et al., 2001). Thus, definition of other specific phenotype clusters could be one way to further explore the SERT gene as a candidate for a component of G*E interaction. As the majority of the measures used in the present research were anxiety- and cognitive ability-centric, it is possible that a G*E interaction may exist in other areas of developmental delay that weren't detectable in the paradigms used.

Identification of specific traits may also be important for the converse reason. McCauley et al (2004) found a general over transmission of the "s" *5-HTTLPR* allele in ASD families (families including members diagnosed with ASD) but no such association when looking at Rigid-Compulsive behaviour as a phenotypic subset (McCauley et al., 2004). When collating data from 352 ASD families Ramoz et al (2006) found no consistent link

between ASD and *5-HTTLPR* polymorphism nor any of another nine SNPs located on the *SLC6A4* gene, even when looking specifically at Rigid-Compulsive and Obsessive-Compulsive Behaviours as phenotypic subsets (Ramoz et al., 2006). Taken together, this means that while there was a generally significant trend for *5-HTTLPR* to be associated with ASD, specific traits weren't automatically so.

It is possible that the current research, focusing on aspects of cognition and anxiety, may have focused on behaviours in which this particular G*E interaction is not a major contributing factor. Considering the previous research highlighting the importance of phenotypic subsets (Buxbaum et al., 2001; Ramoz et al., 2006), it is possible that other specific types of behaviours such as aberrant communication, social behavior or repetitive behaviours would lead to a significant G*E interaction. A current enigma in the literature on the association between *5-HTTLPR* and ASD is the overtransmission of "s" and "l" common VNTRs in different studies. As yet, no satisfactory conclusion has been reached on why some studies report overtransmission of the "l" allele (Devlin et al., 2005).One theory gaining momentum is that *5-HTTLPR* polymorphisms may influence phenotypic expression of ASD rather than susceptibility to development, as one study showed that the two common VNTRs did not convey risk but rather modified severity of social and communication (Tordjman et al., 2001). The "s" and "l" alleles were associated with more and less severe patterns of impairment, respectively (Tordjman et al., 2001). Population ethnicity may also play a role in the development of ASD and the association with *SLC6A4.*

In contrast to the substantial evidence implicating the SERT gene or the chromosomal region in which it resides as risk inducing (Veenstra-VanderWeele et al., 2012; Yang & Gill, 2007) several studies have produced results failing to support this hypothesis (Ramoz et al., 2006). Using family based association studies and detection of three SNPs located on *SLC6A4,* no association to ASD was found in Chinese Han population (Wu et al., 2005), although these authors did not include the *5-HTTLPR* VNTR polymorphism in their analysis. Furthermore, no linkage disequilibrium was found in Japanese trios, which indicates no association between *5-HTTLPR* polymorphisms and ASD (Koishi et al., 2006). Conversely, a linkage study comprised of 37 Sardinian families found an even split in the development of ASD and association between the Human Leukocyte Antigen and *5-HTTLPR* loci variants (Guerini et al., 2006). When taken together, these studies suggest a strong population based difference in the susceptibility genes involved in ASD, varying across ethnicity suggesting that actualization or penetrance varies based on as yet unidentified genetic background.

In support of the critical role of gene-gene interaction in the demonstration of phenotype, anxiety- and depression-like behaviours in the SERT KO mouse model has shown to be contingent on co-occurrence of key genes (Holmes, Li, Murphy, Gold, & Crawley, 2003). Serotonin Transporter KO mice were backcrossed onto two different genetic strains and when assessed for anxiety-like behavioural patterns, only the B6 congenic SERT KO mice demonstrated increased anxiety coupled with decreased 5-HT_{1A} binding and function. It was suggested that the higher baseline anxiety displayed by the 129S6 congenic mice, compared to the B6 strain, masked the anxiogenic effect of the SERT KO genotype (Holmes, Li, et al., 2003). Therefore, while no significant interaction was observed between SERT genotype and prenatal VPA in the present study, perhaps a different genetic background may be necessary for phenotypic presentation.

Because numerous genes of minor to moderate effect are thought to interplay to produce ASD, the presence or absence of different genes may obscure identification. Some genes only show effect if other specific polymorphisms are also present in the genome, likewise they may be interchangeable, in the sense that only alteration to certain systems is requisite; not the specific polymorphisms or genes (Bill & Geschwind, 2009). As an example, much like the development of cancer relies on very particular cell regulation systems to be impaired or altered but not any specific mutation in those systems (Evan & Vousden, 2001). However, cancer usually involves mutations of moderate effect in particular cells over time, rather than changes during development caused by the interaction of minor static genes as in ASD.

Research testing candidate genes and gene expression studies emphasized seven chromosomal regions as areas of risk and deserving of further investigation; one of which 17q11.1-q21.2, harbours *SLC6A4* (Yang & Gill, 2007). However, while much convergent research in the past points towards at least partial involvement of the *SLC6A4*/SERT/5-HT system in development of ASD, the hypothesis that anywhere between five and 20 genes are involved in the pathology of ASD (Yang & Gill, 2007) confounds the ability to identify any clear link in a simple G*E investigation. Rather, minor interactions discovered through future research individually accounting for only a small proportion of the variation and phenotype observed in ASD, will accumulate to produce a more complete and realistic model of the etiology.

Changes in the regulation of the serotonin system per se may be the prerequisite to develop ASD rather than any particular polymorphism of *SLC6A4* or *5-HTTLPR*. Given the complexity of the serotonergic system (for instance there are at least 14 different serotonergic receptors (Hoyer, Hannon, & Martin, 2002)); divergent genotypes might produce similar overall changes in the serotonergic system. This would allow for numerous polymorphisms to be partially associated with ASD with none being found to be unequivocally associated; a phenomenon which has been repeatedly produced by numerous studies (Guerini et al., 2006; Ramoz et al., 2006; Wu et al., 2005). Any change which alters the 5-HT system leading to DHS or any long term change in 5-HT availability or concentration could increase risk of ASD and be phenotypically interchangeable even if the gene responsible was intrinsic to another neurotransmitter or hormonal system.

Under usual conditions 5-HT uptake predominantly occurs via SERT with relatively small proportions binding with other neurotransmitter transporters. When SERT expression or function is reduced for any reason, be it genotype, epigenetic changes or drug actions, 5-HT uptake can occur not only via the remaining SERTs (indicating redundancy in the system) but also via these other neurotransmitter transporters (NTTs)(Daws, 2009). This has been referred to in the past as 'transporter promiscuity' and can present a confounding factor in the present study. Indeed, it has been shown that in the homozygous SERT KO rats, 5-HT is partly removed from the extracellular space by the Norepinephrine transporter (Homberg et al., 2007). Given the apparent significant degree of redundancy in the SERT system, it may be that the reduction in SERT due to the HZ genotype is not severe enough to elicit the deleterious behavioural phenotype and the overlap in 5-HT uptake with other neurotransmitter transporter may exacerbate this.

Given the 'promiscuity' of NTTs (Daws, 2009), an aberration in function or regulation of at least one other NTT may be necessary for SERT HZ genotype to eventuate in deleterious behavioural consequences. Under this theory, the uptake of 5-HT via other NTTs, coupled with the high degree of redundancy in the SERT system could mask any behavioural effects otherwise observable in this G*E model. Without resorting to use of the homozygous SERT KO genotype, minor disruptions to other NTTs known to be bound by excess 5-HT, such as norepinephrine- transporters (Daws, 2009; Homberg et al., 2007), may be more successful in producing an interaction or main effect.

Autism Spectrum Disorder is recognized as a complex and pervasive developmental disorder and as such lends itself to the theory that multiple neurotransmitter, cellular and hormonal systems may converge in its production. If multiple genes of small effect all converge on neurodevelopment, then this would explain the inconsistencies found in previous genome-wide studies; no single gene of large effect is present but rather interchangeable genes of only moderate individual consequence which act on specific developmental pathways (Bill & Geschwind, 2009). Numerous genes from seemingly discreet pathways have been implicated as risks for the development of ASD; cell adhesion molecules, extracellular matrix proteins, and 5-HT (Persico & Napolioni, 2013), each of which is involved in neuronal development, function, migration or distribution (Cochrane, Tansey, Gill, Gallagher, & Anney, 2010; Whitaker-Azmitia, 2001; Zhang et al., 2002). Furthermore, environmental factors like VPA which have been linked to changes in neurotransmitter concentration and long term epigenetic alterations and thereby present a risk of disruption to proper neuronal development (Roullet, Lai, & Foster., 2013).

Given the temporally and hormone/NT sensitive gradients crucial to proper neuronal synthesis, migration and distribution, exposure to any sufficient dose of VPA or other significant environmental factor (like an immune challenge) at a critical period of development could lead to an increased risk of ASD. Simply put, these systems are vulnerable to changes during critical periods of development (Rice & Barone, 2000) and during this temporal window any significant disruptions may cause long lasting impairment phenotypically classified as ASD even though causative factors may be dissimilar. This may also explain why there is individual variation in the experience and manifestation of ASD; because it is not caused by homogenous changes. Likewise, disruption to the 5-HT system may only represent a single (substantial) cog in the underlying machine causing development of ASD.

One way to discern whether this hypothetical challenge has any merit aside from face value, would be for genome studies to account for gene-gene interactions/reliance by including more than one susceptibility gene in their analysis and using a so-called network approach; much like the congenic mouse model discussed previously (Holmes, Li, et al., 2003). Thus, rather than looking at genes individually, using bio-informatics techniques, the relationship between genes and their resulting proteins are studied. This approach will become very helpful in identifying the functional commonalities of individual genetic changes. If any genes are found to interact to a significant extent, then animal models might

be developed with them in mind. For example, SERT KO rats bred with other genetically controlled animals and investigated for key ASD traits, behavioural and cellular. Combining consistent risk genes such as those identified by reviews, for example the list compiled by Yang & Gill (2007), with genome wide association or linkage studies may shed light on the validity of this theory (Yang & Gill, 2007).

While the present study failed to find a statistically significant effect or interaction on measures of anxiety and cognitive function for SERT genotype, it still presents a clear risk factor in need of better understanding. When factoring the implication of *SLC6A4* from GWLS, gene expression studies, previous testing as a candidate gene along with its role in neurodevelopment remains a salient target of further investigation (Yang & Gill, 2007). Coupled with previous research repeatedly linking SERT as a potential pathway for development in ASD, further study is required to define the degree and necessity of 5-HTTLPRs' contribution (Veenstra-VanderWeele & Blakely, 2012).

An important shortcoming of most genetics studies, including previous literature that found no reliable association between *SLC6A4* and *5-HTTLPR* polymorphisms (Koishi et al., 2006; Ramoz et al., 2006; Tordjman et al., 2001; Wu et al., 2005), is that in most studies no attention was paid to environmental factor. For instance the studies of Ramoz et al (2006) collated information from a large sample of ASD families and found no reliable link between the aforementioned genetic variants and ASD when looking at specific areas of developmental delay or abnormal behaviour, but did not factor environmental interaction into their analysis (Ramoz et al., 2006). If any environmental factors, such as VPA, acted as a mediating factor in the development of ASD then failing to include measures which would assimilate exposure to such chemicals could potentially obscure any genetic factors which may otherwise be detectable.

Only by including information about environmental exposures in large scale studies on information resourced from databases like the Autism Genetic Resource Exchange is it possible to get a more accurate picture on the role played by genes and environmental factors on the development of ASD. A prime example is the investigation into the interaction between the catechol O-methyltransferase (*COMT*) and *5-HTTLPR* polymorphisms with maternal smoking on ASD symptoms in children diagnosed with ADHD. While no main effect was found for *5-HTTLPR* s/s polymorphism or maternal smoking, a significant interaction was found and was associated with increased impairment in social interaction, low

birth weight and rigid behaviour (Nijmeijer et al., 2010). Furthermore, the Val/Val *COMT* polymorphism was found to interact with maternal smoking; an interaction which was associated with increased stereotyped behaviour, and again this was not accompanied by significant main effects of either factor (Nijmeijer et al., 2010). Therefore, not only were two G*E interactions found, but phenotypic expression of the comprising factors was entirely contingent on the presence of the other.

Interestingly, the behaviours which were affected by the interaction between maternal smoking and COMT or *5-HTTLPR* polymorphism were divergent (Nijmeijer et al., 2010). This suggests that the phenotypic effect of the maternal smoking risk factor is conditional on the susceptibility genes present, and also compounds the need to identify specific endophenotypes when investigating G*E interactions.

While it is now generally acknowledged that both genes and environmental factors play key roles in the development of ASD and host of other neuropsychiatric disorders (Caspi & Moffitt, 2006; Caspi et al., 2005), the acceptance of this model presents us with an inescapable challenge: each factor may confound the discovery of the other. It is naïve to expect that something as complex ASD to be caused by a single G*E interaction. The numerous environmental factors suspected of influencing the emergence of ASD must be considered along with their potentially reliance on the complex interplay of genes of small effect which ultimately culminate in vulnerability and complicates the task of uncovering the etiology of ASD. Adding to the challenge is the as yet poorly understood mechanism by which genetic and environmental risks influence each other (Tsuang, Bar, Stone, & Faraone, 2004). It has been suggested that any interaction may not necessarily be purely linear, but rather genetic vulnerabilities may alter not only susceptibility to environmental factors but also likelihood of exposure (Tsuang et al., 2004). Therefore, it is important not to write off potential G*E interactions too hastily, but rather expand theories to include additional factors, both genetic and environmental.

Prenatal exposure to VPA is one of many environmental risk factors which have been identified. While no interaction was found in with HZ SERT KOs animals in the present study, there is no reason to expect that SERT would not interact with other environmental factors. Maternal infection during pregnancy has previously been linked with increased risk of ASD (Pardo, Vargas, & Zimmerman, 2005). Likewise, animal models of prenatal immune challenge through exposure to maternal immune activation (MIA) have shown impaired

patterns of behaviour and have been explored as models of both ASD and Schizophrenia (Patterson, 2009). While a return to the drawing board may be unavoidable, the inclusion of other environmental factors such as MIA and maternal smoking may need to be incorporated into the future G*E designs to make further progress in the discovery of the underlying contributors to ASD. Different combinations of genetic and environmental factors may interact potentially explaining the heterogeneity and varied symptomology typical of ASD.

Inclusion of environmental factors and specific phenotypic subsets may hold the key to unraveling the next avenue of research to explore. At this juncture, there are numerous combinations of risks factors which could interact to produce ASD, and mounting evidence that there is likely more than one pathway to develop ASD (Geschwind, 2008).Along with the inclusion of environmental factors in gene association studies, the reverse is also a viable path to explore; factoring risk genes into studies of environmental effect on ASD pathogenesis. At this stage, while not painting the whole picture, identifying new combinations of risk factors which interact could provide valuable insight into the pathogenesis of ASD.

The present study adds further validation to previous research showing significant changes to behaviour of animals prenatally exposed to VPA on the EPM and PPI as well as adding two more paradigms to the repertoire; contributing LI and NSF to the body of literature on the VPA rat model of ASD. The convergent findings of all four paradigms suggests the validity of adding these two previously unused measures to the researchers tool boxes while investigating the development of ASD. These two new paradigms explore and expose different patterns of behaviour. Due to the nature of ASD as a complex neurodevelopmental disorder, every clue is integral.

Autism Spectrum Disorder is a life-long pervasive developmental disorder with the potential to present severe behavioural, cognitive and affective challenges. The effect on quality of life for both individual and Whanau can be extensive, impacting the family unit both emotionally and financially. As rates of ASD are on the increase, with substantial impacts on society, both economic and social, it is imperative that the underlying contributing factors are investigated. Genes and environmental factors, interacting in numerous combinations, are thought to be causative in ASD; and when they are properly understood, the implications for prevention and symptom mitigating are boundless.

While no interaction between the prenatal VPA and HZ SERT KO rats on some measure of anxiety and cognitive ability in the present study, this by no means concludes this avenue of research. Each combination of likely risk factors investigated brings us one step closer to deciphering the ASD puzzle; in turn bringing us closer to effective treatment and better prevention.

In summary, the present research further corroborates the existing literature that a single moderate *in utero* dose of VPA during the critical period of embryogenesis leads to long term aberrations in anxiety- and cognitive ability centric behaviour. No interaction was found between HZ SERT KO genotype and 400mg/kg G12.5 VPA on some measures of anxiety and cognition which was contrary to predictions. However, this study represents a starting point for subsequent research into the potential G*E interaction between SERT genetic reduction and prenatal exposure to teratogens. Numerous possible reasons exist for these interaction null results, including the genotype and genetic background, dose and administration schedule for the VPA, and also the specific behaviours measured. Future studies on different times of VPA administration, dose, genotype, phenotypic subsets and eventually different combinations of genetic and environmental factors.

References

Adamec, R., Burton, P., Blundell, J., Murphy, D. L., & Holmes, A. (2006). Vulnerability to mild predator stress in serotonin transporter knockout mice. *Behavioural Brain Research, 170*(1), 126-140. doi: 10.1016/j.bbr.2006.02.012

Alarcon, M., Abrahams, B. S., Stone, J. L., Duvall, J. A., Perederiy, J. V., Bomar, J. M., . . . Geschwind, D. H. (2008). Linkage, association, and gene-expression analyses identify CNTNAP2 as an autism-susceptibility gene. *American Journal of Human Genetics, 82*(1), 150-159. doi: 10.1016/j.ajhg.2007.09.005

American Psychiatric Association. (2013). Diagnostic and statistical manual of mental disorders(5th ed.). Arlington, VA: American Psychiatric Publishing.

Aronson, M., Hagberg, B., & Gillberg, C. (1997). Attention deficits and autistic spectrum problems in children exposed to alcohol during gestation: a follow-up study. *Develpmental Medicine and Child Neurology, 39*(9), 583-587.

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. doi: 10.1016/j.brainres.2011.06.015

Bauman, M. L., & Kemper, T. L. (2005). Neuroanatomic observations of the brain in autism: a review and future directions. *International Journal of Devevelopmental Neuroscience, 23*(2-3), 183-187. doi: 10.1016/j.ijdevneu.2004.09.006

Berger, M., Gray, J. A., & Roth, B. L. (2009). The expanded biology of serotonin. *Annual Review of Medicine, 60*, 355-366. doi: 10.1146/annurev.med.60.042307.110802

Bill, B. R., & Geschwind, D. H. (2009). Genetic advances in autism: heterogeneity and convergence on shared pathways. *Current Opinion in Genetics & Development, 19*(3), 271-278. doi:10.1016/j.gde.2009.04.004

Birnbaum, A. K., Ahn, J., Brundage, R. C., Conway, J. M., Hardie, N. A., Bowers, S. E., & Leppik, I. E. (2004). Population pharmacokinetics of total valproic acid (VPA) concentrations in elderly nursing home residents. *Epilepsia, 45*, 118-119.

Bonnin, A., Goeden, N., Chen, K., Wilson, M. L., King, J., Shih, J. C., . . . Levitt, P. (2011). A transient placental source of serotonin for the fetal forebrain. *Nature, 472*(7343), 347-U246. doi:10.1038/Nature09972

Bonnin, A., & Levitt, P. (2011). Fetal, Maternal, and Placental Sources of Serotonin and New Implications for Developmental Programming of the Brain. *Neuroscience, 197*, 1-7. doi:10.1016/j.neuroscience.2011.10.005

Buxbaum, J. D., Silverman, J. M., Smith, C. J., Kilifarski, M., Reichert, J., Hollander, E., . . . Davis, K. L. (2001). Evidence for a susceptibility gene for autism on chromosome 2 and for genetic heterogeneity. *American Journal of Human Genetics, 68*(6), 1514-1520. doi:10.1086/320588

Caspi, A., & Moffitt, T. E. (2006). Opinion - Gene-environment interactions in psychiatry: joining forces with neuroscience. *Nature Reviews Neuroscience, 7*(7), 583-590. doi: 10.1038/Nrn1925

Caspi, A., Moffitt, T. E., Cannon, M., McClay, J., Murray, R., Harrington, H., . . . Craig, I. W. (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. doi:10.1016/j.biopsych.2005.01.026

Chugani, D. C., Muzik, O., Rothermel, R., Behen, M., Chakraborty, P., Mangner, T., . . . Chugani, H. T. (1997). Altered serotonin synthesis in the dentatothalamocortical

pathway in autistic boys. *Annals of Neurology*, *42*(4), 666-669. doi: 10.1002/ana.410420420

Clarke, M. C., Tanskanen, A., Huttunen, M. O., Murray, R., & Cannon, M. (2009). The Developmental Trajectory to Schizophrenia-Evidence for Genetic and Environmental Influences. *Schizophrenia Bulletin, 35*, 70-71.

Cochrane, L. E., Tansey, K. E., Gill, M., Gallagher, L., & Anney, R. J. L. (2010). Lack of Association Between Markers in the ITGA3, ITGAV, ITGA6 and ITGB3 and Autism in an Irish Sample. *Autism Research, 3*(6), 342-344. doi: 10.1002/Aur.157

Croen, L. A., Grether, J. K., Yoshida, C. K., Odouli, R., & Hendrick, V. (2011). Antidepressant Use During Pregnancy and Childhood Autism Spectrum Disorders. *Archives of General Psychiatry, 68*(11), 1104-1112. doi: 10.1001/archgenpsychiatry.2011.73

Davis, E., Fennoy, I., Laraque, D., Kanem, N., Brown, G., & Mitchell, J. (1992). Autism and developmental abnormalities in children with perinatal cocaine exposure. *Journal of the National Medical Association, 84*(4), 315-319.

Daws, L. C. (2009). Unfaithful neurotransmitter transporters: Focus on serotonin uptake and implications for antidepressant efficacy. *Pharmacology & Therapeutics, 121*(1), 89-99. doi: 10.1016/j.pharmthera.2008.10.004

Dendrinos, G., Hemelt, M., & Keller, A. (2011). Prenatal VPA Exposure and Changes in Sensory Processing by the Superior Colliculus. *Frontiers in Integrative Neuroscience, 5*, 68. doi: 10.3389/fnint.2011.00068

Devlin, B., Cook, E. H., Coon, H., Dawson, G., Grigorenko, E. L., McMahon, W., . . . Network, C. G. (2005). Autism and the serotonin transporter: the long and short of it. *Molecular Psychiatry, 10*(12), 1110-1116. doi: 10.1038/sj.mp.4001724

DiCicco-Bloom, E., Lord, C., Zwaigenbaum, L., Courchesne, E., Dager, S. R., Schmitz, C., . . . Young, L. J. (2006). The developmental neurobiology of autism spectrum disorder. *Journal of Neuroscience, 26*(26), 6897-6906. doi:10.1523/Jneurosci.1712-06.2006

Dufour-Rainfray, D., Vourc'h, P., Le Guisquet, A. M., Garreau, L., Ternant, D., Bodard, S., . . . Guilloteau, D. (2010). Behavior and serotonergic disorders in rats exposed prenatally to valproate: A model for autism. *Neuroscience Letters, 470*(1), 55-59. doi: 10.1016/j.neulet.2009.12.054

Evan, G. I., & Vousden, K. H. (2001). Proliferation, cell cycle and apoptosis in cancer. *Nature, 411*(6835), 342-348. doi: 10.1038/35077213

Fombonne, E. (1999). The epidemiology of autism: a review. *Psychological Medicine, 29*(4), 769-786.

Fombonne, E. (2003). The prevalence of autism. *Journal of the American Medical Association, 289*(1), 87-89.

Frith, C. D., & Frith, U. (1999). Cognitive psychology - Interacting minds - A biological basis. *Science, 286*(5445), 1692-1695. doi:10.1126/science.286.5445.1692

Ganz, M. L. (2007). The lifetime distribution of the incremental societal costs of autism. *Archives of Pediatrics and Adolescent Medicine, 161*(4), 343-349. doi: 10.1001/archpedi.161.4.343

Geschwind, D. H. (2008). Autism: Many Genes, Common Pathways? *Cell, 135*(3), 391-395. doi: 10.1016/j.cell.2008.10.016

Göttlicher, M. (2003). Valproic acid: an old drug newly discovered as inhibitor of histone deacetylases. *Annals of Hematology, 83*, S91-92.

Grunstein, M. (1997). Histone acetylation in chromatin structure and transcription. *Nature, 389*(6649), 349-352. doi:10.1038/38664

Guerini, F. R., Manca, S., Sotgiu, S., Tremolada, S., Zanzottera, M., Agliardi, C., . . . Ferrante, P. (2006). A family based linkage analysis of HLA and 5-HTTLPR gene

polymorphisms in Sardinian children with autism spectrum disorder. *Human Immunology, 67*(1-2), 108-117. doi: 10.1016/j.humimm.2006.02.033

Hadjikhani, N. (2010). Serotonin, pregnancy and increased autism prevalence: Is there a link? *Medical Hypotheses, 74*(5), 880-883. doi: 10.1016/j.mehy.2009.11.015

Herbert, M. R. (2010a). Contributions of the environment and environmentally vulnerable physiology to autism spectrum disorders. *Current Opinion in Neurology, 23*(2), 103-110. doi: 10.1097/WCO.0b013e328336a01f

Herbert, M. R. (2010b). Environment and vulnerable physiology in autism. *European Neuropsychopharmacology, 20*, S177-S177.

Herbert, M. R., Russo, J. P., Yang, S., Roohi, J., Blaxill, M., Kahler, S. G., . . . Hatchwell, E. (2006). Autism and environmental genomics. *Neurotoxicology, 27*(5), 671-684. doi: 10.1016/j.neuro.2006.03.017

Hill, E. L. (2004). Executive dysfunction in autism. *Trends in Cognitive Sciences, 8*(1), 26-32. doi: 10.1016/j.tics.2003.11.003

Hogg, S. (1996). A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. *Pharmacology Biochemistry and Behavior, 54*(1), 21-30. doi:10.1016/0091-3057(95)02126-4

Holmes, A., Li, Q., Murphy, D. L., Gold, E., & Crawley, J. N. (2003). Abnormal anxiety-related behaviour in serotonin transporter null mutant mice: the influence of genetic background. *Genes Brain and Behavior, 2*(6), 365-380. doi: 10.1046/j.1601-183X.2003.00050.x

Holmes, A., Murphy, D. L., & Crawley, J. N. (2003). Abnormal behavioral phenotypes of serotonin transporter knockout mice: Parallels with human anxiety and depression. *Biological Psychiatry, 54*(10), 953-959. doi:10.1016/j.biopsych.2003.09.003

Homberg, J. R., Olivier, J. D. A., Smits, B. M. G., Mul, J. D., Mudde, J., Verheul, M., . . . Clippen, 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: 10.1016/j.neuroscience.2007.03.030

Hoyer, D., Hannon, J. P., & Martin, G. R. (2002). Molecular, pharmacological and functional diversity of 5-HT receptors. *Pharmacology Biochemistry and Behavior, 71*(4), 533-554. doi: Pii S0091-3057(01)00746-8

Humphries, S. E., Talmud, P. J., Hawe, E., Bolla, M., Day, I. N. M., & Miller, G. J. (2001). Apolipoprotein E4 and coronary heart disease in middle-aged men who smoke: a prospective study. *Lancet, 358*(9276), 115-119. doi: 10.1016/S0140-6736(01)05330-2

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. doi: 10.1016/S0892-0362(99)00083-5

Jarbrink, K., & Knapp, M. (2001). The economic impact of autism in Britain. *Autism, 5*(1), 7-22.

Joeyen-Waldorf, J., Edgar, N., & Sibille, E. (2009). The roles of sex and serotonin transporter levels in age- and stress-related emotionality in mice. *Brain Research, 1286*, 84-93. doi: 10.1016/j.brainres.2009.06.079

Kahne, D., Tudorica, A., Borella, A., Shapiro, L., Johnstone, F., Huang, W., & Whitaker-Azmitia, P. M. (2002). Behavioral and magnetic resonance spectroscopic studies in the rat hyperserotonemic model of autism. *Physiology & Behavior, 75*(3), 403-410. doi: Pii S0031-9384(01)00673-4

Kalueff, A. V., Fox, M. A., Gallagher, P. S., & Murphy, D. L. (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. doi: 10.1111/j.1601-183X.2006.00270.x

Kaminsky, Z., Wang, S. C., & Petronis, A. (2006). Complex disease, gender and epigenetics. *Annals of Medicine, 38*(8), 530-544. doi: 10.1080/07853890600989211

Kanner, L. (1943). Autistic Disturbances of Affective Contact. *Nervous Child, 2*(3), 217-250.

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. *International Journal of Neuropsychopharmacology, 16*(1), 91-103. doi: 10.1017/S1461145711001714

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.

Kim, P., Kim, K. C., Jeon, S. J., Han, S. Y., & Shin, C. Y. (2011). Gender differences in social interaction but not crooked tail phenotype in prenatally valproic acid exposed rats. *Molecular Biology of the Cell, 22*.

Kim, S. J., Cox, N., Courchesne, R., Lord, C., Corsello, C., Akshoomoff, N., . . . Cook, E. H. (2002). Transmission disequilibrium mapping at the serotonin transporter gene (SLC6A4) region in autistic disorder. *Molecular Psychiatry, 7*(3), 278-288. doi: 10.1038/sj/mp/4001033

Kinast, K., Peeters, D., Kolk, S. M., Schubert, D., & Homberg, J. R. (2013). Genetic and pharmacological manipulations of the serotonergic system in early life: neurodevelopmental underpinnings of autism-related behavior. *Frontiers in Cellular Neuroscience, 7*. doi: 10.3389/Fncel.2013.00072

Knapp, M., Romeo, R., & Beecham, J. (2009). Economic cost of autism in the UK. *Autism, 13*(3), 317-336. doi: 10.1177/1362361309104246

Kogan, M. D., Blumberg, S. J., Schieve, L. A., Boyle, C. A., Perrin, J. M., Ghandour, R. M., . . . van Dyck, P. C. (2009). Prevalence of parent-reported diagnosis of autism spectrum disorder among children in the US, 2007. *Pediatrics, 124*(5), 1395-1403. doi: 10.1542/peds.2009-1522

Kohane, I. S., McMurry, A., Weber, G., MacFadden, D., Rappaport, L., Kunkel, L., . . . Churchill, S. (2012). The Co-Morbidity Burden of Children and Young Adults with Autism Spectrum Disorders. *Plos One, 7*(4). doi: 10.1371/journal.pone.0033224

Kohl, S., Wolters, C., Gruendler, T. O. J., Vogeley, K., Klosterkotter, J., & Kuhn, J. (2014). Prepulse Inhibition of the Acoustic Startle Reflex in High Functioning Autism. *Plos One, 9*(3). DOI 10.1371/journal.pone.0092372

Koishi, S., Yamamoto, K., Matsumoto, H., Koishi, S., Enseki, Y., Oya, A., . . . Yamazaki, K. (2006). Serotonin transporter gene promoter polymorphism and autism: A family-based genetic association study in Japanese population. *Brain & Development, 28*(4), 257-260. doi: 10.1016/j.braindev.2005.09.003

Lagace, D. C., O'Brien, W. T., Gurvich, N., Nachtigal, M. W., & Klein, P. S. (2004). Valproic acid: how it works. Or not. *Clinical Neuroscience Research, 4*(3-4), 215-225. doi: 10.1016/j.cnr.2004.09.013

Lam, K. S. L., Aman, M. G., & Arnold, L. E. (2006). Neurochemical correlates of autistic disorder: A review of the literature. *Res Dev Disabil, 27*(3), 254-289. doi:10.1016/j.ridd.2005.03.003

Le Couteur, A., Bailey, A., Goode, S., Pickles, A., Robertson, S., Gottesman, I., & Rutter, M. (1996). A broader phenotype of autism: the clinical spectrum in twins. *J Child Psychol Psychiatry, 37*(7), 785-801.

Leslie, D. L., & Martin, A. (2007). Health care expenditures associated with autism spectrum disorders. *Archives of Pediatrics and Adolescent Medicine, 161*(4), 350-355. doi: 10.1001/archpedi.161.4.350

Levy, S. E., Mandell, D. S., & Schultz, R. T. (2009). Autism. *Lancet, 374*(9701), 1627-1638. doi: 10.1016/S0140-6736(09)61376-3

Lira, A., Zhou, M. M., Castanon, N., Ansorge, M. S., Gordon, J. A., Francis, J. H., . . . Gingrich, J. A. (2003). Altered depression-related behaviors and functional changes in the dorsal raphe nucleus of serotonin transporter-deficient mice. *Biological Psychiatry, 54*(10), 960-971. doi: 10.1016/S0006-3223(03)00696-6

Loscher, W. (1999). Valproate: A reappraisal of its pharmacodynamic properties and mechanisms of action. *Progress in Neurobiology, 58*(1), 31-59. doi: 10.1016/S0301-0082(98)00075-6

Makkonen, I., Riikonen, R., Kokki, H., Airaksinen, M. M., & Kuikka, J. T. (2008a). Serotonin and dopamine transporter binding in children with autism determined by SPECT. *Developmental Medicine and Child Neurology, 50*(8), 593-597. doi: 10.1111/j.1469-8749.2008.03027.x

Markram, K., Rinaldi, T., La Mendola, D., Sandi, C., & Markram, H. (2008). Abnormal fear conditioning and amygdala processing in an animal model of autism. *Neuropsychopharmacology, 33*(4), 901-912. doi: 10.1038/sj.npp.1301453

Matson, J. L., & Nebel-Schwalm, M. S. (2007). Comorbid psychopathology with autism spectrum disorder in children: an overview. *Research in Developmental Disabilities, 28*(4), 341-352. doi: 10.1016/j.ridd.2005.12.004

Mawhinney, E., Campbell, J., Craig, J., Russell, A., Smithson, W., Parsons, L., . . . Morrow, J. (2012). Valproate and the risk for congenital malformations: Is formulation and dosage regime important? *Seizure, 21*(3), 215-218. doi: 10.1016/j.seizure.2012.01.005

McAlonan, G. M., Daly, E., Kumari, V., Critchley, H. D., van Amelsvoort, T., Suckling, J., . . . Murphy, D. G. M. (2002). Brain anatomy and sensorimotor gating in Asperger's syndrome. *Brain, 125*, 1594-1606. doi: 10.1093/Brain/Awf150

McCauley, J. L., Olson, L. M., Dowd, M., Amin, T., Steele, A., Blakely, R. D., . . . Sutcliffe, J. S. (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, 127B*(1), 104-112. doi: 10.1002/Ajmg.B.20151

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), 993-1000.

McLaren, I. P. L., & Mackintosh, N. J. (2000). An elemental model of associative learning: I. Latent inhibition and perceptual learning. *Animal Learning & Behavior, 28*(3), 211-246. doi: 10.3758/Bf03200258

McNamara, I. M., Borella, A. W., Bialowas, L. A., & Whitaker-Azmitia, P. M. (2008). Further studies in the developmental hyperserotonemia model (DHS) of autism: social, behavioral and peptide changes. [Research Support, Non-U.S. Gov't]. *Brain Res, 1189*, 203-214. doi: 10.1016/j.brainres.2007.10.063

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*(2-3), 287-297. doi: 10.1016/j.ijdevneu.2004.05.004

Moffitt, T. E., Caspi, A., & Rutter, M. (2005). Strategy for investigating interactions between measured genes and measured environments. *Archives of General Psychiatry, 62*(5), 473-481. doi: 10.1001/archpsyc.62.5.473

Moore, S. J., Turnpenny, P., Quinn, A., Glover, S., Lloyd, D. J., Montgomery, T., & Dean, J. C. (2000). A clinical study of 57 children with fetal anticonvulsant syndromes. *Journal of Medical Genetics, 37*(7), 489-497.

Narita, M., Oyabu, A., Imura, Y., Kamada, N., Yokoyama, T., Tano, K., . . . Narita, N. (2010). Nonexploratory movement and behavioral alterations in a thalidomide or valproic acid-induced autism model rat. *Neuroscience Research, 66*(1), 2-6. doi: 10.1016/j.neures.2009.09.001

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. doi: 10.1203/00006450-200210000-00018

Nijmeijer, J. S., Hartman, C. A., Rommelse, N. N. J., Altink, M. E., Buschgens, C. J. M., Fliers, E. A., . . . Hoekstra, P. J. (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. doi: 10.1111/j.1469-7610.2010.02277.x

Olivier, J. D. A., Van Der Hart, M. G. C., Van Swelm, R. P. L., Dederen, P. J., Homberg, J. R., Cremers, T., . . . Ellenbroek, B. A. (2008). A study in male and female 5-HT transporter knockout rats: An animal model for anxiety and depression disorders. *Neuroscience, 152*(3), 573-584. doi: 10.1016/j.neuroscience.2007.12.032

Oranje, B., Lahuis, B., van Engeland, H., van der Gaag, R. J., & Kemner, C. (2013). Sensory and sensorimotor gating in children with multiple complex developmental disorders (MCDD) and autism. *Psychiatry Research, 206*(2-3), 287-292. doi: 10.1016/j.psychres.2012.10.014

Orekhova, E. V., Stroganova, T. A., Prokofyev, A. O., Nygren, G., Gillberg, C., & Elam, M. (2008). Sensory gating in young children with autism: Relation to age, IQ, and EEG gamma oscillations. *Neuroscience Letters, 434*(2), 218-223. doi: 10.1016/j.neulet.2008.01.066

Pardo, C. A., Vargas, D. L., & Zimmerman, A. W. (2005). Immunity, neuroglia and neuroinflammation in autism. *International Review of Psychiatry, 17*(6), 485-495.

Patterson, P. H. (2009). Immune involvement in schizophrenia and autism: Etiology, pathology and animal models. *Behavioural Brain Research, 204*(2), 313-321. doi: 10.1016/j.bbr.2008.12.016

Pellow, S., Chopin, P., File, S. E., & Briley, M. (1985). Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *Journal of Neuroscience Methods, 14*(3), 149-167.

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: 10.1016/j.biopsych.2005.09.025

Persico, A. M., & Bourgeron, T. (2006). Searching for ways out of the autism maze: genetic, epigenetic and environmental clues. *Trends Neurosci, 29*(7), 349-358. doi: 10.1016/j.tins.2006.05.010

Persico, A. M., & Napolioni, V. (2013). Autism genetics. *Behavioural Brain Research, 251*, 95-112. doi: 10.1016/j.bbr.2013.06.012

Pessah, I. N., & Lein, P. J. (2008). Evidence for environmental susceptibility in autism *Autism* (pp. 409-428): Springer.

Pessah, I. N., Seegal, R. F., Lein, P. J., LaSalle, J., Yee, B. K., Van De Water, J., & Berman, R. F. (2008). Immunologic and neurodevelopmental susceptibilities of autism. *Neurotoxicology, 29*(3), 532-545. doi: 10.1016/j.neuro.2008.02.006

Ploeger, A., Raijmakers, M. E. J., van der Maas, H. L. J., & Galis, F. (2010). The Association Between Autism and Errors in Early Embryogenesis: What Is the Causal Mechanism? *Biological Psychiatry, 67*(7), 602-607. doi: 10.1016/j.biopsych.2009.10.010

Preissler, M. A. (2008). Associative learning of pictures and words by low-functioning children with autism. *Autism, 12*(3), 231-248. doi: 10.1177/1362361307088753

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. doi: 10.1016/j.biopsych.2006.01.009

Rasalam, A. D., Hailey, H., Williams, J. H. G., Moore, S. J., Turnpenny, P. D., Lloyd, D. J., & Dean, J. C. S. (2005). Characteristics of fetal anticonvulsant syndrome associated autistic disorder. *Developmental Medicine and Child Neurology, 47*(8), 551-555. doi: 10.1017/S0012162205001076

Rice, D., & Barone, S. (2000). Critical periods of vulnerability for the developing nervous system: Evidence from humans and animal models. *Environmental Health Perspectives, 108*, 511-533. doi: 10.2307/3454543

Roullet, F. I., Lai, J. K. Y., & Foster, J. A. (2013). In utero exposure to valproic acid and autism - A current review of clinical and animal studies. *Neurotoxicology and Teratology, 36*, 47-56. doi: 10.1016/j.ntt.2013.01.004

Sabers, A., Bertelsen, F. C. B., Scheel-Kruger, J., Nyengaard, J. R., & Moller, A. (2014). Long-term valproic acid exposure increases the number of neocortical neurons in the developing rat brain. A possible new animal model of autism. *Neuroscience Letters, 580*, 12-16. doi: 10.1016/j.neulet.2014.07.036

Schneider, T., & Przewlocki, R. (2005). Behavioral alterations in rats prenatally exposed to valproic acid: Animal model of autism. *Neuropsychopharmacology, 30*(1), 80-89. doi: 10.1038/sj.npp.1300518

Schneider, T., Roman, A., Basta-Kaim, A., Kubera, M., Budziszewska, B., Schneider, K., & Przewtocki, 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. doi: 10.1016/j.psyneuen.2008.02.011

Schneider, T., Turczak, J., & Przewlocki, R. (2005). Environmental enrichment reverses behavioral alterations in rats prenataly exposed to valproic acid. *Behavioural Pharmacology, 16*, S33-S33. doi: 10.1097/00008877-200509001-00105

Schneider, T., Ziolkowska, B., Gieryk, A., Tyminska, A., & Przewlocki, R. (2007). Prenatal exposure to valproic acid disturbs the enkephalinergic system functioning, basal hedonic tone, and emotional responses in an animal model of autism. *Psychopharmacology, 193*(4), 547-555. doi: 10.1007/s00213-007-0795-y

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 and Adolescent Psychiatry, 47*(8), 921-929. doi: 10.1097/Chi.0b013e318179964f

Smits, B. M. G., Mudde, J. B., van de Belt, J., Verheul, M., Olivier, J., Homberg, J., . . . Cuppen, E. (2006). Generation of gene knockouts and mutant models in the laboratory rat by ENU-driven target-seletted mutagenesis. *Pharmacogenetics and Genomics, 16*(3), 159-169.

Stokstad, E. (2001). New hints into the biological basis of autism. *Science, 294*(5540), 34-37. doi: 10.1126/science.294.5540.34

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. doi: 10.1016/j.rasd.2008.04.006

Swerdlow, N. R., Geyer, M. A., & Braff, D. L. (2001). Neural circuit regulation of prepulse inhibition of startle in the rat: current knowledge and future challenges. *Psychopharmacology, 156*(2-3), 194-215.

Tordjman, S., Gutknecht, L., Carlier, M., Spitz, E., Antoine, C., Slama, F., . . . Anderson, G. M. (2001). Role of the serotonin transporter gene in the behavioral expression of autism. *Molecular Psychiatry, 6*(4), 434-439. doi: 10.1038/sj.mp.4000873

Tsuang, M. T., Bar, J. L., Stone, W. S., & Faraone, S. V. (2004). Gene-environment interactions in mental disorders. *World Psychiatry, 3*(2), 73.

Veenstra-VanderWeele, J., & Blakely, R. D. (2012). Networking in Autism: Leveraging Genetic, Biomarker and Model System Findings in the Search for New Treatments. *Neuropsychopharmacology, 37*(1), 196-212. doi: 10.1038/Npp.2011.185

Veenstra-VanderWeele, J., Muller, C. L., Iwamoto, H., Sauer, J. E., Owens, W. A., Shah, C. R., . . . Blakely, R. D. (2012). Autism gene variant causes hyperserotonemia, serotonin receptor hypersensitivity, social impairment and repetitive behavior. *Proceedings of the National Academy of Sciences of the United States of America, 109*(14), 5469-5474. doi: 10.1073/pnas.1112345109

Volkmar, F. R., & Pauls, D. (2003). Autism. *Lancet, 362*(9390), 1133-1141. doi: 10.1016/S0140-6736(03)14471-6

Whitaker-Azmitia, P. M. (2001). Serotonin and brain development: Role in human developmental diseases. *Brain Research Bulletin, 56*(5), 479-485. doi: 10.1016/S0361-9230(01)00615-3

Whitaker-Azmitia, P. M. (2005). Behavioral and cellular consequences of increasing serotonergic activity during brain development: a role in autism? *International Journal of Developmental Neuroscience, 23*(1), 75-83. doi: 10.1016/j.ijdevneu.2004.07.022

Whitaker-Azmitia, P. M., & Azmitia, E. C. (1986). Autoregulation of fetal serotonergic neuronal development: role of high affinity serotonin receptors.*Neuroscience Letters, 67*(3), 307-312.

White, S. W., Oswald, D., Ollendick, T., & Scahill, L. (2009). Anxiety in children and adolescents with autism spectrum disorders. *Clinical Psychology Review, 29*(3), 216-229. doi: 10.1016/j.cpr.2009.01.003

Williams, G., King, J., Cunningham, M., Stephan, M., Kerr, B., & Hersh, J. H. (2001). Fetal valproate syndrome and autism: additional evidence of an association. *Developmental Medicine and Child Neurology, 43*(3), 202-206.

Wohr, M., & Scattoni, M. L. (2013). Behavioural methods used in rodent models of autism spectrum disorders: Current standards and new developments. *Behavioural Brain Research, 251*, 5-17. doi: 10.1016/j.bbr.2013.05.047

Wu, S. P., Guo, Y. Q., Jia, M. X., Ruan, Y., Shuang, M., Liu, J., . . . Zhang, D. (2005). Lack of evidence for association between the serotonin transporter gene (SLC6A4) polymorphisms and autism in the Chinese trios. *Neuroscience Letters, 381*(1-2), 1-5. Doi: 10.1016/j.neulet.2005.01.073

Yamamoto, T., Shimura, T., Sako, N., Yasoshima, Y., & Sakai, N. (1994). Neural Substrates for Conditioned Taste-Aversion in the Rat. *Behavioural Brain Research, 65*(2), 123-137. doi: 10.1016/0166-4328(94)90097-3

Yang, C. J., Tan, H. P., & Du, Y. J. (2014). The Developmental Disruptions of Serotonin Signaling May Involved in Autism during Early Brain Development. *Neuroscience, 267*, 1-10. doi: 10.1016/j.neuroscience.2014.02.021

Yang, M. S., & Gill, M. (2007). A review of gene linkage, association and expression studies in autism and an assessment of convergent evidence. *International Journal of Developmental Neuroscience, 25*(2), 69-85. doi: 10.1016/j.ijdevneu.2006.12.002

Zhang, H., Liu, X., Zhang, C., Mundo, E., Macciardi, F., Grayson, D. R., . . . Holden, J. J. A. (2002). Reelin gene alleles and susceptibility to autism spectrum disorders. *Molecular Psychiatry, 7*(9), 1012-1017. doi: 10.1038/sj.mp.4001124

Appendix

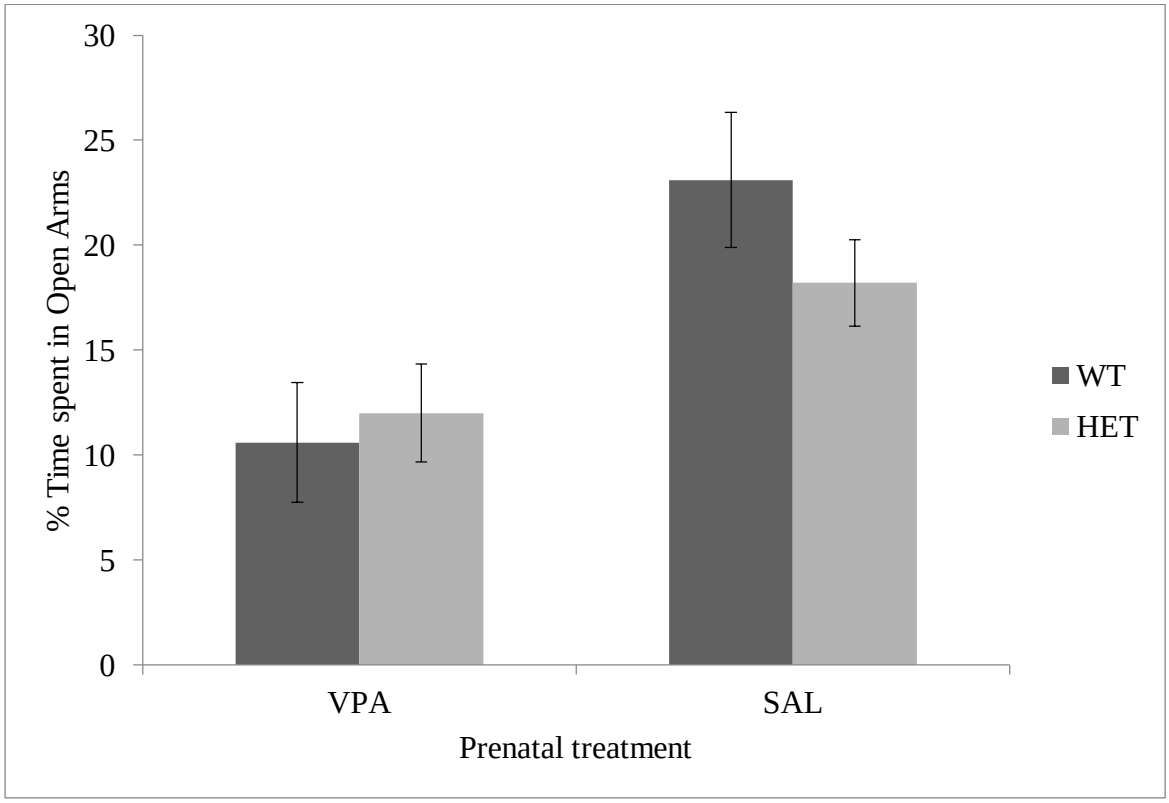

Figure 1. Average percentage of time spent in the Open Arms of the Elevated Plus-Maze for rats prenatally exposed to Valproate (VPA) or saline with heterozygous (HZ) or wildtype (WT) serotonin transporter knockout genotypes. Data shows mean ± SEM.

Table 1

Average Performance on the Elevated Plus-Maze for Rats by Prenatal Treatment and Genotype

	VPA on G12.5			Saline on G12.5		
	M	*SD*	*n*	*M*	*SD*	*n*
Entries made into Open Arms						
Wildtype	59.4%	45.7	12	73.8%	47.8	13
Heterozygous	59.8%	52.6	18	44.2%	31.2	22
Latency to first emerge						
Wildtype	37.9s	82.7	12	12.8s	4.2	13
Heterozygous	38.5s	76.8	18	10.3s	4.0	22

Note. Data shown represents average scores for heterozygous and wildtype serotonin transporter knockout rats prenatally treated with VPA or saline on gestational day 12.5 (G12.5). Number of entries made into open arms is presented as a percentage of the total number of arms entered, both open and closed, while in the Elevated Plus-Maze. Latency to first emerge represents the time taken for the rat to first venture out of the initial arm it was placed in. *M*=Mean, *SD*= Standard Deviation, and *n*= group size.

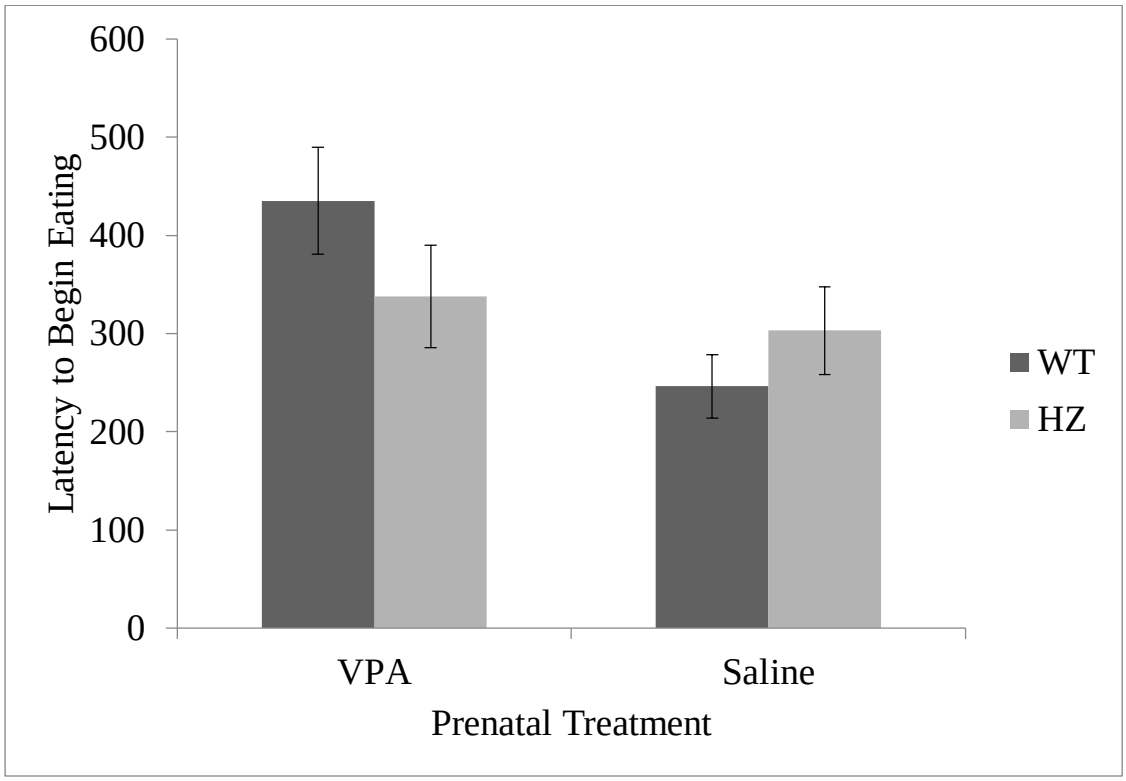

Figure 2. Average latency for rats prenatally exposed to Valproate (VPA) or saline, with Heterozygous (HZ) or wildtype (WT) serotonin transporter knockout genotypes, to begin eating in the Novelty Suppressed-Feeding test. Data shows the mean ± SEM.

Table 2

Average Conditioned Taste-Aversion in Rats by Prenatal Treatment, Genotype and Pre-exposure

	VPA on G12.5			Saline on G12.5		
	M	*SD*	*n*	*M*	*SD*	*n*
Wildtype						
Pre-exposed to sucrose	46.5%	14.8	6	29.5%	15.6	7
Non pre-exposed	19.1%	6.1	6	13.4%	3.8	6
Heterozygous						
Pre-exposed to sucrose	30%	20.1	9	22.2%	9.3	9
Non pre-exposed	22.6%	23.8	9	10.6%	5.6	9

Note. Data shows average sucrose solution consumed for heterozygous and wildtype serotonin transporter knockout rats prenatally treated with Valproate (VPA) or saline on gestational day 12.5 (G12.5). Groups split into pre-exposed or not pre-exposed to sucrose solution during preconditioning. Average sucrose solution consumed is presented as the percentage consumed out of the total liquid consumed (water and sucrose solution) on the testing day. *M*= Mean, *SD*= standard Deviation, and *n*= group size

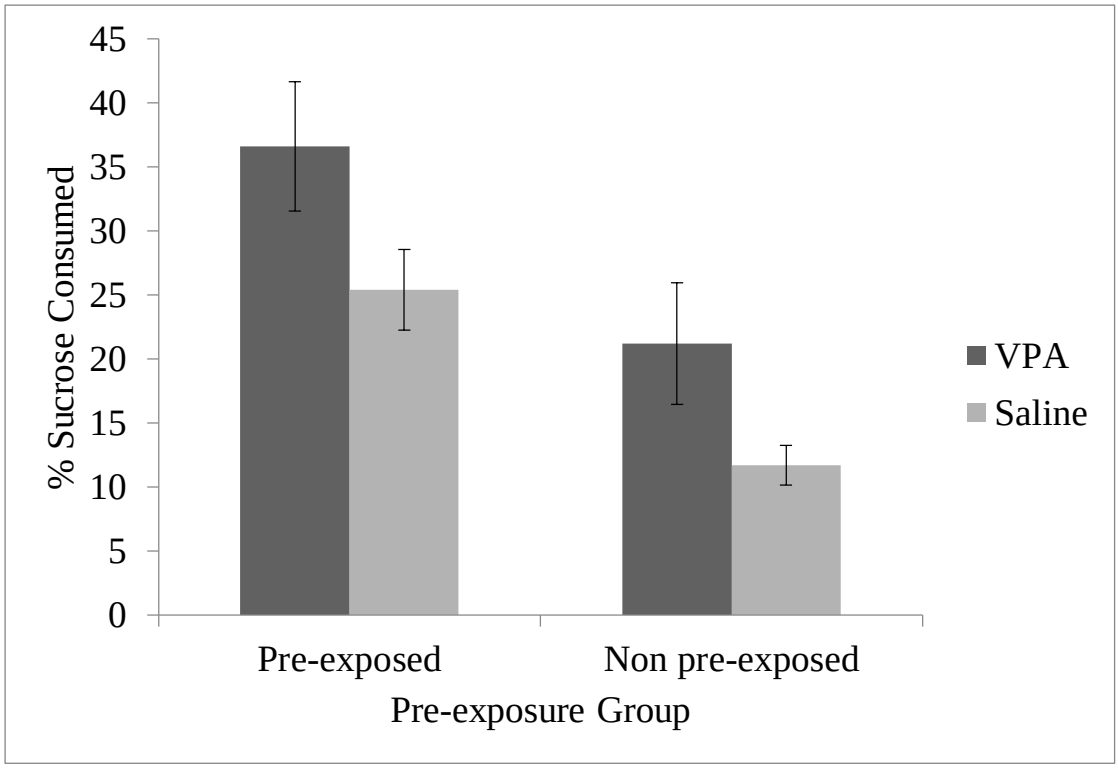

Figure 3. The percentage of sucrose solution consumed by rats prenatally exposed to Valproate (VPA) or saline, either pre-exposed to sucrose solution or not. Data shows mean ± SEM.

Table 3

Average Startle Response and Habituation for Rats by Prenatal Treatment and Genotype

	VPA on E12.5			Saline on E12.5		
	M	*SD*	*n*	*M*	*SD*	*n*
P120						
Wildtype	316.2	291.8	13	892.7	1199.2	15
Heterozygous	560.8	614.8	12	917	1547.6	12
Habituation (%)						
Wildtype	57.7	38.8	13	51.3	39	15
Heterozygous	82.1	158.9	12	45.6	33.9	12

Note. Data shows average basal startle response (P120) and total percent habituation for heterozygous and wildtype serotonin transporter knockout rats prenatally treated with Valproate (VPA) or saline on gestational day 12.5 (G12.5). Basal startle response shows the average displacement for the first block of startle stimulus presentation only trials. Habituation shows the percent decrease in startle response between the first and second blocks of startle stimulus only trials. *M*= Mean, *SD*= Standard Deviation, and *n*=group size.

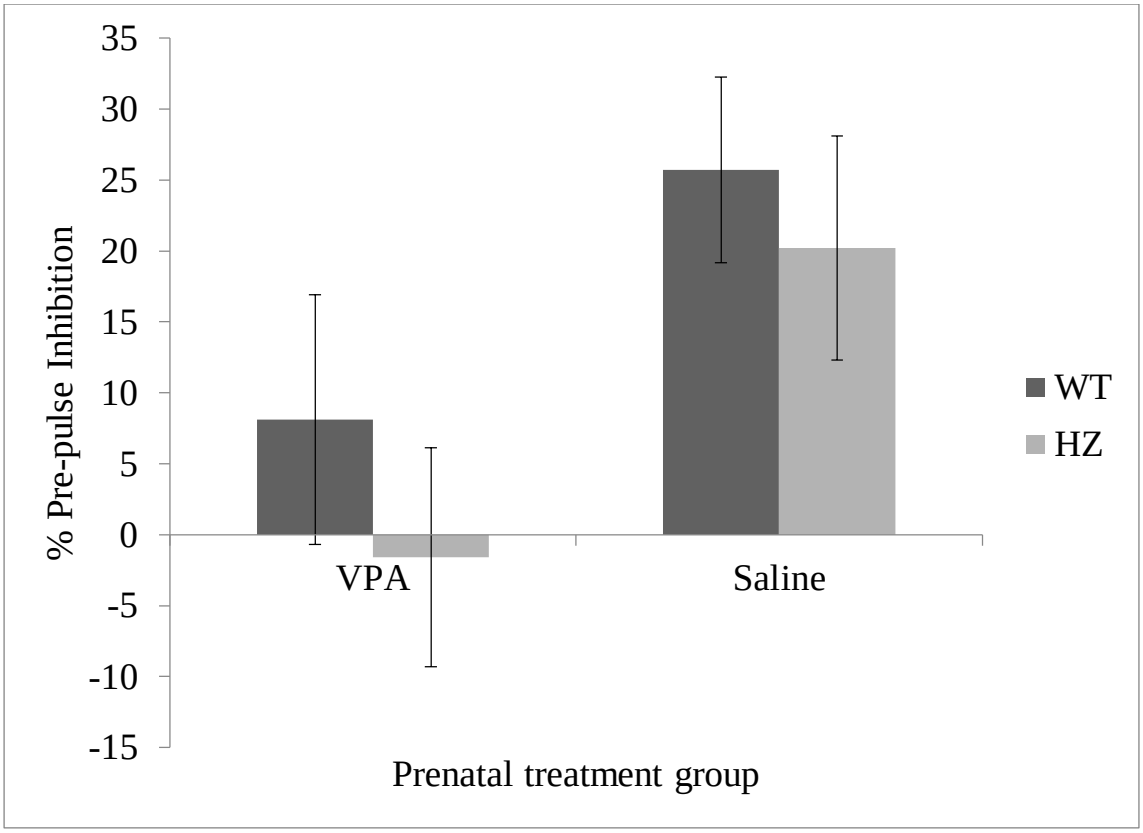

Figure 4. Average percent prepulse inhibition to acoustic startle for rats prenatally exposed to Valproate (VPA) or saline, with heterozygous (HZ) or wildtype (WT) serotonin transporter knockout genotypes. Data shows the mean ± SEM.