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The effects of pre- and postnatal administration of propionic acid and lipopolysaccharide on the behaviour of adolescent male and female rats

Kelly A. Foley
The University of Western Ontario

Supervisor
Peter Ossenkopp
The University of Western Ontario Joint Supervisor
Dr. Martin Kavaliers
The University of Western Ontario

Graduate Program in Neuroscience
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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THE EFFECTS OF PRE- AND POSTNATAL ADMINISTRATION OF PROPIONIC ACID
AND LIPOPOLYSACCHARIDE ON THE BEHAVIOUR OF ADOLESCENT MALE AND
FEMALE RATS

(Thesis format: Integrated Article)

by

Kelly A. Foley

Graduate Program in Neuroscience

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Neuroscience

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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ABSTRACT

Increasing evidence suggests the gut microbiome plays an important role in immune functioning and neurodevelopment. Metabolic products of enteric bacteria, such as short chain fatty acids and lipopolysaccharides, may alter development and subsequent behaviour. Altered microbiome composition, including elevated short chain fatty acids, and/or immune system dysfunction has been observed in children with autism spectrum disorders (ASD). This thesis describes the effects of prenatal propionic acid (PPA), a short chain fatty acid and metabolic fermentation product of antibiotic resistant enteric bacteria, and of prenatal lipopolysaccharide (LPS), a bacterial mimetic and product of enteric bacteria, on a range of behaviours in male and female neonatal, adolescent and adult rats. Long-Evans rats were administered PPA or LPS during pregnancy and rat pups were administered PPA in the second week of life. The first study evaluated the effects of prenatal PPA and LPS, and postnatal PPA, on developmental milestones in early life and on locomotor activity, repetitive behaviour, and anxiety-related behaviour in adolescent rats. Secondly, sensorimotor behaviours were examined using the acoustic startle response and prepulse inhibition. The final study investigated the effects of prenatal PPA and LPS on social and related behaviours in neonatal, adolescent, and adult rats. Overall, prenatal and postnatal treatments subtly altered behaviour in a sex- and test-specific manner. Male and female rats showed developmental delay in day of eye opening and in acquiring a nest seeking odor discrimination. Prenatal and postnatal treatments increased anxiety-related behaviour and altered acoustic startle responses in male and female adolescent rats. Male rats displayed alterations in social behaviour and locomotor activity that was not observed in female rats, supporting the male bias seen in ASD. However, female rats showed sensitivity to PPA, displaying repetitive behaviour, altered acoustic startle response, and decreased prepulse inhibition. There is evidence to suggest that these behaviours are more severe in females diagnosed with ASD. These findings demonstrate that the metabolic products of enteric bacteria, PPA and LPS, may alter development in ways resembling ASD and contribute to the growing literature on the importance of the gut microbiome and its components on influencing brain and behaviour.

Keywords: autism; sex differences; neurodevelopment; microbiome; short chain fatty acid; maternal immune activation; locomotor activity; anxiety; acoustic startle response; prepulse inhibition; novelty response; social behaviour; adolescence

Statement of Co-Authorship

All experimental work was carried out by Kelly Foley with the exception of assistance from Alisha Vaz who aided in the collection and analysis of the data presented in Chapter 4. Drs. Klaus-Peter Ossenkopp, Martin Kavaliers, and Derrick MacFabe contributed to the design of the experiments presented here (Chapters 2-4) and the editing of the manuscripts. When submitted for publication, the authors for Chapter 2 will be: Foley, Ossenkopp, Kavaliers, MacFabe; the authors for Chapter 3 will be: Foley, MacFabe, Kavaliers, Ossenkopp; the authors for Chapter 4 are: Foley, MacFabe, Vaz, Ossenkopp, Kavaliers.

Acknowledgments

Thank you to Dr. Peter Ossenkopp and Dr. Martin Kavaliers for your support and guidance over many years of being in the lab and allowing me to develop as a researcher. This would not have been completed without you and words cannot express my appreciation. Thanks to Dr. Derrick MacFabe for initiating my involvement in the Kilee Patchell-Evans Research Group and for your advice over the years. Thank you to Dr. Susanne Schmid for advice on organizing the acoustic startle and prepulse inhibition data.

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Abbreviations

ASD – autism spectrum disorders	VPA – valproic acid
ASR – acoustic startle response	VT – vertical time
BBB – blood brain barrier	
CNS – central nervous system	
dB – decibel	
EPM – elevated plus maze	
G – gestational day	
GABA – gamma-aminobutyric acid	
GI – gastrointestinal	
GPR – G-protein coupled receptor	
HDAC – histone deacetylase	
IL – interleukin	
ISI – inter-stimulus interval	
ITI – inter-trial interval	
LPS – lipopolysaccharide	
MIA – maternal immune activation	
MT – horizontal movement time	
NM – number of horizontal movements	
NMDA – N-methyl-D-aspartate	
NOR – novel object recognition	
P – postnatal day	
Poly I:C – polyinosinic: polycytidylic acid	
PPA – propionic acid	
PPI – prepulse inhibition	
SC – subcutaneously	
SCFA – short chain fatty acid	
TD – total distance travelled	
VEH – vehicle (phosphate buffered saline)	
VM – number of vertical movements	

Chapter 1
General Introduction

1.1. Microbiome

The gastrointestinal tract (GI) is home to over a trillion commensal bacteria, known as the microbiome, that have a symbiotic relationship with their human hosts. Microbiota benefit from the nutrient-rich environment provided, while the metabolic activities of the microbiota provide essential products and increase our ability to harvest nutrients from food (e.g., vitamins, amino acids, short chain fatty acids). These products can communicate with the central nervous system (CNS) through the enteric nervous system or via metabolites and/or neurotransmitters released into the bloodstream. The microbiome plays an important role in maintaining homeostasis and in the development and functioning of the immune system (Cryan and Dinan, 2012; Nicholson et al., 2012; Round and Mazmanian, 2009). Various environmental factors can influence microbiome composition diversity over time during early life. For example, introducing new foods and antibiotic treatment can alter the bacterial composition of the microbiome, leading to changes in metabolic activity (Bennet et al., 2002; Cho et al., 2012; Koenig et al., 2011).

Increasingly, attention has focused on how the microbiome and gut bacteria influence the CNS and the health of the host. Studies using germ-free mice illustrate the relationship between the microbiome and the CNS. These mice are void of the commensal bacterial populations that conventional mice have. Alterations in brain and behaviour include changes in immediate early gene expression, neurotransmitter turnover and stress responses, and reduced anxiety and social behaviour (Desbonnet et al., 2013; Foster and Neufeld, 2013; Heijtz et al., 2011; Neufeld et al., 2011; Sudo et al., 2004). These and other studies with rodents show that a normal bacterial population is necessary for the development of appropriate humoral and immunological functions.

Maintaining the correct balance of bacterial species is also essential for normal behavioural, metabolic, and neural functioning. For example, introducing Gram-negative bacterial species, such as those of food-borne pathogens, increases anxiety and early gene expression in limbic brain regions involved in anxiety (Bercik et al., 2011; Goehler et al., 2008; Lyte et al., 2006). Antibiotic treatment has been shown to alter the microbiome composition and reverse infection induced anxiety (Bercik et al., 2011). With bidirectional communication between the CNS, immune, and GI systems, GI dysbiosis has been implicated in inflammatory diseases, obesity, and neuropsychiatric health, such

as inflammatory bowel disease, depression, and as discussed here, possibly autism (Cryan and Dinan, 2012; Nicholson et al., 2012).

1.2. Autism spectrum disorders

The prevalence of autism spectrum disorders (ASD) has increased to approximately 1 in 88 children with a male predominance of approximately 4:1 over females (Autism and Developmental Disabilities Monitoring Network Surveillance Year 2008 Principal Investigators, 2012). ASD are currently diagnosed through aberrant behaviours, widely encompassing impairments in communication, social behaviour, and restricted and repetitive behaviour (DiCicco-Bloom et al., 2006). In the Diagnostic and Statistical Manual of Mental Disorders (Fifth Edition, DSM-5), this triad will become a dyad consisting of social communication and repetitive, restrictive behaviour, with sensory abnormalities being incorporated as a core criterion into the repetitive, restrictive behaviour domain. Both hyper- and hypo-sensitivities to stimuli across multiple modalities are reported in ASD (Leekam et al., 2007; Marco et al., 2011). Multiple co-morbidities have been observed in patients with ASD, including anxiety disorders, seizures, and gastrointestinal disturbance, suggesting that ASD is not merely a disorder of the brain but of dysfunction in multiple organ systems (Herbert et al., 2006; Tuchman and Rapin, 2002; Simonoff et al., 2008; Williams et al., 2011).

1.2.1. Etiology of ASD

While the exact causes of ASD are unknown, there is clearly a genetic component to the development of the disorders. Multiple candidate genes have been identified through linkage analyses and *de novo* copy number variations that have been found, implicating a number of processes including synaptic development and neurotransmission, cell signalling and transcription, and immune and mitochondrial function (Cook, Jr. and Scherer, 2008; Geschwind, 2011; Szatmari et al., 2007). Despite these discoveries, known genetic factors exclusively account for approximately 10-20% of ASD cases (Hallmayer et al., 2011; Scherer and Dawson, 2011). Furthermore, concordance rates among monozygotic twins range from 50-90% (Bailey et al., 1995; Hallmayer et al., 2011). This has led to the acceptance that the environment influences the development of ASD, likely acting on underlying genetic susceptibilities during prenatal or early postnatal life (Herbert, 2010).

Multiple risk factors have been suggested to increase the risk for ASD such as preterm birth, maternal and paternal age, and pre-eclampsia/eclampsia (Kolevzon et al., 2007; Mann et al., 2010). A number of environmental agents have been identified which can increase the risk of ASD, including thalidomide, ethanol, and valproic acid (Arndt et al., 2005). Lastly, the immune system has garnered much attention as prenatal infections increase the risk of developing ASD, with viral infection in the first trimester and bacterial infection in the second trimester being associated with ASD (Atladottir et al., 2010).

1.3. Immune abnormalities in ASD

Immune system abnormalities, both central and peripheral, have been found in children with autism and their families. A family history of autoimmune disease has been associated with families of children with ASD (Atladottir et al., 2009; Croen et al., 2005). Plasma of children with ASD has shown an increase in a number of cytokines and chemokines that have been associated with severity of behaviours, including interleukin (IL)-1 β , IL-6, IL-17, IL-23, and monocyte chemoattractant protein (MCP)-1 (Ashwood et al., 2011a; Ashwood et al., 2011b; Enstrom et al., 2009). Additionally, alterations in the adaptive and innate cellular immune responses have been observed in children (see Onore et al., 2012 for review). Infections during pregnancy or early life in genetically susceptible populations may contribute to altered neurodevelopmental processes via release of pro-inflammatory cytokines. The balance between pro- and anti-inflammatory cytokines is important for normal development (e.g., neural cell differentiation and migration), and dysregulation in this balance may have adverse consequences (Deverman and Patterson, 2009). Postmortem analysis of brain tissue of autistic patients has revealed a neuroinflammatory response with increased activated microglia and astrocytes and proinflammatory cytokines (Li et al., 2009; Vargas et al., 2005). Increases in the proinflammatory cytokine IL-6 has been suggested to alter neural processes in autism, mediating the effects of maternal immune infection and neuroinflammatory responses (Parker-Athill and Tan, 2010; Wei et al., 2013).

1.4. Gastrointestinal abnormalities in ASD

A subset of patients with ASD have gastrointestinal (GI) symptoms, with the severity of autistic behaviours associated with the severity of GI dysfunction (Adams et

al., 2011b). These symptoms may be lessened by alterations in diet in some cases (Knivsberg et al., 2002; Pennesi and Klein, 2012). GI symptoms can include increased permeability or inflammation of the intestinal tract, alterations in gut motility, and food allergies/sensitivities (deMagistris et al., 2010; Horvath and Perman, 2002; Jyonouchi et al., 2002; White, 2003). Multiple changes in immune and GI functioning have been observed in children with ASD and GI symptoms, including increases in intestinal pro-inflammatory cytokines (Ashwood et al., 2004; Jyonouchi et al., 2005; Jyonouchi et al., 2011).

Additionally, immune responses can alter the composition of the microbiome of the GI tract, which may contribute to intestinal inflammation and development or maintenance of ASD (Bartlett and Gerding, 2008; Bennet et al., 2002). Indeed, abnormal levels of bacteria flora have been found in the intestinal tract and feces of children with ASD and GI symptoms, including *Clostridia*, *Bacteroidetes*, and *Desulfovibrio* (Finegold et al., 2002; Finegold et al., 2012; Parracho et al., 2005), with other bacterial species implicated as well (Williams et al., 2011; Williams et al., 2012). Interestingly, these anaerobic bacteria are antibiotic-resistant. As such, repeated infections in early life treated with antibiotics may provide an enteric environment that promotes overgrowth of these bacteria (Cho et al., 2012; Finegold et al., 2012) and an association has been made between antibiotic use and autism (Parracho et al., 2005). Alternatively, prenatal infection and/or, in addition, GI disturbances in pregnancy may alter gut bacteria composition. Products of these bacteria as a result of their metabolic activities include elevated levels of a number of compounds, including lipopolysaccharide and short chain fatty acids (SCFA), apart from the amounts of these compounds released from normal carbohydrate metabolism by bacteria in general (Finegold et al., 2010). These compounds may induce inflammatory responses, alter neurodevelopment, and act on the CNS to influence behaviour.

1.5. Environmental animal models of ASD

1.5.1. Maternal immune activation (MIA)

Studies that administer agents that induce an immune response to pregnant rodents are used to investigate the role of prenatal infectious processes in the development of neurodevelopmental disorders such as autism and schizophrenia

(reviewed in Meyer, 2013; Patterson, 2011). To activate the maternal immune response in the absence of a pathogen, the viral mimetic, polyinosinic:polycytidylic acid (poly I:C) or the bacterial mimetic, lipopolysaccharide (LPS) are widely used. These agents activate Toll-like receptors 3 and 4, respectively, to induce an inflammatory response and trigger the release of pro-inflammatory cytokines (Akira and Takeda, 2004).

LPS is the major component of the cell wall of Gram-negative bacteria but is present in low amounts in the GI tract as a product of enteric bacteria. It has been shown that the composition of the gut microbiome and increased levels of LPS can have detrimental effects. For example, infection of germ-free mice with the Gram-negative enteric pathogen (*Campylobacter jejuni*) altered anxiety-like behaviour and was associated with increased early gene expression in brain regions implicated in anxiety (Goehler et al., 2008; Lyte et al., 2006). Additionally, systemic LPS administration, that results in clinically relevant plasma concentrations, induced intestinal inflammation and increased intestinal cell wall permeability in rats and mice similar to that seen in gut and intestinal disorders (Ge et al., 2000; Guo et al., 2013; Tokes et al., 2011; Yue et al., 2012). Inflammation and increased permeability of the GI tract can result in the further release of cytokines and LPS into the bloodstream to exact effects on the CNS (de Theije et al., 2011), including triggering brain endothelial cells to release cytokines (Verma et al., 2006).

Prenatal administration of LPS and poly I:C produces changes in behaviour in adult male and female offspring, with limited studies, to date, in adolescent offspring. Timing and dose of MIA can influence the nature of the behavioural change and cytokine release (Boksa, 2010; Meyer et al., 2006). Results of a variety of studies have shown that offspring of MIA dams displayed increased anxiety behaviour, decreased exploratory behaviour, decreased social interaction and approach, and decreased sensorimotor gating (Fortier et al., 2007; Shi et al., 2003; Smith et al., 2007). Alterations in dopamine and GABA neurotransmission and white matter changes have also been observed (Baharnoori et al., 2013; Meyer, 2013; Oskvig et al., 2012; Vuillermot et al., 2010). Giovanoli et al. (2013) prenatally administered a low dose of the viral mimetic, poly I:C, which did not result in changes to behaviour in adult offspring unless combined with a stress protocol in early adolescence (P30-40). It is becoming apparent that prenatal immune activation may

act to predispose individuals to a number of neuropsychiatric conditions, or act on pre-existing genetic predispositions (Meyer, 2013). Termed the double hit hypothesis, the idea of genetic predisposition leaving individuals vulnerable to an environmental trigger later in life that results in manifestation of a disorder was originally proposed for schizophrenia (Bayer et al., 1999).

The mechanisms associated with MIA induced alterations in brain and behaviour of offspring are beginning to be elucidated. It appears that toxins and/or cytokines gain access to the fetus, as maternal virus is not detected in the fetus (Bloise et al., 2013; Shi et al., 2005). Cytokines, particularly IL-6, have been shown to induce further cytokine release and activate intracellular pathways in the placenta to alter gene expression and growth protein levels (Hsiao and Patterson, 2011), while blocking IL-6 has reversed behavioural impairments in MIA mice (Smith et al., 2007). Gene expression changes in offspring include upregulation of cellular stress genes and downregulation of genes associated with neurogenesis, neural migration, and neurotransmission (Garbett et al., 2012; Oskvig et al., 2012).

1.5.2. Propionic acid (PPA) model

Propionic acid (PPA) is a short chain fatty acid (SCFA), and is a metabolic product of enteric gut bacteria (Finegold et al., 2002). Physiologically, PPA can cross the gut-blood and blood-brain barriers (BBB). G-protein coupled receptors, GPR41 and GPR43, are specific for SCFAs and located in intestine, immune and blood endothelial cells (Brown et al., 2003; Bindels et al., 2013), while monocarboxylate transporters are located at the BBB for SCFAs (Pierre and Pellerin, 2005). SCFAs are endogenous and in normal physiological conditions, PPA and other SCFAs fulfill a number of important roles in cell metabolism. For example, SCFA are involved in energy utilization from the diet via activation of GPR43, are used as substrates themselves in cell metabolism, and regulation of the immune system (Al-Lahham et al., 2010; Brestoff and Artis, 2013; Kimura et al., 2013).

It has been proposed that elevated levels of PPA may act as an environmental trigger and contribute to the development of ASD. Elevated levels of fecal SCFAs have been measured in children with ASD (Wang et al., 2012) and a recent case report was published of a child with propionic acidemia and autism (Al-Owain et al., 2013). Altered

propionic acid (PPA) metabolism occurs in propionic acidemia, a neurodevelopmental disorder that clinically resembles some aspects of autism. Children with this disorder have a deficient enzyme resulting in elevated levels of PPA along with developmental and cognitive delay, seizures, and stereotyped movements (Feliz et al., 2003).

PPA can elicit diverse effects on the CNS that could drastically alter neural processes, including changes in neurotransmitter synthesis and release, oxidative stress and mitochondrial function, immune activation, and gene expression (DeCastro et al., 2005; Inoue et al., 2012; Le Poul et al., 2003; Parab et al., 2007; Wajner et al., 2004). Metabolic dysfunction in mitochondria and oxidative stress have been found in children with ASD, and associated with severity of behavioural symptoms (Adams et al., 2011a; Rossignol and Frye, 2012).

PPA, similar to valproic acid (VPA), is capable of acting as a histone deacetylase inhibitor to elicit epigenetic changes in gene expression, and both are fatty acids that can interfere with mitochondria cell metabolism (Brass, 1992; Coulter, 1991; Frye et al., 2013). VPA is a common antiepileptic drug and with use in pregnancy, there is a risk for congenital malformations (e.g., spina bifida) and for ASD to develop in children, specifically when used in the first trimester (Bromley et al., 2008; Jentink et al., 2010). Animal studies administering VPA during a comparable window of vulnerability (gestation day 12 in rats) produce offspring that display physical malformations, developmental delay, and behavioural deficits, including sensory impairments, and decreases in exploratory behaviour, social play/interaction, and prepulse inhibition (Favre et al., 2013; Roullet et al., 2013). VPA through its effects as a histone deacetylase inhibitor and can alter gene expression, with administration in rodents both increasing and decreasing expression of ASD-implicated genes (Kolozi et al., 2009; Okada et al., 2005; Yu et al., 2009).

Changes in brain and behaviour resembling those observed in ASD have been shown in rats receiving central PPA, providing face validity for a rodent model. The majority of previous studies investigating the effects of PPA on brain and behaviour have been in adult male rats administered PPA centrally into the lateral ventricles with some studies of peripheral administration. Repeated infusions of PPA produced kindled seizures, increased locomotor activity, decreased social behaviour, and impaired reversal

of the Morris water maze in adult male rats (Shultz et al., 2008; Shultz et al., 2009; Thomas et al., 2012), with decreased social behaviour and impaired maze reversal in adolescent male rats (MacFabe et al., 2011).

Peripheral administration of PPA in adolescent and male adult rats has aversive properties, and produced decreased social behaviour and increased anxiety (Benzaquen et al., 2010; Ossenkopp et al., 2011; Shams et al., 2009). Brains of animals that received central PPA showed an innate neuroinflammatory response, oxidative stress and mitochondrial dysfunction, and lipid alterations, all of which have been associated with ASD (Frye et al., 2013; MacFabe et al., 2007; MacFabe et al., 2008; Thomas et al., 2010; Thomas et al., 2012). As ASD are childhood disorders and are more prevalent in males, the next step in garnering evidence for PPA as an environmental trigger of ASD is to conduct studies on younger animals and investigate both males and females for possible sex differences.

1.6. Present studies

The goal of my thesis was to investigate the effects of metabolic products, associated with enteric bacteria and an altered gut microbiome composition, on neurodevelopment and subsequent behaviour. Immune system activation can alter the composition of the microbiome of the gastrointestinal tract (Bartlett and Gerding, 2008; Bennet et al., 2002). Repeated environmental insults (e.g., immune) in prenatal and/or early postnatal life may lead to chronic inflammation, altering the gut microbiome, and contribute to the development of ASD. Thus, I administered the enteric metabolic products PPA and LPS prenatally to rats to mimic subtle alterations in the production of gut metabolites that may result from a low-grade bacterial infection and the resulting inflammatory response.

To investigate the effects of an early life infection or insult that could alter the gut microbiome, a second 'hit' of PPA in the second postnatal week was given to mimic postnatal production of SCFA from the developing gut microbiota (Midtvedt and Midtvedt, 1992; Nafday et al., 2005). Manipulations in development leaving animals susceptible to later environmental insults are not unheard of, with evidence for the double hit hypothesis observed in neonatal immune research. Neonatal LPS in the first week of life produced altered behaviour in rats upon a second environmental insult during

adolescence or adulthood (Tenk et al., 2008; Walker et al., 2009). This approach has not, to date, been used in previous animal models of ASD. However, not all maternal infections result in neurodevelopmental disorders and repeated insults throughout development may be required. It is thus possible that prenatal treatment with LPS or PPA may leave offspring vulnerable to the effects of postnatal PPA exposure.

Specifically, in this thesis I addressed the following questions: 1. Does administration of propionic acid in development alter subsequent behaviour in adolescent male and female rats? 2. Does prenatal administration of a low dose of lipopolysaccharide alter subsequent behaviour in adolescent male and female rats? and 3. Based on the double-hit hypothesis, will a combination of prenatal and postnatal treatments exacerbate subsequent behaviour altered by prenatal treatment or allow alterations in behaviour to appear that would not otherwise occur with either treatment alone? I used a range of behavioural tests to characterize the effects of PPA and LPS on offspring.

Developmental milestones, locomotor activity, and anxiety (Chapter 2), reactivity to acoustic startle and sensorimotor gating (Chapter 3), and social and related behaviour (Chapter 4) were assessed. I hypothesized that prenatal PPA and LPS would produce developmental delay in milestones, decrease sensorimotor gating, and decrease social behaviour. I also predicted alterations in locomotor activity and acoustic startle responses with prenatal PPA, either increases or decreases. Where prenatal effects occurred, I hypothesized that a second hit with postnatal PPA would exacerbate behavioural changes. These studies demonstrate that metabolic products of the gut microbiome, PPA and LPS, alter neurodevelopment to produce sexually dimorphic behavioural changes in offspring that resemble some of the behaviours observed in ASD.

1.7. References

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Chapter 2

Sex differences in the effects of prenatal lipopolysaccharide or the bacterial metabolic product, propionic acid on postnatal development, and locomotor activity and anxiety in male and female adolescent rats

2.0. Summary

Alterations in the composition of the gut microbiome and/or immune system function may have a role in the development of autism spectrum disorders (ASD). The current study examined the effects of prenatal and early life administration of lipopolysaccharide (LPS), a bacterial mimetic, and the short chain fatty acid, propionic acid (PPA), a metabolic fermentation product of enteric bacteria, on developmental milestones, locomotor activity, and anxiety-like behaviour in adolescent male and female offspring. Pregnant Long-Evans rats were injected once a day with PPA (500 mg/kg SC) on gestation days G12-16, LPS (50 µg/kg SC) on G15-16, or vehicle control on G12-16 or G15-16. Male and female offspring were injected with PPA (500 mg/kg SC) or vehicle twice a day, every second day from postnatal days P10-18. Physical milestones and reflexes were monitored in early life with prenatal PPA and LPS inducing delays in eye opening. Locomotor activity and anxiety was assessed in adolescence (P40-42) in the elevated plus maze and open-field. Prenatal and postnatal treatments altered behaviour in a sex-specific manner. Prenatal PPA decreased time spent in the centre of the open-field in males and females while prenatal and postnatal PPA increased anxiety behaviour on the EPM in female rats. Prenatal LPS did not influence locomotor activity or anxiety-like behaviour. Evidence for the double hit hypothesis was seen as females receiving a double hit of PPA (prenatal and postnatal) displayed increased repetitive behaviour in the open-field. These results provide evidence for the hypothesis that by-products of enteric bacteria metabolism may contribute to ASD, altering development and behaviour in adolescent rats similar to that observed in ASD and other neurodevelopmental disorders.

2.1. Introduction

Autism spectrum disorders (ASD) are neurodevelopmental disorders with roughly 4 males diagnosed for every 1 female. ASD comprise a number of behavioural symptoms, including impairments in communication, social behaviour, sensory abnormalities, and restricted and repetitive behaviour (DiCicco-Bloom et al., 2006). Repetitive behaviour can include motor patterns such as hand flapping or repetitive use of objects while restricted interests may include an insistence on sameness or resistance to change (Leekam et al., 2011; Richler et al., 2007). In many children and adults with ASD, psychiatric disorders, gastrointestinal symptoms and epilepsy comorbidly occur (Tuchman and Rapin, 2002; Simonoff et al., 2008; Williams et al., 2011).

It is becoming well established that both genetics and environmental factors contribute to the development and expression of ASD. A number of genes involved in immune function, mitochondrial function, and neural circuit formation have been implicated (Cook, Jr. and Scherer, 2008; Szatmari et al., 2007). However, known genetic factors discovered thus far account for 10-20% of ASD and concordance rates among monozygotic twins are less than 100%, suggesting an important role for environmental risk factors which act on the genetic susceptibilities (Hallmayer et al., 2011; Anderson et al., 2008).

The gastrointestinal tract (GI) is home to over a trillion commensal bacteria, known as the microbiome, that have a bidirectional relationship with the central nervous system and contribute to normal immune system development and homeostasis in both humans and rodents (see Foster and Neufeld, 2013). GI dysbiosis has been implicated in inflammatory diseases and neuropsychiatric health (Brestoff and Artis, 2013; Cryan and Dinan, 2012; Nicholson et al., 2012). There is suggestive evidence that imbalances in the composition of the microbiome may also contribute to the development or maintenance of ASD in children with findings of abnormal levels of bacteria flora, including *Clostridia*, *Bacteroidetes*, and *Desulfovibrio*, in the GI tract of autistic children (Finegold et al., 2002; Finegold et al., 2012; Parracho et al., 2005). These anaerobic bacteria are antibiotic-resistant. As such, repeated early infections in postnatal life treated with antibiotics may provide an enteric environment that promotes overgrowth of these bacteria resulting in intestinal inflammation (Cho et al., 2012; Finegold et al., 2012).

Metabolic products of these enteric bacteria include the short chain fatty acids (SCFA, from carbohydrate metabolism) (Finegold et al., 2010), which are able to enter circulation and may alter immune function and/or exacerbate ASD behaviours. In fact, propionic acidemia is a neurodevelopmental metabolic disorder characterized by elevated levels of the SCFA, propionic acid, (PPA) that clinically resembles some aspects of autism (Feliz et al., 2003). A case study of autism occurring comorbidly with propionic acidemia has been reported (Al-Owain et al., 2013) while elevated fecal levels of SCFA have been found in ASD children (Wang et al., 2012). Although PPA is necessary for normal immune and physiological functioning, elevated levels may result in disruptive effects (Brestoff and Artis, 2013). It has been proposed that PPA, produced by enteric bacteria, may be a potential environmental factor in the development of ASD. Central administration of PPA has produced hyperactivity and decreased social behaviour in adult male rats (MacFabe, 2012). High levels of SCFA in the hindgut of rats and peripheral PPA injections have also produced changes in activity, anxiety-like, and social behaviour, consistent with ASD (Hanstock et al., 2004; Ossenkopp et al., 2012). Neuroinflammatory and metabolic changes, implicating oxidative stress and mitochondrial dysfunction, have been observed in a subset of patients with ASD and in rats given central PPA (Frye et al., 2013; MacFabe et al., 2008; Rossignol and Frye, 2012).

Immune dysfunction may increase the risk for ASD with alterations in the adaptive and innate cellular immune responses having been observed in children (see Onore et al., 2012 for review). Viral infection in the first trimester and bacterial infection in the second trimester have also been associated with ASD (Atladottir et al., 2010). Maternal immune activation (MIA) in rodents is used to investigate the role of the immune system in development, including its role in anxiety, schizophrenia and ASD. A variety of agents, including poly I:C (a viral mimetic) and lipopolysaccharide (LPS), induce an inflammatory response. LPS, a bacterial mimetic, is the major component of the cell wall of Gram-negative bacteria and is also a by-product of enteric bacteria metabolism. Valproic acid (VPA), a common epilepsy treatment, has been shown to increase the risk of ASD. MIA and prenatal administration of VPA produces developmental delay and behavioural deficits in rodents (see Boksa, 2010; Roullet et al.,

2013). Brusque et al., (1999) administered daily PPA throughout postnatal life (days 6-28 of life) and reported developmental delay and motor impairment. Interestingly, VPA is converted to PPA. However, to date, there have been no studies examining the effects of prenatal PPA administration on behaviour in either male or female offspring.

Prenatal and postnatal administration of immune stimulants (such as LPS) has shown to result in changes in anxiety-like and exploratory behaviour in adult male and female rats. Increased anxiety in the elevated plus maze have been shown in male and female adult offspring (Enayati et al., 2012; Lin et al., 2012; Walker et al., 2009). Assessment of open-field activity has yielded mixed results, with decreased exploration (Shi et al., 2003; Smith et al., 2007), no change in activity (Fortier et al., 2004; Vorhees et al., 2012), or hyperactivity (Howland et al., 2012) being observed. Studies of MIA with adolescent rats are fewer in numbers. Examination of possible sex differences in younger animals is particularly relevant in view of the predominance of ASD in young males. Studies report increased anxiety on the elevated plus maze in adolescent males and decreased exploration in the open-field in adolescent males and females, with some studies reporting no change in behaviour (Enayati et al., 2012; Howland et al., 2012; Oskvig et al., 2012; Schwendener et al., 2009; Vorhees, 1987). While there is increasing attention on investigating possible sex differences following MIA, most published studies, to date, are with male rodents.

Results of a number of studies have also shown that the effects of postnatal LPS on subsequent behaviour do not manifest themselves unless a second environmental insult (e.g., restraint stress or LPS injection) is experienced in adulthood (e.g., Tenk et al., 2008, Walker et al., 2009). Postnatal immune activation confers a susceptibility to later systemic insults that result in abnormal behaviour. This idea, termed the double hit hypothesis, was put forward to describe the genetic predisposition in schizophrenia that may confer vulnerability to an environmental trigger later in life that results in emergence of the disorder (Bayer et al., 1999). Genetics may also confer a susceptibility to prenatal or postnatal environmental insults in ASD, or it may be that more than one insult may be required as is the case in repeated infections in early life. Acute or repeated immune responses may alter the composition of the gut microbiome, increasing production of potential aversive metabolic products (Bartlett and Gerding, 2008; Bennet et al., 2002).

Thus, it is possible that prenatal treatment with PPA may leave offspring vulnerable to the effects of postnatal PPA treatment. Prenatal LPS may also leave offspring vulnerable to postnatal PPA, as LPS is also a product of enteric bacteria. This approach of multiple environmental insults has not been used in animal models of ASD thus far.

The present study investigated the effects of prenatal treatment with the immune stimulant, LPS, and the microbiome associated gastrointestinal factors, PPA, on postnatal developmental milestones, open-field activity and anxiety-like behaviour in adolescent male and female offspring. In addition, the effects of a second ‘hit’ of PPA in the second postnatal week were examined. Specifically, the effects of prenatal LPS, prenatal PPA, and postnatal PPA administration on the development and behaviour of adolescent male and female rats are compared. It was hypothesized that prenatal LPS would increase anxiety-like behaviour in offspring and that prenatal PPA would increase locomotor activity and anxiety-like behaviour in offspring. Combinations of prenatal and postnatal treatments, prenatal LPS with postnatal PPA and prenatal PPA with postnatal PPA, are included to assess whether behavioural effects will be magnified compared to that seen after either treatment alone. Developmental delay was observed in male and female rats with prenatal PPA and LPS, while increased anxiety-like behaviour was sex- and treatment-specific.

2.2. Method

2.2.1. Animals

Twelve primiparous female Long-Evans rats weighing between 270-310 g were mated with adult male Long-Evans rats (375-550 g, Charles River, Canada) for a total of 12 litters. Females were paired overnight with a male the night before behavioural estrus. Sperm present on a vaginal smear (hematoxylin & eosin stain) the morning after pairing indicated successful mating and this was designated gestational day 0 (G0). Dams were housed individually in standard polypropylene cages (45 x 22 x 20 cm) with *ad libitum* access to both food (ProLab RMH 3000) and water. A 12:12 h light:dark cycle (lights on at 0700 h) was maintained in a temperature controlled colony room ($21 \pm 2^\circ\text{C}$). Litters were born on G22 (designated as postnatal day (P) 0), toe-clipped for identification, and were weaned at P21 ($M = 14.17$ pups, $SD = 2.41$). On P21, pups were weaned and randomly culled to a maximum of 10 animals per litter (5 males, 5 females). Weaned rats

were housed in same-sex, same-postnatal drug groups of 2 or 3 in standard polypropylene cages under the same conditions as dams. All behavioural testing took place during the light phase and animals were monitored (e.g., body weight) during testing. Procedures were approved by the University of Western Ontario Animal Use Subcommittee and were in accordance with the Canadian Council of Animal Care (CCAC) guidelines.

2.2.2. Prenatal LPS and PPA administration

Sodium propionate (PPA, P1880, Sigma Chemical, St. Louis, MO, USA) was dissolved in 0.1 M phosphate buffered saline and administered at a dose of 500 mg/kg subcutaneously (SC, pH corrected to 7.4 with concentrated HCl) once a day on G12-16 for a total of 5 injections. Injections started on G12 to mimic the VPA and MIA models of ASD (Schneider and Przewlocki, 2005). Multiple injections were administered given the short half-life of PPA (20 min) (Brusque et al., 1999). A low dose (compared to previous studies) was used in order to be comparable to the relatively low dose of elevated PPA resulting from an altered microbiome composition. Lipopolysaccharide (LPS from *E. coli* serotype 0111:B4, L2630, Sigma Chemical, St. Louis, MO, USA) was dissolved in 0.1 M phosphate buffered saline and administered SC at a low dose of 50 µg/kg on G15 and G16. Prenatal LPS administered at this time has been previously shown to increase anxiety-like behaviour (Enayati et al., 2012) and decrease sensorimotor gating (Fortier et al., 2007). An equivalent volume of phosphate buffered saline was injected SC as a vehicle control (2 mL/kg) to yield two control groups, either on G15 and G16 (2VEH) or on G12-16 (5VEH). All maternal injections were administered between the shoulder blades.

2.2.3. Postnatal PPA administration

As synaptogenesis occurs during the first 3 weeks of postnatal life in rats (Rice and Barone Jr, 2000), on P10, 12, 14, 16, and 18, male and female pups were injected twice a day SC with either PPA (500 mg/kg, pH = 7.4) or equivalent volumes of phosphate buffered saline vehicle (VEH, 5mL/kg) to correspond with an environmental insult in early human life. Half of each litter was injected with postnatal PPA and the rest with VEH. Injections took place at 0930 h (between the shoulder blades) and 1530 h (between the haunches).

2.2.4. Experimental procedure

Table 2.1 provides a summary of drug treatments and group numbers of pups monitored for developmental milestones. Litters were weaned to a maximum of 10 animals and these animals underwent behavioural testing in adolescence on P40, 42 (Table 2.2). The prenatal and postnatal injection schedule yielded the following treatment combinations for each sex: No drug treatment except vehicle (prenatal 2VEH or 5VEH with postnatal VEH); Prenatal treatment alone (prenatal LPS or PPA with postnatal VEH); Postnatal PPA alone (prenatal 2VEH or 5VEH with postnatal PPA); Prenatal and Postnatal treatment combined (prenatal LPS or PPA with postnatal PPA).

2.2.4.1. Developmental milestones

The body weights of pups were monitored daily for the first 20 days of life. The following developmental milestones were assessed for day of appearance: Righting reflex (P2-6): pups were placed on their back and given 30 s to turn onto stomach with all limbs outstretched from body; Pinna detachment (P2-4): bilateral pinna unfolding completely from head; Incisor eruption (P7-13): both upper and lower; Eye opening (P12-16): scored as 0 = both eyes closed, 1 = one eye open, 2 = both eyes open; Negative geotaxis (P7-10): time (s) to rotate 180° on a 30° incline when placed head down (assesses vestibular function and motor development) (Altman and Sudarshan, 1975) with each pup given a maximum of 3 – 60 s trials to complete the task; Free-fall righting reflex (P15): pups were held 35 cm above a padded surface with back facing down and released. A successful trial occurred when the pup landed on its stomach and all limbs were outstretched, with 3 successive trials (15 s apart) yielding a possible maximum score of 3.

2.2.4.2. Elevated plus maze (EPM) – P40

The EPM was made of wood and painted grey with non-toxic paint. The apparatus consisted of two opposite open arms (54 x 12 cm) with no sides or ends and orthogonal to two enclosed arms with sides and ends (54 x 12 x 48 cm). The four arms extended from a centre platform (12 x 12 cm) and the apparatus was raised 50 cm from the floor. An overhead camera connected to a television and DVD-R recorded behaviour for later scoring.

Table 2.1. Summary of treatment groups tested for developmental milestones

		Prenatal treatment					
		2VEH	LPS	5VEH	PPA		
Postnatal treatment	VEH	(M)	8	8	8	12	36
		(F)	9	12	12	10	43
	PPA	(M)	10	8	11	14	43
		(F)	11	14	12	11	48
Total			38	42	43	47	170

Note: Numbers in the table represent number of animals per group for males (M) and females (F). There were 3 litters in each of the 4 prenatal groups (2VEH, 5VEH: 2 or 5 injections of phosphate buffered saline vehicle on G15-16 or G12-16, respectively; LPS: Lipopolysaccharide, 50 ug/kg on G15-16; PPA: Propionic acid, 500 mg/kg on G12-16). Postnatal treatment during the second week of rat pups' life consisted of phosphate buffered saline vehicle (VEH) or propionic acid (PPA).

Table 2.2. Summary of treatment groups used for behavioural testing

		Prenatal treatment				
		2VEH	LPS	5VEH	PPA	
Postnatal treatment	VEH (M)	6	6	6	6	24
	(F)	6	6	6	6	24
	PPA (M)	8	8	9	9	34
	(F)	8	9	9	9	35
Total		28	29	30	30	117

Note: Numbers in the table represent number of animals per group for males (M) and females (F). There were 3 litters in each of the 4 prenatal groups (2VEH, 5VEH: 2 or 5 injections of phosphate buffered saline vehicle on G15-16 or G12-16, respectively; LPS: Lipopolysaccharide, 50 ug/kg on G15-16; PPA: Propionic acid, 500 mg/kg on G12-16). Postnatal treatment during the second week of rat pups' life consisted of phosphate buffered saline vehicle (VEH) or propionic acid (PPA). A maximum of 5 males and 5 females (3 postnatal PPA, 2 postnatal VEH for each sex) per litter were included in behavioural testing. Testing took place on P40 and P42.

Testing took place on the afternoon of P40. Animals were recorded for 5 minutes and placed on the centre platform facing an open arm to begin the test. After each animal, the maze was cleaned with a 20% alcohol solution. Measures assessed included the number of entries onto open and closed arms and time spent (s) on open and closed arms. Percent time in open arms was taken as a measure of anxiety (time spent in open arms/time spent in open arms + time spent in closed arms x 100).

2.2.4.3. Open-field test – P42

Locomotor activity was monitored using eight Versamax Animal Activity Monitors (AccuScan Model DCM-8, Columbus, OH, USA), each consisting of a Plexiglas open field chamber (40 cm x 40 cm x 30.5 cm), and a Plexiglas lid with air holes. Infrared beams surrounding each chamber recorded horizontal and vertical locomotor activity as beam breaks, from which locomotor measures were compiled (Ossenkopp and Kavaliers, 1996). There were 16 infrared beam sensors on each side (2.54 cm apart, 4.5 cm from the floor) for horizontal movements, while on two opposite sides, 16 upper beams were located 15 cm above the chamber floor to assess vertical movements. Additionally, the VersaMax software separated the open-field into discrete periphery (7.5 cm wide border) and centre (30 x 30 cm square) zones to measure thigmotaxis (tendency of animals to stay close to the walls, an indication of anxiety) (Treit and Fundytus, 1988).

Animals were placed in the novel open-field for 60 min on P42 to assess any changes in locomotor activity. Horizontal activity measures analyzed were: total distance (TD) – total horizontal distance (cm); horizontal movement time (MT) – amount of time (s) an animal was engaged in horizontal movement; number of horizontal movements (NM) – number of horizontal movements separated by 1 s stop time. Vertical activity measures analyzed were: vertical movement time (VT) – amount of time (s) an animal spent in a vertical position; number of vertical movements (VM) – number of vertical movements separated by 1 s stop time. Repetitive activity was measured as number of revolutions (clockwise and counterclockwise) - number of times an animal runs in a clockwise or counterclockwise circle of at least 2 inches in diameter. Duration spent (s) in the periphery and centre was measured, and locomotor activity was corrected for time spent in each zone (TD, MT, VM, VT).

2.2.5. Statistical analysis

All analyses were performed with IBM Statistics 20 (formerly Statistical Package for the Social Sciences, SPSS). As pups within a litter are not independent samples, the effects associated with belonging to a litter and being raised in a litter must be accounted for. To do this, linear mixed models were used for each of the dependent variables, with Litter used as a subject variable. Fixed factors in all models were: Sex, Prenatal drug, Postnatal drug while litter size was used as a covariate. For body weight, negative geotaxis, and eye opening, Day was also included as a factor. LSD post-hocs were performed. Significance was set to $\alpha = 0.05$.

2.3. Results

2.3.1. Development

2.3.1.1. Body Weight: Postnatal

Weight was monitored daily for the first 20 days of life (P0-P19). All pups gained weight across days, $F(19,3072) = 3077$, $p < 0.001$, and male pups weighed significantly more than female pups, $F(1,3072) = 88.02$, $p < 0.001$ (Figure 2.1A-D). There was a significant Sex x Prenatal drug x Postnatal drug interaction, $F(3,3072) = 6.20$, $p < 0.001$. Postnatal PPA treated male pups were significantly heavier than postnatal VEH treated male pups ($p < 0.001$) on P13-19 in the prenatal PPA and 2VEH groups, $ps < 0.05$ (Figure 2.1A-B). Postnatal PPA treated female pups were significantly lighter than postnatal VEH treated female pups ($p < 0.001$) with individual days failing to reach significance in the prenatal LPS group (Figure 2.1C-D).

2.3.1.2. Body Weight: Adolescence

Weight was monitored on P39, P45, 47, 49, and 51 (data from P45-51 presented in Chapter 3). All animals gained weight across days, $F(4,419) = 298.04$, $p < 0.001$, with males weighing significantly more than females, $F(1,417) = 1841.06$, $p < 0.001$. A significant Sex x Prenatal drug x Postnatal drug interaction, $F(3,417) = 3.36$, $p = 0.019$, indicated that in prenatal 2VEH animals, postnatal PPA adolescent males weighed significantly more than postnatal VEH males, $p < 0.001$ (P39 NS, P45-51 $ps < 0.05$), with no significant effect of postnatal drug in adolescent females (see Figure 2.2).

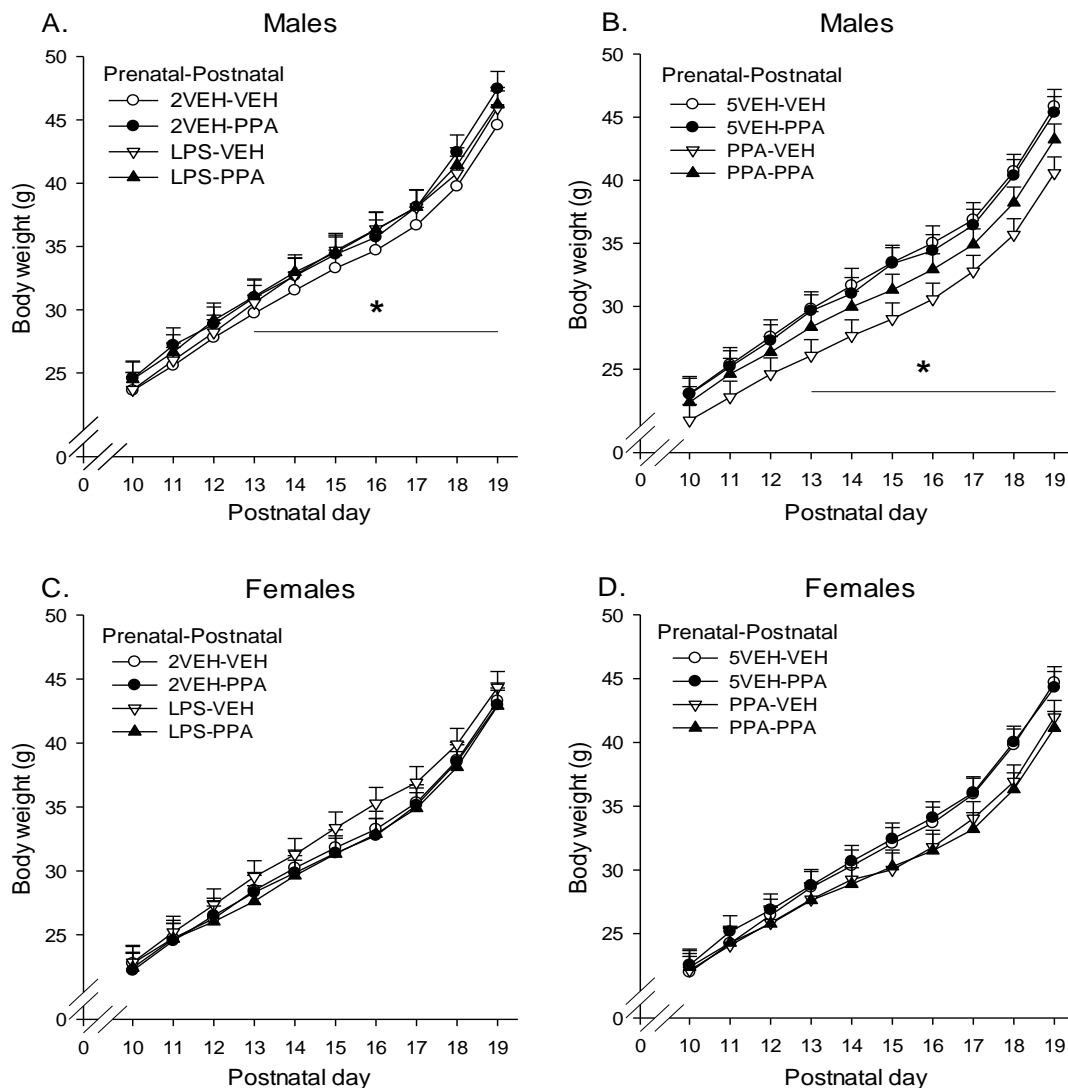


Figure 2.1. Body weight (g) for male and female offspring from postnatal days 10-19.

A-B: Males. C-D: Females. Rats were prenatally exposed to either lipopolysaccharide (LPS) on G15-16, propionic acid (PPA) on G12-16, or their respective phosphate buffered saline controls (2VEH and 5VEH). Postnatal treatment, either PPA or VEH, was administered 2x/day every other day from P10-18. Males receiving postnatal PPA weighed significantly more than postnatal VEH treated males within prenatal PPA and 2VEH groups, while females receiving postnatal PPA weighed significantly less than postnatal VEH treated females within the prenatal LPS group ($p < .05$). Error bars represent S.E.M. Refer to Table 2.1 for sample sizes.

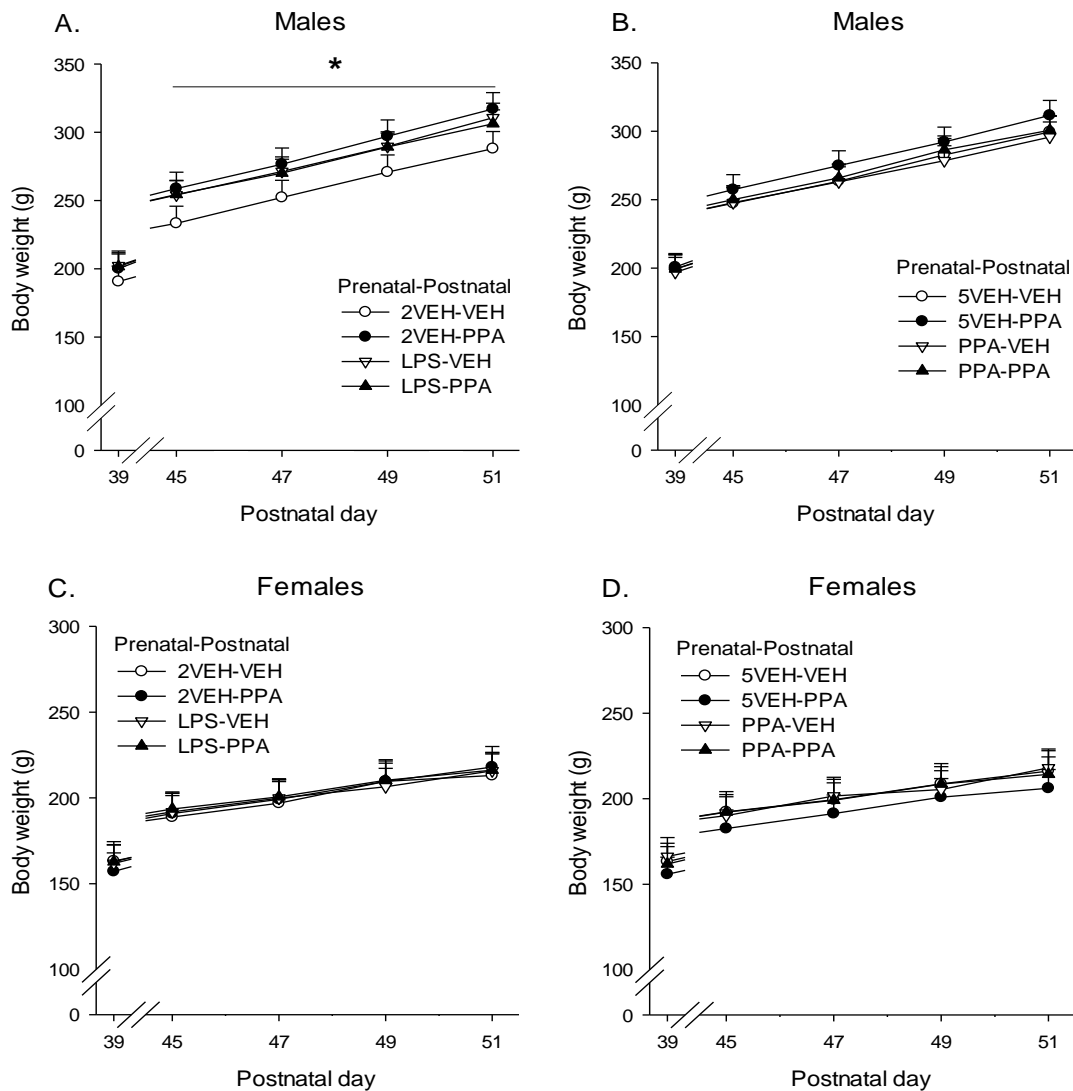


Figure 2.2. Body weight (g) for male and female offspring during adolescence.

A-B: Males. C-D: Females. Rats were prenatally exposed to either lipopolysaccharide (LPS) on G15-16, propionic acid (PPA) on G12-16, or their respective phosphate buffered saline controls (2VEH and 5VEH). Postnatal treatment, either PPA or VEH, was administered 2x/day every other day from P10-18. Postnatal PPA males treated prenatally with 2VEH weighed significantly more than prenatal 2VEH-postnatal VEH males on P45-51 ($p < 0.05$). There were no significant differences in female offspring. Error bars represent S.E.M. Refer to Table 2.2 for sample sizes.

2.3.1.3. Physical Milestones

There was no developmental delay and no sex differences in pups as a result of either prenatal PPA or LPS treatment for eruption of top and bottom incisors, and pinna detachment (Figure 2.3A-C). There was developmental delay observed in day of eye opening in both prenatal PPA and prenatal LPS treated male and female pups (Figure 2.4). A significant Day x Prenatal drug interaction, $F(12,762) = 13.0$, $p < 0.001$, indicated that prenatal PPA and LPS treated male and female pups were significantly different from both 5VEH ($ps < 0.01$) and 2VEH treated pups ($ps < 0.05$) on P14, while on P15, prenatal PPA treated male and female pups were significantly different from 5VEH and LPS ($ps < 0.05$).

2.3.1.4. Reflexes

The day at which rat pups could perform a righting reflex was monitored. A sex difference was present, $F(1,147) = 6.70$, $p = 0.011$, with males performing the reflex significantly earlier than females, with prenatal drug treatment having no significant effect on this reflex (Figure 2.5A). Negative geotaxis was monitored daily on P7-10 to ensure motor reflex development (Figure 2.5B). There was no delay among treatment groups and all animals showed improvement across days, $F(3,588) = 3.66$, $p = 0.012$. Lastly, on P15, a free-fall righting reflex test was performed with a higher score indicating good performance. There was a significant Sex x Prenatal drug x Postnatal drug interaction, $F(3,146) = 4.95$, $p = 0.003$. Males receiving prenatal LPS and postnatal PPA had significantly higher scores on the free-fall righting reflex test than males receiving prenatal LPS and postnatal VEH, $p = 0.002$. Females receiving prenatal LPS and postnatal PPA had significantly lower scores on the reflex test than females receiving prenatal LPS and postnatal VEH ($p = 0.031$) and lower scores than females receiving prenatal 2VEH and postnatal PPA ($p = 0.029$) or prenatal PPA and postnatal PPA ($p = 0.042$, Figure 2.5C).

2.3.2. Behavioural Tests in Adolescence

2.3.2.1. Open-Field Locomotor Activity

Generally, regardless of drug treatment, female offspring were more active than male offspring for total distance traveled, $F(1,100) = 8.55$, $p = 0.004$, horizontal

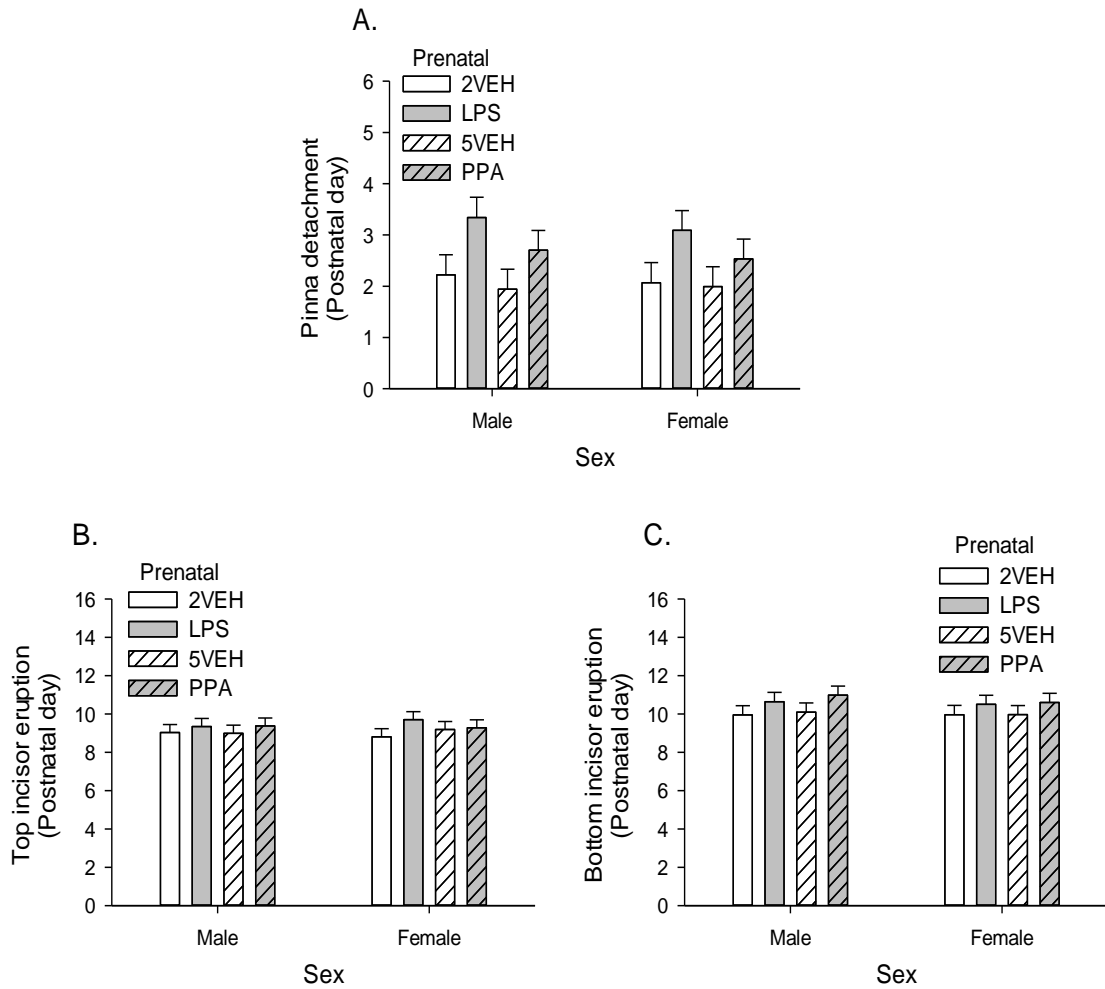


Figure 2.3. Physical developmental milestones for male and female offspring.

Pups were monitored daily and postnatal day of milestone emergence was recorded. A: Pinna detachment, B: Top incisor eruption, and C: Bottom incisor eruption. There were no significant effects of prenatal treatment and no sex differences. Error bars represent S.E.M. Refer to Table 2.1 for group designations and sample sizes.

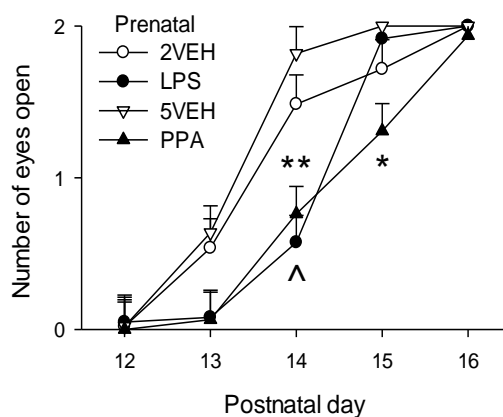


Figure 2.4. Eye opening across postnatal days for prenatal treatment groups.

Data is collapsed across sex and postnatal treatment as there were no significant effects of these on eye opening. On P14, both prenatal LPS (\wedge , $p < 0.05$) and PPA treated animals were delayed compared to vehicle treated controls ($* p < 0.05$, $** p < 0.01$). On P15, prenatal PPA treated pups continued to exhibit delayed eye opening. Error bars represent S.E.M. Refer to Table 2.1 for group designations and sample sizes.

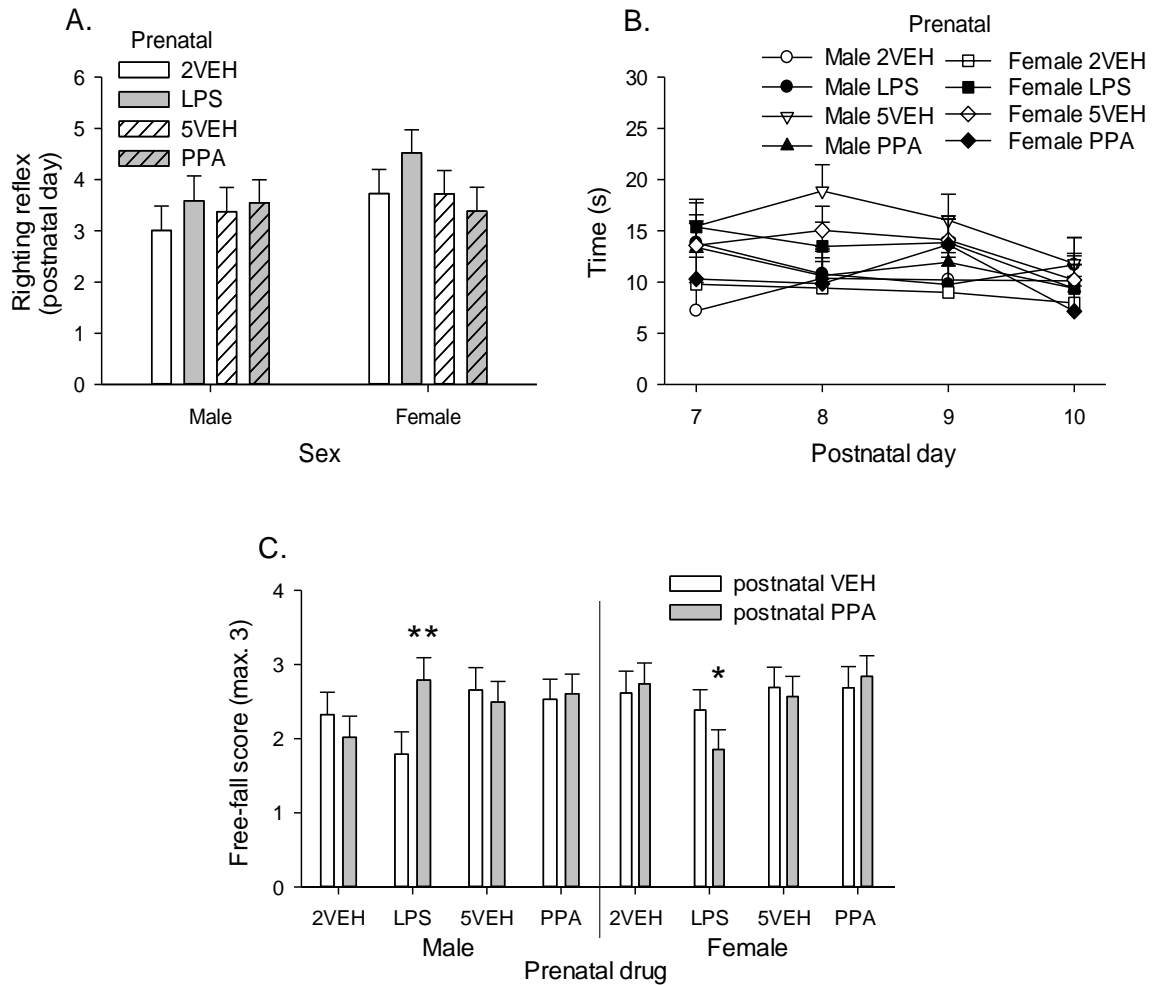


Figure 2.5. Reflexes during early life for male and female offspring.

A. Righting reflex: the day at which animals could right themselves from a supine position with all limbs outstretched. Male pups righted significantly earlier than females. B. Negative geotaxis: on postnatal days 7-10, the time in seconds that it took animals to rotate 180° on a 30° incline when placed head down. An effect of Day indicated that pups completed a 180° turn more quickly across postnatal days. There were no significant effects of sex or prenatal drug. C. Free-fall righting reflex: 3 trials occurred, with a successful trial given a score of 1. In prenatal LPS treated pups, postnatal PPA produced sex differences, with higher scores in postnatal PPA treated males and lower scores in postnatal PPA treated females compared to postnatal VEH treated males and females, respectively. Error bars represent S.E.M. Refer to Table 2.1 for group designations and sample sizes. * $p < 0.05$, ** $p < 0.01$

movement time, $F(1,100) = 6.31$, $p = .014$, and number of revolutions, $F(1,93) = 11.86$, $p = 0.001$). Significant main effects of Prenatal drug were found for total distance traveled, $F(3,100) = 3.23$, $p = 0.026$, and number of horizontal movements, $F(3,100) = 4.37$, $p = 0.006$, but not for horizontal movement time. Animals prenatally exposed to PPA or 5VEH moved a significantly greater total distance than animals in both the LPS and 2VEH control group, $ps < 0.05$ (Figure 2.6A, B). Further analysis showed this difference to be present in only female offspring as prenatal PPA and 5VEH treated females were significantly more active than prenatal LPS treated females, $ps < 0.01$. Animals prenatally exposed to 5VEH performed a significantly greater number of horizontal movements than the other 3 prenatal drug groups (PPA $p = 0.040$, 2VEH $p = 0.002$, LPS $p = 0.003$), with the same effect in both male (5VEH significantly greater than 2VEH $p = 0.014$) and female offspring (5VEH significantly greater than 2VEH, LPS $ps < 0.05$, Figure 2.6C).

There were no effects of prenatal LPS or PPA, or postnatal PPA treatment on vertical activity measures (number of vertical movements and vertical movement time, data not shown). For number of revolutions, the Sex x Prenatal drug x Postnatal drug interaction was nearly significant, $F(3,93) = 2.68$, $p = 0.051$. Further analysis showed that a double hit of prenatal and postnatal PPA increased the number of revolutions made in the female offspring, but not in the male offspring (Figure 2.6D). Female offspring exposed to prenatal and postnatal PPA displayed significantly more revolutions than their male counterparts ($p = 0.001$), prenatal PPA-postnatal VEH treated females ($p = 0.046$), and female offspring exposed to prenatal LPS or 2VEH and postnatal PPA ($ps < 0.05$).

Overall, prenatal PPA, prenatal LPS, and postnatal PPA alone did not produce hyper- or hypo-activity in male and female adolescent offspring. Prenatal PPA and postnatal PPA combined significantly increased repetitive behaviour (number of revolution) in female, not male offspring.

2.3.2.2. Open-Field Thigmotaxis

There was a significant main effect of Prenatal drug, $F(3,100) = 4.83$, $p = 0.004$, for percent time spent in the centre (Figure 2.7A). Prenatal PPA treated animals spent significantly less time in the centre of the open-field compared to prenatal 5VEH treated

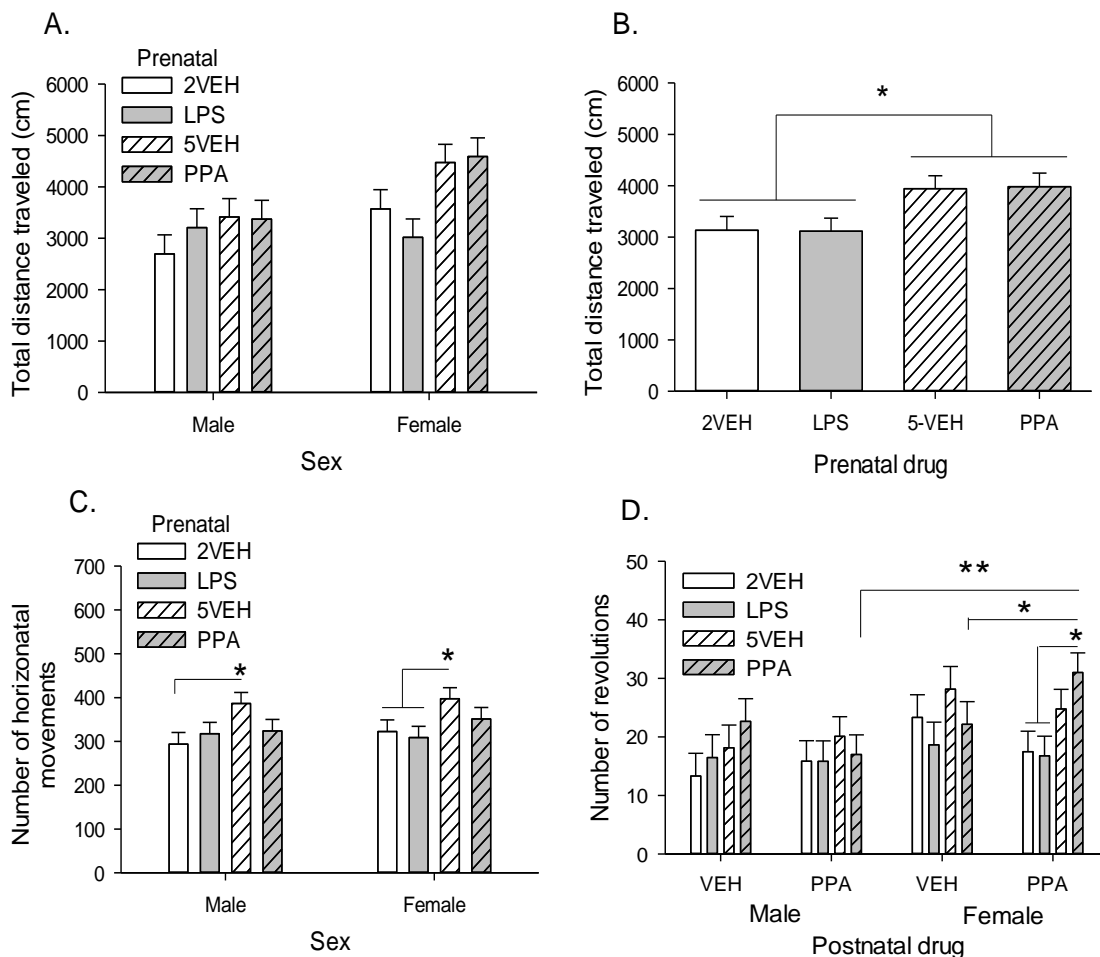


Figure 2.6. Locomotor activity in a novel open-field (P42) in male and female offspring.

Adolescent females moved significantly more than males. Total distance traveled (cm): A: Sex x Prenatal treatment and B: Prenatal treatment. Prenatal PPA and 5VEH treated animals moved significantly more than prenatal LPS and 2VEH treated animals. C: Number of horizontal movements. An effect of prenatal treatment showed animals in the prenatal 5VEH group made significantly more horizontal movements than the other 3 prenatal treatment groups. D: Number of revolutions. Females made significantly more revolutions than males. A double hit of prenatal PPA and postnatal PPA produced significantly more revolutions in female offspring compared to prenatal PPA alone in females and a double hit of PPA in males. Error bars represent S.E.M. Refer to Table 2.2 for group designations and sample sizes. * $p < .05$, ** $p < .01$

controls, $p = 0.005$. Prenatal 2VEH and LPS treated animals also spent significantly less time in the centre than animals treated with 5VEH (2VEH $p = 0.023$, LPS $p = 0.001$). There were no significant effects of drug on the number of entries into the centre or perimeter of the open-field and postnatal PPA as compared to postnatal VEH had no significant effect on percent time in the centre or perimeter of the open-field (Figure 2.7B).

Locomotor activity measures were corrected for the amount of time spent in the perimeter or centre of the open-field. Prenatal LPS, prenatal PPA, and postnatal PPA did not significantly affect locomotor activity in the perimeter of the open-field on any horizontal or vertical activity measures. Females traveled significantly greater total distances, Sex $F(1,100) = 11.53$, $p = 0.001$, and spent more time moving horizontally than males, Sex $F(1,100) = 8.41$, $p = 0.005$ (data not shown).

Postnatal PPA and prenatal LPS also did not affect central locomotor activity. However, prenatal PPA resulted in increased activity in the centre of the open-field. There were significant main effects of Sex, $F(1,100) = 5.82$, $p = 0.018$, and Prenatal drug, $F(3,100) = 5.14$, $p = 0.002$, for total distance traveled in the centre (Figure 2.7C) and a significant main effect of Prenatal drug, $F(3,100) = 2.82$, $p = 0.043$, for horizontal movement time in the centre (data not shown). Animals in the prenatal PPA group traveled significantly greater total distances than all other prenatal groups (5VEH $p < 0.001$, LPS $p = 0.006$, 2VEH $p = 0.008$). This effect was present in both females (prenatal PPA significantly greater than other 3 groups, $ps < 0.01$) and in males (prenatal PPA significantly greater than 5VEH, $p = 0.021$). For horizontal movement time, prenatal PPA treated animals spent significantly more time moving in the centre than 5VEH treated animals, $p = 0.005$.

While in the centre of the open-field, females generally performed more vertical movements, Sex $F(1,93) = 6.56$, $p = 0.012$, and spent more time engaged in rearing than males, Sex $F(1,100) = 4.48$, $p = 0.037$. A significant Sex x Prenatal drug interaction for number of vertical movements in the centre of the open-field, $F(3,93) = 2.89$, $p = 0.040$, indicated that females prenatally exposed to PPA performed a significantly greater number of vertical movements than prenatal PPA treated males, $p = 0.001$ (Figure 2.7D). No significant effects of prenatal drugs (LPS or PPA) were found for vertical time.

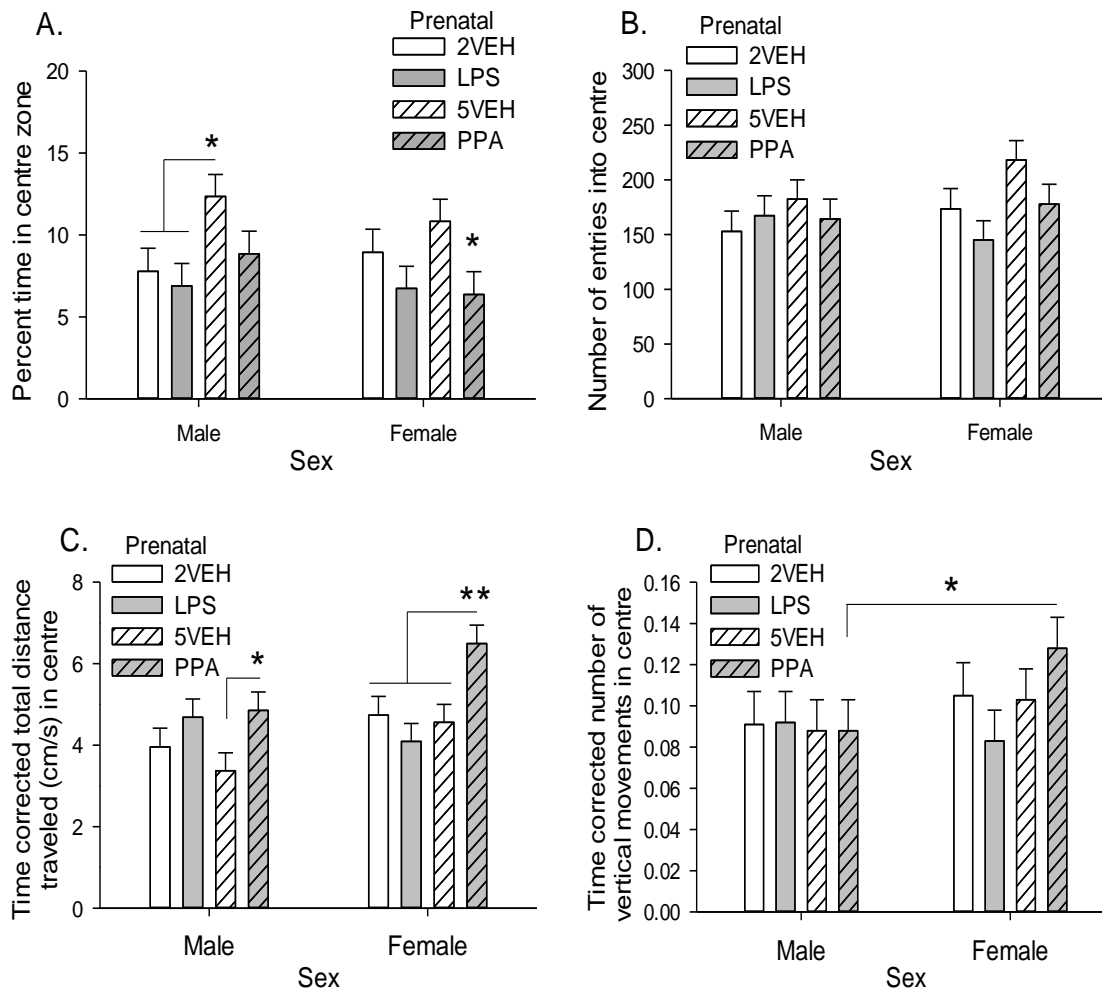


Figure 2.7. Thigmotaxis measures in a novel open-field (P42) in male and female offspring.

Activity measures were time corrected. Females moved significantly more than males. A: Percent time in the centre of the open-field. Animals in the prenatal PPA treated group spent significantly less time in the centre of the open field than the prenatal 5VEH group. B: Number of entries into the centre. There were no significant differences in entries into the centre. C: Total distance traveled (cm/s). Male and female offspring in the prenatal PPA treated group traveled significantly greater distances than the 5VEH treated control group. D: Number of vertical movements. Prenatal PPA treated female offspring reared significantly more than prenatal PPA treated male offspring. Error bars represent S.E.M. Refer to Table 2.2 for group designations and sample sizes. * $p < .05$, ** $p < .01$

Overall, prenatal LPS and postnatal PPA did not significantly alter time spent, or locomotor activity, in the perimeter or centre of the open-field. However, prenatal PPA significantly increased locomotor activity in the centre of the open-field in both males and females.

2.3.2.3. Elevated Plus Maze

The number of entries into the closed arm was used as a measure of locomotion. There were no significant differences among groups in closed arm entries (Figure 2.8A). Number of entries into the open arm, percent time spent in the open arm, and closed arm time were used as traditional measures of anxiety-like behaviour.

For number of entries into the open arm, there was a Prenatal drug x Postnatal drug interaction, $F(3,92) = 2.99, p = 0.035$. Postnatal PPA treated animals in the prenatal 5VEH group entered the open arm significantly fewer times than postnatal VEH animals ($p = 0.01$). A significant Sex x Prenatal drug interaction for both open arm entries, $F(3,92) = 9.45, p < 0.001$, and percent time in the open arm, $F(3,99) = 7.14, p < 0.001$, showed there were no differences in open arm entries among male offspring. Female offspring prenatally exposed to PPA entered the open arm significantly less often ($p = 0.001$) and spent significantly less time in the open arm than prenatal 5VEH treated female offspring ($p < 0.001$, Figure 2.8B, C). Prenatal LPS and 2VEH treated females were also significantly less than prenatal 5VEH treated females for open arm entries and percent time in the open arm ($ps < 0.001$). The significant Sex x Prenatal drug interaction, $F(3,92) = 4.10, p = 0.009$, for time spent in the closed arm showed that female offspring in the prenatal 5VEH group spent significantly less time in the closed arm than female offspring in the other 3 prenatal groups ($ps < 0.01$, Figure 2.8D). Postnatal PPA treated animals also spent significantly more time in the closed arm than postnatal VEH treated animals, Postnatal drug $F(1,92) = 6.74, p = 0.011$, but this was only in female offspring ($p = 0.037$).

In summary, females in the prenatal PPA group made significantly less open arm entries, and spent less percent time in the open arm with more time in the closed arm than controls. Postnatal PPA significantly increased closed arm time in female offspring.

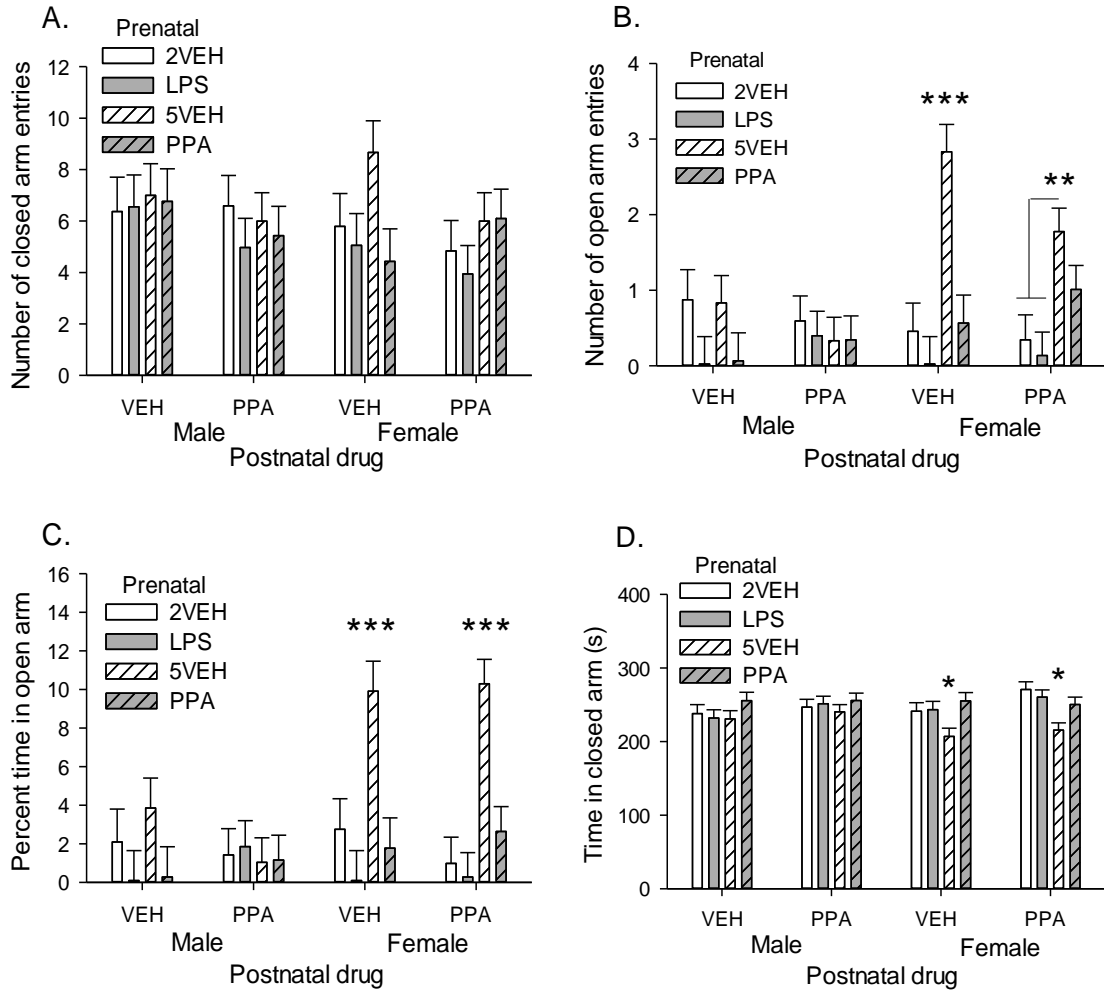


Figure 2.8. Elevated plus maze (P40) for male and female offspring.

A: Number of closed arm entries was not significant. B: Number of open arm entries, C: Percent time in the open arm, D: Time in closed arm. Female offspring in the prenatal PPA group made less open arm entries, spent less time in the open arm, and spent more time in the closed arm than the prenatal 5VEH group. Error bars represent S.E.M. Refer to Table 2.2 for group designations and sample sizes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

2.4. Discussion

Until relatively recently, limited attention has been given to possible developmental effects of microbiome associated, GI metabolic products in rodents. Likewise, investigations of the effects of prenatal and postnatal immune activation with LPS and poly I:C have been mostly limited to adult male rodents. The present study investigated the effects of prenatal PPA and prenatal LPS in the emergence of postnatal developmental milestones, locomotor activity and on anxiety-like behaviour in both male and female adolescent offspring. Additionally, a PPA regimen in the second week of life was used to evaluate if a subsequent postnatal insult would exacerbate any behavioural effects of prenatal treatment.

Prenatal LPS and PPA treatment resulted in developmental delays in male and female offspring, suggesting altered neurodevelopmental effects. Prenatal PPA alone did not influence open-field activity, but as expected, prenatal PPA produced increased anxiety-like behaviour as evidenced by decreased time spent in the centre of the open-field in male and female adolescent rats, and less time on the open arm of the elevated plus maze (EPM) in females. Prenatal LPS did not alter behaviour in the open-field and EPM. Postnatal PPA alone did not alter open-field activity, yet increased anxiety-like behaviour in the EPM. Evidence for the double hit hypothesis was present in female adolescent rats, with the combination of PPA treatments increasing repetitive behaviour. As a whole, the present results provide evidence that prenatal LPS or PPA and postnatal PPA can alter neurodevelopmental processes and that these changes manifest as sex- and test-specific alterations in activity and anxiety-like behaviour in adolescent rats.

2.4.1. Prenatal and postnatal treatments produce developmental delay

Prenatal and postnatal treatments influenced body weight and developmental milestones. Body weight in male and female offspring was affected slightly by postnatal PPA treatment administered in the second week of life. Male pups were heavier and female pups were lighter than control pups, with the effects in males reaching significance across days. PPA administered in the first week of life and postnatal VPA in prior studies had no effect on body weight (Brusque et al., 1999; Reynolds et al., 2012). However, while postnatal LPS has been demonstrated to not influence body weight in the first 3 weeks of life, there is some evidence of increasing body weight in males

throughout adolescence (Iwasa et al., 2010; Spencer et al., 2007). There were no differences in females with postnatal PPA and differences in males only in the prenatal 2VEH group at adolescence, suggesting minimal long-lasting effects on body weight.

Motor reflexes and development also developed normally in early life, consistent with previous studies in rats receiving LPS and VPA (Baharnoori et al., 2010; Schneider and Przewlocki, 2005). In female offspring, there was a deficit in the ability to right mid-air on P15 in animals receiving a combination of prenatal LPS and postnatal PPA compared to prenatal vehicle treated controls. Prenatal VPA impaired free-fall righting in male and female offspring (Wagner et al., 2006), while daily postnatal administration of PPA impaired free-fall righting in male offspring (Brusque et al., 1999). However, in males, prenatal LPS males receiving postnatal PPA were found to have higher righting ability scores compared to prenatal LPS-postnatal VEH treated males. These findings suggest that changes in activity in prenatal LPS and PPA treated adolescents are not due to developmental impairment in motor functions, but rather are associated with developmental changes in underlying neurobiological processes.

Physical developmental milestones monitored were normal in prenatal PPA and LPS treated pups compared to vehicle controls, with the exception of eye opening, which was delayed in both prenatal PPA and LPS treated male and female pups. These results are consistent with previous studies showing VPA delayed eye opening (Roullet et al., 2010; Schneider and Przewlocki, 2005). In contrast, prenatal LPS on G15-16 at a higher dose than this study found no delay in eye opening (Baharnoori et al., 2010), which may suggest that Long-Evans rats are more susceptible to developmental delay with prenatal LPS than Sprague-Dawley rats. Eye opening has been shown to be important for initiating glutamatergic synapse maturation (Zhao et al., 2013). As such, prenatal LPS and PPA treatment and postnatal PPA appear to have the capacity to affect some developmental processes.

2.4.2. Prenatal PPA and postnatal PPA combined produces repetitive behaviour in female offspring

Overall, female adolescent rats displayed greater levels of basal locomotor activity than males, consistent with what is seen in adults (Lynn and Brown, 2009; Lynn and Brown, 2010). Previously, central administration of PPA directly into the brain

ventricles of adult males increased locomotion and repetitive behaviour (MacFabe et al., 2007; Thomas et al., 2012). While these acute administrations are not directly comparable to prenatal effects, they do point to possible direct locomotory effects with prenatal PPA. However, prenatal PPA or postnatal PPA alone did not affect locomotor activity. In fact, a ‘double hit’ of PPA in female offspring was required to produce an increase in repetitive movement, as measured by number of revolutions. This is similar to the results of Schneider et al. (2008) who reported a single injection of prenatal VPA increased duration and number of stereotypic movements in adult female rats, but not male rats.

Prenatal VPA has been shown to produce hyperactivity in male and female juvenile rats (P22-28) and adolescent male rats in an open-field (Dendrinis et al., 2011; Schneider and Przewlocki, 2005). The present findings suggest that PPA at the present dosage may not be as effective as VPA and/or exert its effects through different mechanisms (Dawson, 1991). MIA offspring challenged with dopamine and NMDA drugs show increased locomotor activity compared to MIA controls challenged with the drugs (Fortier et al., 2004; Howland et al., 2012). It is possible that PPA also affected dopamine neurotransmission (DeCastro et al., 2005; Rorig et al., 1996), and that changes are subtle and might only appear if animals were challenged with psychomimetic drugs at the time of testing.

General activity in a novel open-field was greater in prenatal PPA and 5VEH treated offspring compared to LPS and 2VEH treated offspring. Receiving five injections may have induced a mild stress response and been enough of a stressor to affect development. Prenatally stressed rats have been shown to be hyperactive in a novel open-field, as well as displaying anxiety-like behaviour (Fride and Weinstock, 1988; Wilson et al., 2013). Repeated injections can alter baseline levels of plasma corticosterone (Drude et al., 2011; Ryabinin et al., 1999) and prenatal stress can compromise the placental barrier, exposing developing animals to corticosterone (O'Donnell et al., 2009). This may have led to greater activity in a potentially stressful situation, in this case, a novel open-field.

2.4.3. Prenatal LPS did not influence locomotor activity and anxiety-like behaviour

General activity in an open-field does not seem to be altered by MIA alone in adolescent or adult offspring, as observed in this and prior studies (Fortier et al., 2004; Howland et al., 2012; Lin et al., 2012; Vorhees et al., 2012). This does not necessarily indicate that there are no developmental changes in neural functioning. For example, Fortier et al. (2004) found no change in open-field activity with a low dose of prenatal LPS until adult rats were challenged with amphetamine. Alternatively, the open-field used in this study may have been too small to detect aversiveness to the centre of the open-field in prenatal LPS-treated rats. In a study with a larger novel open-field, prenatal LPS induced decreases in locomotor activity and time spent in the centre (Lin et al., 2012).

The low dose of LPS (50 µg/kg) administered during mid-late gestation (G15-16) may help explain why there were no effects of prenatal LPS on anxiety measures in the open-field and EPM. Increased anxiety-like behaviour in the EPM was observed in adolescent male rats prenatally exposed to LPS on G16 or G17 at higher doses (100 and 150 µg/kg), and in adult mice offspring exposed to a similar dose of LPS as this study, but earlier prenatally (G10), or postnatally (Enayati et al., 2012; Lin et al., 2012; Majidi-Zolbanin et al., 2013). While prenatal LPS produced developmental delay in rats, it was not sufficient to alter anxiety-like behaviour.

2.4.4. Prenatal PPA increased anxiety-like behaviour in the open-field

When the open field was divided into centre and perimeter zones, prenatal PPA treatment increased anxiety-like behaviour in both male and female offspring. Reduced time in the centre is indicative of increased anxiety, as the open space acts as an aversive space. Adolescent males and females prenatally exposed to PPA were also hyper-active when in the centre of the open-field, which may suggest a level of aversiveness. Adult rats fed a carbohydrate-rich diet exhibited anxiety and elevated levels of SCFAs in the gut (Hanstock et al., 2004) while previous research with MIA and VPA report decreased time in the centre of an open-field in adolescent male and female offspring (Lin et al., 2012; Smith et al., 2007; Vorhees, 1987). Additionally, infection of germ-free mice with a Gram-negative enteric pathogen (*Campylobacter jejuni*) increased anxiety behaviour and was associated with increased early gene expression in brain regions implicated in

anxiety (Goehler et al., 2008; Lyte et al., 2006). However PPA, unlike LPS and *C. jejuni*, is not a pathogen and may not alter developmental processes in the same way. It is possible that similar immune processes are activated (e.g., cytokine release) as central PPA induces an innate neuroinflammatory response in the brain (MacFabe et al., 2007); however, it remains to be seen if PPA in development alters immune function in rat offspring.

2.4.5. Prenatal and postnatal PPA increased anxiety-like behaviour in the EPM

There were sexually dimorphic effects of prenatal and postnatal PPA treatment, with female rats displaying increased anxiety-like behaviour. Compared to 5VEH treated control females, time in the open arm and open arm entries were decreased in prenatal PPA treated females and closed arm time was increased in prenatal PPA and postnatal PPA treated females. An increase in anxiety is consistent with previous reports of prenatal VPA and enteric infection producing anxiety-like behaviour in male and female adult rat offspring (Lyte et al., 1998; Markram et al., 2008; Schneider et al., 2008).

A sex difference was present in the 5VEH control group, with males exhibiting similar levels of behaviour as prenatal PPA treated males and females, suggesting anxiety in these control males. Brief daily periods of maternal stress resulted in increased anxiety in male adolescent rats, but not females (Muhammad and Kolb, 2011). To prevent an effect of prenatal PPA in males from being significant, mild stress associated with the control injections may have increased exposure to corticosterone and altered developmental processes in males. Studies of prenatal stress have shown sex differences in neurogenesis, decreased levels of testosterone and increased levels of corticosterone in offspring that may account for alterations in behaviour (reviewed in Weinstock, 2011).

Similar to prenatal 5VEH males, both males and females treated with LPS and 2VEH displayed low levels of activity on the EPM. There may have been a ceiling effect in the current study that prevented an effect of LPS from being evident. It appears that this effect may be specific to these animals as behavioural testing occurred in a paired fashion (2VEH and LPS animals tested on the same day, and 5VEH and PPA animals tested together), and prenatal 5VEH treated females performed as expected. It is unclear why 2VEH and LPS treated animals produced such low levels of maze exploration. It has been shown that exposure to additional stressors has been required before increased

anxiety-like behaviour on the EPM was observed in rats treated with postnatal LPS (Breivik, 2002; Walker et al., 2009). Additionally, EPM results are sensitive to multiple environmental factors including prior housing condition, illumination levels, and prior handling (reviewed in Carobrez and Bertoglio, 2005). Some aspect of the current EPM set-up may have induced a stressful state that led to decreased levels of exploration in the maze. Future investigations under different conditions may provide insight on this issue and how it relates to results of previous studies.

2.4.6. LPS and PPA have the potential to alter neurodevelopmental processes

An imbalance between excitation and inhibition in the brain has been implicated in autism and anxiety, with changes in GABA and possibly serotonin suggested to be critical. Modifications in GABAergic systems have been reported in various brain regions of patients with ASD (Fatemi et al., 2002; Oblak et al., 2010) and these systems may be vulnerable to environmental agents during development.

SCFAs and VPA can cross the placenta via monocarboxylate transporters and gain access to the developing fetus (Nagai et al., 2010; Ushigome et al., 2001). PPA and VPA can act as histone deacetylase inhibitors and induce changes in gene expression (D'Souza et al., 2009; Phiel et al., 2001) with preliminary results demonstrating that central administration of PPA can alter gene expression in ASD associated genes (unpublished observations). Oral PPA during gestation and early life depleted whole brain GABA, serotonin, and dopamine and increased IL-6 in young rat brains (El-Ansary et al., 2011). Further investigation into the mechanisms associated with PPA-induced alterations in development and behaviour is needed.

While no behavioural effects of LPS were found in the current study, acute and chronic LPS during gestation induces a proinflammatory response and alters the placental barrier, which may allow external agents and/or cytokines to gain access to the fetus (Bloise et al., 2013; Shi et al., 2005). Increases in proinflammatory cytokines, and changes in gene expression and GABAergic neurons have been found following prenatal LPS treatment (Garbett et al., 2012; Gayle et al., 2004; Nouel et al., 2012).

2.4.7. Conclusion

In summary, these results are the first to demonstrate that prenatal PPA, and one of a few to demonstrate that postnatal PPA and a low dose of LPS, alters developmental

processes and subsequent behaviour in male and female adolescent rats, resembling alterations observed in ASD and previous animal models. Prenatal LPS and prenatal PPA produced delay in eye opening and prenatal and postnatal PPA increased anxiety-like behaviour in both male and female offspring, with a greater effect observed in female offspring. Developmental delay and altered temperament are observed in children with ASD, as reduced communication and motor skills or inappropriate emotional responses (e.g., passiveness) are observed (Mitchell et al., 2011; Zwaigenbaum et al., 2005) and anxiety disorders were the most common psychiatric conditions reported in ASD populations (Skokauskas and Gallagher, 2010). There was no male bias in PPA and LPS induced alterations in behaviour, unlike the male predominance seen in ASD. A more balanced male to female ratio and the presence of gastrointestinal abnormalities was observed in children with ASD and mitochondrial disease (MD) (Rossignol and Frye, 2012). It is possible that environmental insults contribute differently to the sex ratio observed in ASD. Additionally, evidence suggests that females with ASD display more severe behavioural symptoms than males and are likely to show more repetitive interests (Fombonne, 2009; Mandy et al., 2012; Russell et al., 2011). The current results support this with a female sensitivity to PPA effects on repetitive behaviour and anxiety. These results provide evidence that by-products of enteric bacteria metabolism can alter development and behaviour in rats resembling that of ASD. Repeated infection or immune insult throughout gestation and early life may induce intestinal inflammation and alter the composition of the gut microbiome. Subsequent production of metabolic products, such as LPS and PPA, has the potential to adversely alter neurodevelopment in susceptible populations.

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Chapter 3

Prenatal exposure to lipopolysaccharide and pre- and postnatal exposure to propionic acid, alters acoustic startle response and prepulse inhibition in male and female adolescent rats

3.0. Summary

Potential environmental risk factors for autism spectrum disorders (ASD) include viral/bacterial infection and an altered microbiome composition. The present study investigated whether administration of immune and gastrointestinal factors during gestation and early life altered startle response and prepulse inhibition in adolescent offspring using lipopolysaccharide (LPS), a bacterial mimetic, and propionic acid (PPA), a short chain fatty acid and enteric metabolic bacterial product. Pregnant Long-Evans rats were injected once a day with PPA (500 mg/kg SC) on gestation days G12-16, LPS (50 µg/kg SC) on G15-16, or vehicle control on G12-16 or G15-16. Male and female offspring were injected with PPA (500 mg/kg SC) or vehicle twice a day, every second day from postnatal days 10-18. Acoustic startle response and prepulse inhibition was measured on postnatal days 45, 47, 49, and 51. Prenatal and postnatal treatments altered startle response characteristics in a sex-specific manner. Prenatal LPS treatment produced hypersensitivity to acoustic startle in males, but not females and did not alter prepulse inhibition. Subtle alterations in startle responses, which disappeared with repeated trials, occurred with prenatal PPA and postnatal PPA treatment in both male and female offspring. Prenatal PPA treatment decreased prepulse inhibition in females, but not males. Females receiving a double hit of PPA (prenatal and postnatal) showed sensitization to acoustic startle, providing evidence for the double hit hypothesis. The current study provides support for the hypothesis that immune activation and metabolic products of enteric bacteria can alter development and behaviour in ways that resemble sensory abnormalities observed in ASD.

3.1. Introduction

The prevalence of autism spectrum disorders (ASD) has increased to approximately 1 in 88 children (Autism and Developmental Disabilities Monitoring Network Surveillance Year 2008 Principal Investigators, 2012). ASD encompasses a wide range of behavioural symptoms, including impairments in communication and social behaviour, and the presence of stereotyped movements and repetitive behaviour (DiCicco-Bloom et al., 2006). In the DSM-5, sensory aspects have been incorporated into the repetitive and restrictive behaviour domain, as both hyper- and hypo-sensitivities to stimuli across multiple modalities are reported (Leekam et al., 2007; Marco et al, 2011). Both genetic and environmental factors contribute to the development of ASD. A number of genes have been implicated as well as de novo copy number variations (Cook and Scherer, 2008; Geschwind, 2011), with heritability estimates of 90%. However, concordance rates among monozygotic twins is reported to range from 50-90% (Bailey et al., 1995; Hallmayer et al., 2011), leaving an important role for environmental risk factors to act on underlying genetic susceptibilities (Herbert, 2010).

Immune dysfunction may increase the risk for ASD as alterations in the adaptive and innate cellular immune responses have been observed in children with ASD (see Onore et al., 2012 for review). Viral infection in the first trimester and bacterial infection in the second trimester have been associated with development of ASD (Atladottir et al., 2010). During an immune insult, the release of proinflammatory cytokines, which act both peripherally and centrally, result in a range of behavioural and physiological responses termed sickness behaviours. This release of cytokines during critical periods may have adverse consequences for neurodevelopmental processes, such as cell differentiation, migration, and synaptogenesis (Bilbo and Schwarz, 2012; Deverman and Patterson, 2009).

There has been an increasing interest in the role of host gut microbial populations, or microbiome, in communicating with the central nervous system and influencing gastrointestinal (GI), immune, and neuropsychiatric health (Cryan and Dinan, 2012; Nicholson et al., 2012). Imbalances in the composition of the microbiome and the immune sequelae may also contribute to the development and/or maintenance of ASD in children. Support for this comes from findings of abnormal levels of bacteria flora,

including augmented *Clostridia*, *Bacteroidetes*, and *Desulfovibrio* subtypes, in the GI tract of autistic children (Finegold et al., 2002; Finegold et al., 2012; Parracho et al., 2005). As these anaerobic bacteria are antibiotic-resistant, repeated early infections in postnatal life treated with antibiotics may provide an enteric environment that promotes the overgrowth of these bacteria and the propensity for intestinal inflammation and associated neuroimmune and neurohormonal changes (Cho et al., 2012; Finegold et al., 2012).

Metabolic products of these bacteria include short chain fatty acids (SCFA, from carbohydrate metabolism) (Finegold et al., 2010), which are able to enter circulation and may alter metabolic and immune function and/or exacerbate ASD behaviours. Indeed, propionic acidemia, a neurodevelopmental metabolic disorder characterized by elevated levels of the SCFA, propionic acid (PPA), clinically resembles some aspects of autism (Feliz et al., 2003) and a case study of comorbidity of propionic acidemia and ASD has been presented (Al-Owain et al., 2013). Our laboratory has proposed that PPA, produced by enteric bacteria, may be a potential environmental factor in the development of ASD. Central administration of PPA in adult male rats has produced a number of brain and behavioural changes including hyperactivity and decreased social behaviour consistent with ASD (MacFabe et al., 2007; MacFabe et al., 2008; MacFabe et al., 2011; MacFabe, 2012; Shultz et al., 2008; Shultz et al., 2009; Thomas et al., 2012) and has predictive value in many metabolic alterations in a subset of ASD patients (Frye et al., 2013).

Maternal immune activation (MIA) in rodents is used to investigate the role of the immune system in ASD. An inflammatory response is induced using a variety of agents, including influenza and polyinosinic:polycytidylic acid (poly I:C- a viral mimetic). Lipopolysaccharide (LPS, a bacterial mimetic) is the major component of the cell wall of Gram-negative bacteria and is also a by-product of many enteric bacteria metabolism. Offspring of dams treated with these immune agents display behavioural deficits in exploratory behaviour and social interaction (Fortier et al., 2007; Romero et al., 2010; Shi et al., 2003; Smith et al., 2007). Valproic acid (VPA), an epilepsy treatment that increases the risk of ASD, shares pharmacological properties with PPA (Brass, 1992; Coulter, 1991), and is also widely used in animal studies. Prenatal administration of VPA produced developmental delay and behavioural deficits (reviewed in Rouillet et al., 2013).

Brusque et al. (1999) administered daily PPA throughout postnatal life (days 6-28 of life) and reported developmental delay with mild behavioural deficits. To date, there are no published studies on the effects of prenatal PPA and one study (Brusque et al., 1999) on the effects of postnatal PPA administration on behaviour in offspring.

Sensory abnormalities reported in children and adults with autism vary in modalities affected and severity. In both self-reports and parental reports, over 90% of those with autism report unusual responses to sensory stimuli (Crane et al., 2009; Leekam et al., 2007). Both hyper-responding (more than typical) and hypo-responding (below normal response) to taste/smell, tactile, visual, and auditory stimuli have been observed, and there is evidence of abnormal sensory integration (Baranek et al., 2007; Iarocci and McDonald, 2006; Leekam et al., 2007; Rogers et al., 2003). Difficulties with habituation to sensory stimuli (decreased responding to repeated stimuli over time) have also been observed (Barry and James, 1988; Ornitz et al., 1993), but are not always present in patients (Baranek et al., 2007; Rogers and Ozonoff, 2005).

In animal models, the acoustic startle response (ASR) is a commonly used measure of sensory responsiveness. The ASR can be modulated in a number of ways, including with a low intensity prepulse and with habituation. Habituation is the reduction in startle response with repeated presentation of the stimulus. Prepulse inhibition refers to a decrease in acoustic startle response level that occurs when the startle stimulus is preceded 30-500 ms by a non-startling stimulus (prepulse). This inhibition effect is presumed to be due to sensory filtering to allow prepulse processing (Koch, 1999).

As ASD are present in childhood and are more prevalent in males, it is important to conduct animal studies on younger animals and investigate both males and females for possible sex differences. Although more attention has been recently focused on sex differences in adults, the majority of animal studies have used male adults (Boksa, 2010), and information on adolescents is still lacking. Also, few studies assess ASR, focusing on other behavioural tests, though prepulse inhibition is more frequently included in behavioural test batteries. While repeated treatment with LPS throughout gestation has been shown to decrease prepulse inhibition in adolescent male and female rats (Romero et al., 2010), there are no reports, to our knowledge, of the effects of prenatal LPS administered at specific gestational time points on ASR, habituation, or prepulse

inhibition in adolescent offspring. Previous MIA research has obtained decreases in prepulse inhibition in adult male and female (Basta-Kaim et al., 2011; Howland et al., 2012) and adolescent male offspring (Wolff and Bilkey, 2010). Prenatal exposure to other toxins, such as valproic acid (VPA), have resulted in mixed ASR results and decreased prepulse inhibition in adult male and female rats and adolescent males (Markram et al., 2008; Schneider and Przewlocki, 2005; Vorhees, 1987).

The present study investigated the effects of prenatal treatment with LPS or PPA, on ASR, habituation, and prepulse inhibition in adolescent male and female offspring. Additionally, a second ‘hit’ of PPA, in the second postnatal week, was given to act as an early life insult to mimic postnatal production of SCFA from the developing gut microbiota (Midtvedt and Midtvedt, 1992; Nafday et al., 2005). This double hit hypothesis has been proposed for schizophrenia, where genetic predisposition leaves individuals vulnerable to an environmental trigger later in life that results in manifestation of the disorder (Bayer et al., 1999). The “double hit” approach has also been applied to animal models of immune activation using two environmental insults. Immune activation early in life may confer susceptibility to disease or psychopathology in adulthood (Giovanoli et al., 2013; Tenk et al., 2008; Walker et al., 2009). Genetics may also confer susceptibility to prenatal or postnatal environmental insults in ASD, or, more than one insult may be required, as in repeated infections in early life. Immune responses can alter the composition of the microbiome of the gastrointestinal tract (Bartlett and Gerding, 2008; Bennet et al., 2002). It is thus possible that prenatal treatment with LPS or PPA may leave offspring vulnerable to the effects of postnatal PPA exposure. To date, this approach has not been used in previous animal models of ASD. Previous animal models of ASD have primarily focused on adult offspring, with few investigations of sex differences in adolescent male and female offspring.

The present study specifically compared the effects of prenatal exposure to LPS and/or PPA, and postnatal exposure to PPA in adolescent male and female rats. Thus, combined treatments of prenatal LPS with postnatal PPA and prenatal PPA with postnatal PPA are considered. These unique combinations of prenatal and postnatal treatment assess the presence of exacerbated behavioural effects compared to treatments alone. It was hypothesized that prenatal PPA would alter the acoustic startle response and that

both prenatal PPA and LPS exposure would result in decreased prepulse inhibition in adolescent offspring. If effects on behaviour were found, postnatal PPA treatment was expected to exacerbate behaviour.

3.2. Method

3.2.1. Animals

The animals used in this experiment are the same animals from Chapter 2, with behavioural testing following the behaviours measured in Chapter 2. Twelve primiparous female Long-Evans rats weighing between 270-310 g were mated with adult male Long-Evans rats (375-550 g, Charles River, Canada) for a total of 12 litters. Females were paired overnight with a male the night before behavioural estrus. Sperm present on a vaginal smear (hematoxylin & eosin stain) the morning after pairing indicated successful mating and this was designated gestational day 0 (G0). Dams were housed individually in standard polypropylene cages (45 x 22 x 20 cm) with *ad libitum* access to both food (ProLab RMH 3000) and water. A 12:12 h light:dark cycle (lights on at 0700 h) was maintained in a temperature controlled colony room ($21 \pm 2^\circ\text{C}$). Litters were born on G22 (designated as postnatal day (P) 0), toe-clipped for identification, and were weaned at P21 ($M = 14.17$ pups, $SD = 2.41$). On P21, pups were weaned and randomly culled to a maximum of 10 animals per litter (5 males, 5 females). Weaned rats were housed in same-sex, same-postnatal drug groups of 2 or 3 in standard polypropylene cages under the same conditions as the dams. All behavioural testing took place during the light phase and body weight was monitored during testing. Procedures were approved by the University of Western Ontario Animal Use Subcommittee and were in accordance with the Canadian Council of Animal Care (CCAC) guidelines.

3.2.2. Prenatal LPS and PPA administration

Sodium propionate (PPA, P1880, Sigma Chemical, St. Louis, MO, USA) was dissolved in 0.1 M phosphate buffered saline and administered at a dose of 500 mg/kg subcutaneously (SC, pH corrected to 7.4 with concentrated HCl) once a day on G12-16 for a total of 5 injections. Injections started on G12 to mimic the VPA and MIA models of ASD (Schneider and Przewlocki, 2005); multiple injections were administered given the short half-life of PPA (20 min, Brusque et al., 1999). Lipopolysaccharide (LPS from *E. coli* serotype 0111:B4, L2630, Sigma Chemical, St. Louis, MO, USA) was dissolved

in 0.1 M phosphate buffered saline and administered SC at a dose of 50 µg/kg on G15 and G16. Administration at this time has been shown to alter adult prepulse inhibition in models of maternal immune activation (Fortier et al., 2007). An equivalent volume of phosphate buffered saline was injected SC as a vehicle control (2 mL/kg) to yield two control groups, either on G15 and G16 (2VEH) or on G12-16 (5VEH). All maternal injections were administered between the shoulder blades.

3.2.3. Postnatal PPA administration

As synaptogenesis occurs during the first 3 weeks of postnatal life in rats (Rice and Barone, 2000), male and female pups were injected twice a day SC with either PPA (500 mg/kg, pH = 7.4) or equivalent volumes of phosphate buffered saline vehicle (VEH, 5mL/kg) on P10, 12, 14, 16, and 18 to correspond with an environmental insult in early human life. Approximately half of each litter was injected with postnatal PPA, the rest with VEH. Injections took place at 0930 h (between the shoulder blades) and 1530 h (between the haunches).

3.2.4. Experimental procedure – Acoustic startle and prepulse inhibition (PPI)

Behavioural testing occurred in late adolescence on P45, 47, 49, and 51. Additional behavioural testing took place prior to startle testing (Chapter 2). A summary of the treatment groups is provided in Table 3.1. The prenatal and postnatal injection schedule yielded the following treatment combinations for each sex: Vehicle only (prenatal 2VEH or 5VEH with postnatal VEH); Prenatal treatment alone (prenatal LPS or PPA with postnatal VEH); Postnatal PPA alone (prenatal 2VEH or 5VEH with postnatal PPA); Prenatal and Postnatal treatment combined (prenatal LPS or PPA with postnatal PPA).

Acoustic startle response and prepulse inhibition (PPI) testing was conducted in 3 separate startle chambers (SRLAB, San Diego Instruments, San Diego, CA). Each chamber consisted of a cylindrical, clear acrylic rat enclosure (10.2 cm outside diameter) mounted on an acrylic platform. The platform sat on a piezoelectric accelerometer which transduced the force of animal movement. This was placed inside a ventilated, sound attenuating box containing a mounted fluorescent light and a speaker which emitted the background noise, prepulse and acoustic startle stimuli. Data were recorded for 100 ms

Table 3.1. Summary of treatment groups

		Prenatal treatment				
		2VEH	LPS	5VEH	PPA	
Postnatal treatment	VEH (M)	6	6	6	6	24
	(F)	6	6	6	6	24
	PPA (M)	8	8	9	9	34
	(F)	8	9	9	9	35
Total		28	29	30	30	117

Note: Numbers in the table represent number of animals per group for males (M) and females (F). There were 3 litters in each of the 4 prenatal groups (2VEH, 5VEH: 2 or 5 injections of phosphate buffered saline vehicle on G15-16 or G12-16, respectively; LPS: Lipopolysaccharide, 50 ug/kg on G15-16; PPA: Propionic acid, 500 mg/kg on G12-16). Postnatal treatment during the second week of rat pups' life consisted of phosphate buffered saline vehicle (VEH) or propionic acid (PPA). A maximum of 5 males and 5 females (3 postnatal PPA, 2 postnatal VEH for each sex) per litter were included in behavioural testing. Testing took place from P45-51.

immediately following the onset of the acoustic startle stimulus. The magnitude of the first peak in the startle response from stimulus onset was taken as the measure of the acoustic startle response. Four testing sessions in the startle apparatus took place, one on each of P45, 47, 49, and 51.

In a testing session, which lasted approximately 22 min, a 5 min acclimation period with background noise (70 dB) was followed by a 17 min (67 trials) testing session in which the 70 dB background noise was maintained. Eleven trial types were used in the testing session; startle-alone trials (consisting of a 115 dB burst of white noise stimulation lasting 40 ms in duration), six different prepulse inhibition trial types (prepulses 3, 6 or 12 dB louder than the 70 dB background noise (73, 76 and 82 dB, respectively), each consisting of a 20 ms burst of white noise presented with an onset either 120 ms prior to the startle pulse (100 ms inter-stimulus interval, ISI) or 80 ms prior to the startle pulse (60 ms ISI)), and four control trial types (no pulse, 73, 76, or 82 dB prepulse only).

The first 10 trials were startle-alone trials which served to reduce the amount of variability measured for the startle response. The middle 52 trials (presented in pseudo-random order) consisted of 10 startle-only, 30 PPI trials (5 each of 6 different PPI trial types), and 12 control trials (3 each of no pulse, 73, 76, or 82 dB only). The session ended with 5 startle-alone trials. All of the trials were separated by an inter-trial interval (ITI) of 8–23 s in length (average ITI = 15 s).

3.2.5. Behavioural measures

The magnitude of the first peak recorded following the startle pulse was used as the measure of acoustic startle response and the average of each trial type was computed. Startle responses on the first 2 trials were taken as a measure of initial startle reactivity. For habituation during each session, the startle response of trials 3-10 was each divided by the average of the first 2 trials to give normalized startle amplitudes. Within each test session, a percent habituation score was also calculated using startle responses at the beginning and end of the session. $\% \text{Habituation} = 100 \times (\text{average of startle response on trials 6-10} - \text{average of startle response on last 5 trials of the session}) / \text{average of trials 6-10}$.

Prepulse inhibition (PPI) was calculated for each prepulse level/ISI. $\%PPI = 100 \times (\text{Startle only magnitude} - \text{PPI startle magnitude}) / \text{Startle only magnitude}$. Similar to other studies (Braff et al., 1999; Lockey et al., 2009), some groups of animals produced prepulse facilitation (increased startle response with prepulse presentation) at the 73 dB prepulse instead of inhibition. This can occur with prepulses that are too close to the background noise. As a result, only 76 dB and 82 dB prepulses were analyzed for prepulse inhibition.

3.2.6. Statistical analysis

All analyses were performed with IBM Statistics 20 (formerly Statistical Package for the Social Sciences, SPSS). Outliers were identified as being ± 2 standard deviations from the mean and were removed from analysis. As pups within a litter are not independent samples, the effects associated with belonging to a litter and being raised in a litter must be accounted for. To do this, linear mixed models were used for each of the dependent variables, with Litter used as a subject variable. Fixed factors in most models were: Session, Sex, Prenatal drug, Postnatal drug. For habituation across the first 10 trials, Session was removed and replaced by Trials, with a model used for each session individually. Additionally, Prepulse level was added as a fixed factor into models for each ISI level to assess prepulse inhibition. The random factor was startle box the animal was tested in and covariates were body weight and litter size in all models. LSD post-hocs were performed. Significance was set to $\alpha = 0.05$.

3.3. Results

3.3.1. Initial startle reactivity

Each startle session began with 10 trials in which the startle pulse alone was presented. Responses can vary greatly across the first few trials as animals acclimate to the startle stimulus and a period of adjustment is desired prior to assessment of average startle response and prepulse inhibition. However, the first trials can also provide a true indication of initial startle reactivity prior to habituation (Geyer and Swerdlow, 2001). The startle response for the first 2 trials of each session was analyzed to determine if initial startle reactivity was influenced by prenatal or postnatal treatments.

Startle responses decreased from trial 1 to 2, with a significant effect of Trial, $F(1,777) = 15.30, p < 0.001$ (data not shown) and decreased across Session,

$F(3,777) = 4.06, p = 0.007$ (Session 1 significantly greater than Sessions 3 and 4, $ps < 0.05$). The Session x Sex x Prenatal drug x Postnatal drug interaction was significant, $F(9,777) = 2.10, p = 0.028$. As there were no significant interactions between trials and drug treatments, startle responses were collapsed across the first 2 trials for the 4 sessions (Figure 3.1).

Effects of postnatal PPA treatment

On Session 1, there was a sex difference in the postnatal effect of PPA on startle responses in the prenatal 2VEH control group. Postnatal PPA treated males in this group showed significant hypo-responsiveness to startle compared to postnatal VEH ($p = 0.007$), while postnatal PPA treated females showed significant hyper-responsiveness to startle compared to postnatal VEH ($p = 0.048$). This effect was not present on any other sessions (Figure 3.1A).

Effects of prenatal LPS treatment

Prenatal LPS treatment produced hyper-responsiveness to startle in female offspring postnatally treated with either PPA or VEH. In postnatal VEH treated females, prenatal LPS produced significantly greater startle responses than 2VEH treatment ($p = 0.013$), while in postnatal PPA treated females prenatal LPS produced significantly greater startle responses than both prenatal PPA ($p = 0.032$) and 5VEH ($p = 0.027$) treatments. These effects of treatment on initial startle responses were limited to Session 1 (Figure 3.1A).

Effects of prenatal PPA treatment

Prenatal PPA treatment alone did not influence startle on the first 2 trials until Session 4. Prenatal PPA treatment produced significant hyper-responsiveness to startle relative to prenatal 5VEH treatment in females for both postnatal PPA ($p = 0.044$) and postnatal VEH ($p = 0.046$) and in males for postnatal VEH ($p = 0.049$, Figure 3.1D).

Effects of combined prenatal LPS or PPA and postnatal PPA treatment

Later test sessions showed effects of combined prenatal and postnatal treatment. Postnatal PPA attenuated the effects of prenatal LPS treatment (Figure 3.1C, D). On Sessions 3 and 4, prenatal LPS treatment produced hyper-responsiveness to startle in male offspring compared to other prenatal treatment groups, but only in postnatal VEH treated males (Session 3: 2VEH, $p = 0.035$, PPA, $p = 0.016$, and 5VEH, $p = 0.023$;

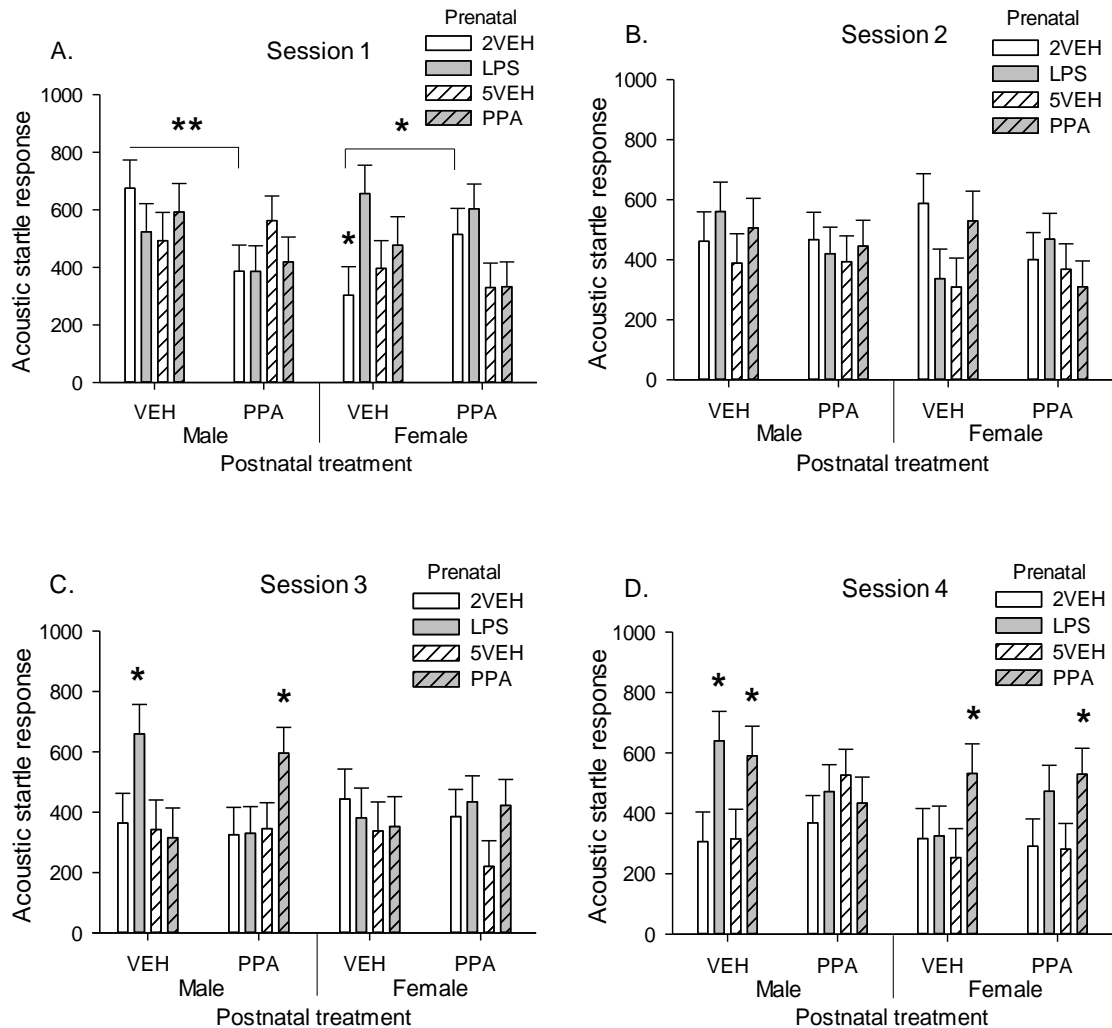


Figure 3.1. Initial acoustic startle response collapsed across the first 2 trials for the 4 sessions.

On Session 1, postnatal PPA decreased startle in males and increased startle in females (prenatal 2VEH group). Prenatal LPS increased startle initially in females (Session 1) and increased startle developed in males (Session 3, 4). In later sessions, prenatal PPA treated offspring also showed increased startle. Error bars represent SEM. Refer to Table 3.1 for group designations and sample sizes. * $p < 0.05$, ** $p < 0.01$

Session 4: 2VEH, $p = 0.018$ and 5VEH, $p = 0.020$). On Session 3, prenatal LPS-postnatal VEH treated males also had significantly greater startle responses than prenatal LPS-postnatal PPA treated males ($p = 0.002$).

On Session 3, an effect of prenatal PPA in combination with postnatal PPA was seen in male offspring. In postnatal PPA treated males, prenatal PPA produced significantly greater startle responses than prenatal 5VEH ($p = 0.043$), 2VEH ($p = 0.041$), and LPS ($p = 0.038$) treatments. This group (prenatal PPA-postnatal PPA) was also significantly greater than males treated with prenatal PPA and postnatal VEH, $p = 0.007$ (Figure 3.1C).

In summary, on Session 1, postnatal PPA decreased startle in males and increased startle in females in the prenatal 2VEH control group. Prenatal LPS treatment also initially increased startle in females, with increased startle developing in later sessions in males. The effect in males was attenuated with postnatal PPA. Lastly, increased startle with prenatal PPA treatment developed in later sessions in both males and females.

3.3.2. Habituation

3.3.2.1. Habituation across the first 10 trials

To assess whether drug treatments affected habituation to the startle stimulus, the first 2 trials of a session were averaged and trials 3-10 were normalized to this average. A linear model was used to analyze each of the 4 sessions. There were significant effects of Trial for all Sessions (1, 2: $p < 0.001$; 3: $p = 0.001$; 4: $p = 0.020$), but no significant interactions with trial and treatments. By trial 10, startle responses were significantly lower than the average response of trials 1 and 2 across all 4 Sessions ($ps < 0.05$). When data were divided into treatment groups, no animals displayed significant habituation across the first 10 trials on Sessions 1, 3, and 4, with 6 of 16 treatment groups on Session 2 showing habituation (trials 9 and 10 each significantly lower than the averaged response of trials 1 and 2, $ps < 0.05$).

On Session 1, 2, and 4, significant Sex x Prenatal drug x Postnatal drug interactions were found (1: $F(3,891) = 7.26$, $p < 0.001$; 2: $F(3,894) = 6.68$, $p < 0.001$; 4: $F(3,896) = 4.69$, $p = 0.003$), and significant Sex x Postnatal drug ($F(1,900) = 5.05$, $p = 0.025$) and Prenatal x Postnatal drug ($F(3,906) = 11.94$, $p < 0.001$) interactions were found on Session 3 (Figure 3.2).

Effects of postnatal PPA treatment

In male offspring, there was an effect of postnatal PPA on normalized startle amplitudes. Postnatal PPA produced significantly greater startle responses compared to postnatal VEH in prenatal PPA treated males on Session 1 ($p = 0.029$), in LPS, 2VEH, and 5VEH treated males on Session 3 ($ps < 0.05$) and in prenatal PPA and LPS treated males on Session 4 ($ps < 0.01$).

Effects of prenatal LPS treatment

Limited to Session 2, prenatal LPS produced significantly greater startle responses compared to the other 3 prenatal treatments in females treated with postnatal VEH (2VEH, 5VEH, PPA, $p < 0.01$), and compared to prenatal 2VEH in females treated with postnatal PPA ($p = .035$, Figure 3.2B).

Effects of combined prenatal PPA and postnatal PPA treatment

Prenatal PPA treatment alone did not significantly affect startle responses across the first 10 trials. Prenatal PPA treatment combined with postnatal PPA treatment to increase the startle response of female offspring on Sessions 1 and 2. Prenatal PPA-postnatal PPA treated females produced significantly greater startle responses than the other 3 prenatal groups treated with postnatal PPA (5VEH, 2VEH, LPS, $p < 0.001$), and prenatal PPA treated females receiving postnatal VEH ($p < 0.001$) on Session 1. On Session 2, this group was significantly greater than 5VEH and 2VEH females treated with postnatal PPA ($ps < 0.01$, Figure 3.2A, B).

Figure 3.3B shows that prenatal PPA-postnatal PPA treated females were not habituating to startle and were in fact sensitized across trials compared to the average of trial 1 and 2 on Session 1 (trials 3-10, $ps < 0.05$). This effect was significant on Session 2, but not as pronounced, with females prenatally exposed to PPA and postnatally to PPA showing significantly greater startle responses than 5VEH and 2VEH females on trials 3, 5-7, $ps < 0.05$ (data not shown). By Session 3, the effects of prenatal and postnatal PPA had disappeared, with Session 4 showing the opposite pattern to Session 1 and 2 (Figure 3.2C, D). Collapsed across trials, prenatal PPA in combination with postnatal PPA treatment in females produced significantly lower startle responses compared to the other prenatal groups combined with postnatal PPA (5VEH $p = 0.018$, 2VEH $p = 0.006$, LPS $p = 0.002$).

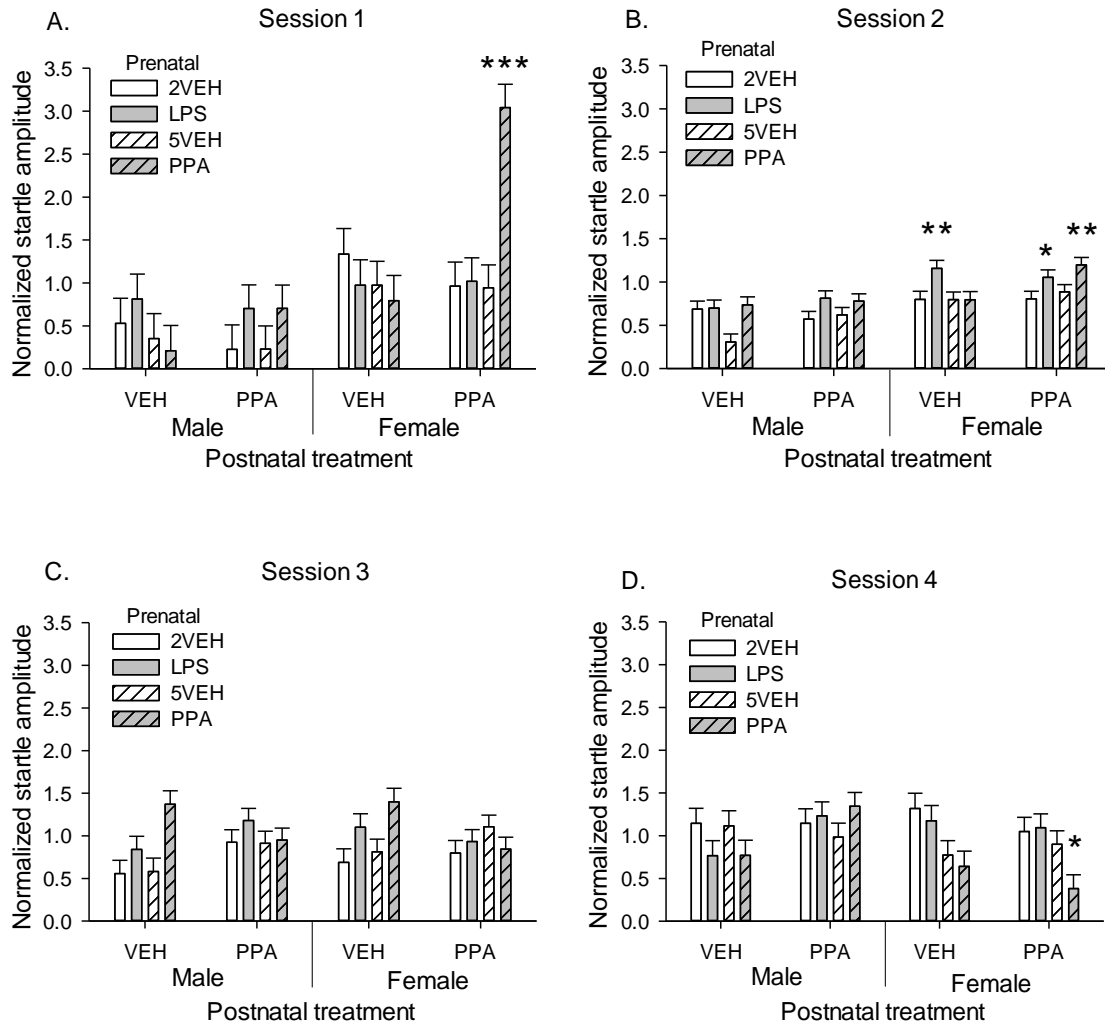


Figure 3.2. Habituation to the acoustic startle pulse across the 4 sessions.

Habituation to the startle pulse was assessed over the first 10 trials of each session. Collapsed across trials, effects of prenatal PPA and postnatal PPA treatment were evident in female, but not male, offspring. Females who received both prenatal and postnatal PPA showed significantly increased startle on Sessions 1 and 2, with a decrease in Session 4 compared to other prenatal treatments. Error bars represent SEM. Refer to Table 3.1 for group designations and sample sizes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

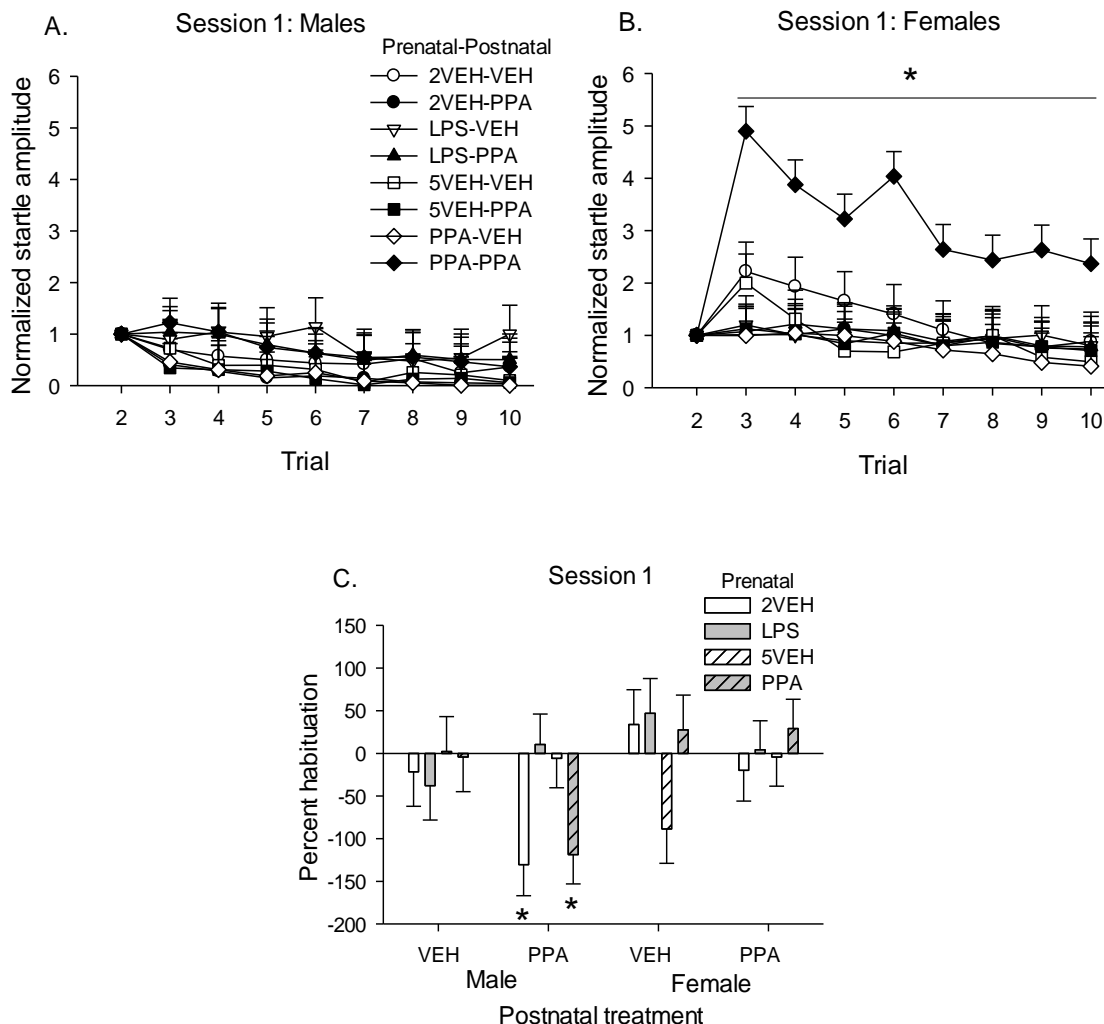


Figure 3.3. Habituation to the acoustic startle pulse on Session 1.

Session 1 habituation across trials 3-10 for A: Males and B: Females. Female offspring who received prenatal and postnatal PPA displayed increased startle amplitudes compared to other prenatal treatment groups. Trial 2 is the average of trial 1 and 2. C: Percent habituation score between the beginning and end of the session. Animals performed similarly at the beginning and end of the startle session, with the exception of postnatal PPA treated male offspring in the prenatal 2VEH and PPA treated group. These groups showed negative habituation, or sensitization, to startle at the end of the session. This effect was present only on Session 1. Error bars represent SEM. Refer to Table 3.1 for group designations and sample sizes. * $p < 0.05$

3.3.2.2. Percent habituation within session

The last 5 trials of each startle session were pulse alone trials. The average of the peak startle response was taken along with the average of the peak startle response of trials 6-10 in order to compute a percent habituation measure within each startle session. A significant Session x Sex x Prenatal x Postnatal interaction was found, $F(9,369) = 1.98$, $p = 0.041$, with some treatment groups showing sensitization (increased levels) at the end of a startle session compared to the beginning.

On Session 1, an effect of postnatal PPA was found in male offspring prenatally exposed to PPA or 2VEH, as these animals showed significant sensitization compared to prenatal LPS and 5VEH ($ps < 0.05$) and to prenatal PPA and 2VEH males postnatally treated with VEH ($ps < 0.05$, Figure 3.3C). This effect was only present on Session 1 as the remaining sessions showed similar startle responses among male offspring. On Session 2, female offspring in the prenatal 2VEH group postnatally treated with PPA showed sensitization compared to the prenatal LPS, PPA, and 5VEH groups ($ps < 0.01$), with no differences during the other 3 sessions (data not shown).

In summary, postnatal PPA treatment produced slight sensitization to startle in male offspring. Prenatal LPS treatment increased startle responses in female offspring only on Session 2. Most striking is the sensitization to startle in female offspring that received both prenatal and postnatal PPA. This was present on Sessions 1 and 2, with habituation occurring by Session 4 compared to other prenatal treatment groups.

3.3.3. Acoustic startle response

Following habituation to the first 10 trials, each startle session contained 10 startle alone trials interspersed with prepulse-pulse, prepulse only, or no pulse trials. These 10 trials provided a measure of average startle response. There was a significant effect of Session, $F(3,361) = 6.06$, $p < 0.001$, with startle responses during Session 1 greater than the other 3 sessions. There were no interactions with Session. However, a significant Sex x Prenatal drug x Postnatal drug interaction was found, $F(3,376) = 5.08$, $p = 0.002$ (Figure 3.4). Prenatal treatment with LPS produced significant hyper-reactivity to startle in postnatal-VEH treated male offspring as compared to prenatal 2VEH ($p = 0.025$) or 5VEH treatments ($p = 0.034$). This was not found in male offspring postnatally treated with PPA where startle responses were significantly lower in prenatal LPS-postnatal PPA

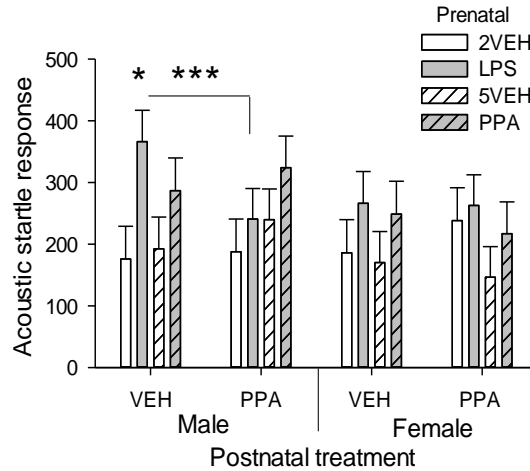


Figure 3.4. Acoustic startle response collapsed across the 4 sessions.

There were no significant effects of prenatal or postnatal treatment in female offspring. In male offspring, prenatal LPS produced a hyper-responsiveness to startle in postnatal VEH treated animals, while this effect was attenuated in postnatal PPA treated animals. Error bars represent SEM. Refer to Table 3.1 for group designations and sample sizes.

* $p < 0.05$, *** $p < 0.001$

treated males as compared to prenatal LPS-postnatal VEH treated males ($p < 0.001$). No significant effects of prenatal LPS in females, or prenatal and postnatal PPA in males and females, on acoustic startle response were found.

3.3.4. Percent prepulse inhibition (%PPI)

Percent prepulse inhibition was assessed for the 76 dB and 82 dB prepulses at each inter-stimulus interval (ISI, 60 ms and 100 ms). Inclusion of trials consisting of each prepulse in the absence of the 115 dB pulse verified that animals were not startling to the prepulses alone and that the drug treatments were not significantly different.

At the 60 ms ISI, %PPI increased across session, $F(3,730) = 68.02$, $p < 0.001$, and increased as the prepulse intensity increased, $F(1,730) = 82.93$, $p < 0.001$. There were no other significant effects of session or prepulse. The Sex x Prenatal drug interaction, $F(3,751) = 7.67$, $p < 0.001$, indicated that while there were no differences in %PPI for male offspring, female offspring prenatally exposed to PPA exhibited a significant decrease in %PPI compared to females in the 5VEH control group, $p = 0.037$ (Figure 3.5). Further analysis showed that prenatal PPA resulted in a deficit in female offspring on Sessions 2 (76 dB, $p = 0.028$), 3 (82 dB, $p = 0.018$), and 4 (76 dB, $p = 0.023$).

As with a 60 ms ISI, the 100 ms ISI also produced %PPI that increased across session, $F(3,728) = 32.70$, $p < 0.001$, and increased as the prepulse intensity increased, $F(1,728) = 250.05$, $p < 0.001$. There was a significant Sex x Prenatal drug x Postnatal drug interaction, $F(3,746) = 3.57$, $p = 0.014$ (Figure 3.6). In prenatal 2VEH treated male offspring, postnatal PPA produced significantly greater %PPI than postnatal VEH, $p = 0.003$. This effect was present on Sessions 1 (82 dB, $p = 0.026$), 2 ($p = 0.049$, NS on each prepulse), and 4 (82 dB, $p = 0.045$). In female offspring, postnatal PPA had the reverse effect in the 2VEH group, with postnatal PPA significantly decreasing %PPI compared to postnatal VEH, $p = 0.017$ (Session 3, 82 dB, $p = 0.042$; Session 4, 76 dB, $p = 0.039$). Limited to Session 1, there was a difference in the prenatal control groups in female offspring postnatally treated with PPA as females in the 2VEH group had significantly lower %PPI than the 5VEH group (76 dB, $p = 0.020$). The 100 ms ISI also produced a Session x Sex x Prenatal drug interaction, $F(9,728) = 2.07$, $p = 0.030$. On Session 2, females in the prenatal PPA group showed significantly less %PPI than 5VEH females, $p = 0.006$ (76 dB, $p = 0.011$).

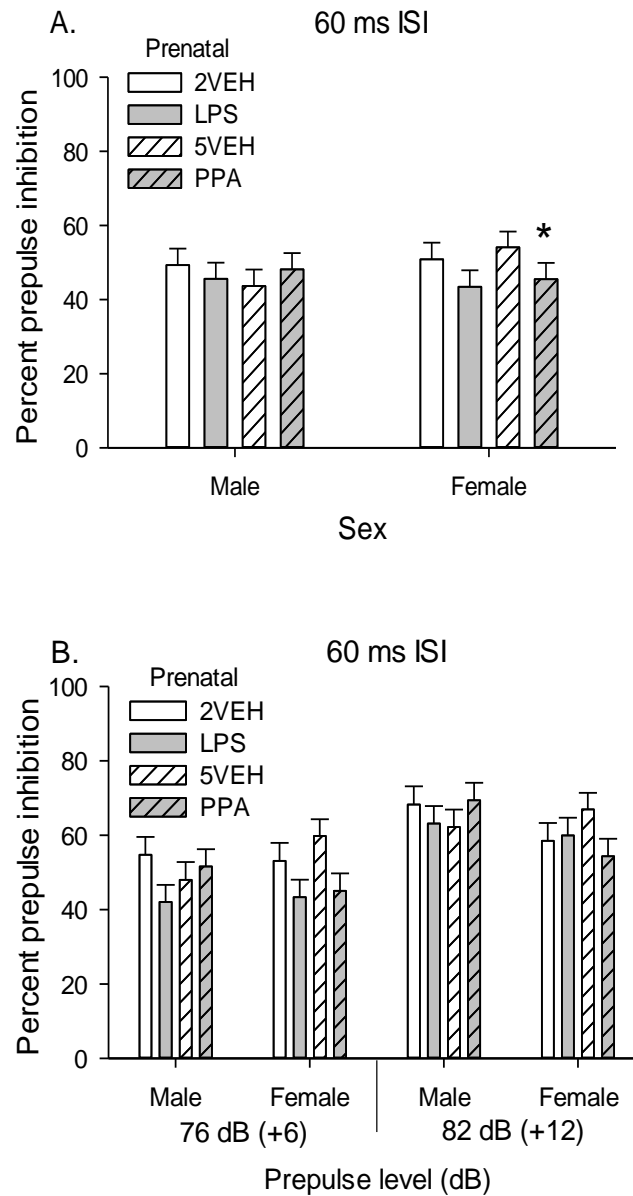


Figure 3.5. Percent prepulse inhibition (%PPI) with 60 ms inter-stimulus interval.

The amount of prepulse inhibition increased as prepulse intensity increased, no interactions with treatments were found. A: Sex x Prenatal drug interaction was significant. Females in the prenatal PPA treated group displayed significantly decreased prepulse inhibition compared to 5VEH treated controls. B: Data displayed across prepulse level. Error bars represent SEM. Refer to Table 3.1 for group designations and sample sizes. * $p < 0.05$

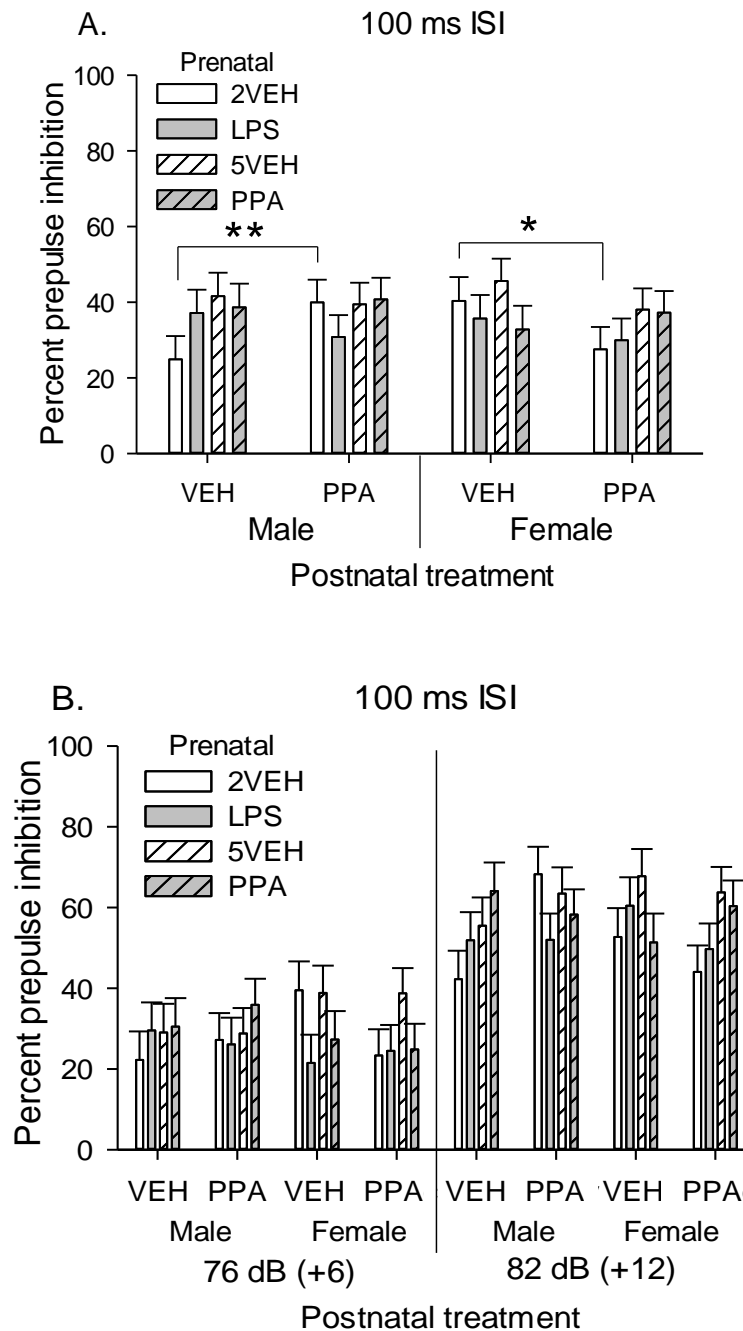


Figure 3.6. Percent prepulse inhibition (%PPI) with 100 ms inter-stimulus interval.

The amount of prepulse inhibition increased as prepulse intensity increased, no interactions with treatments were found. A: Sex x Prenatal drug x Postnatal drug interaction was significant. In the prenatal 2VEH treated group, postnatal PPA decreased prepulse inhibition in female offspring and increased PPI in male offspring. B: Data displayed across prepulse level. Error bars represent SEM. Refer to Table 3.1 for group designations and sample sizes. * $p < 0.05$, ** $p < 0.01$

Overall, prenatal LPS did not significantly affect prepulse inhibition. Prenatal PPA treatment produced a %PPI deficit in female, but not male, offspring at both ISIs. This effect was more evident at the 60 ms ISI. Additionally, at the 100 ISI, there was a sex difference in the effects of postnatal PPA, with males showing increased %PPI and females showing decreased %PPI.

3.4. Discussion

Prior investigations of the effects of prenatal and postnatal immune activation and the effects of GI microbial metabolites have been limited to adult rodents. In the present study, the acoustic startle response (ASR), habituation to this response, and prepulse inhibition in male and female adolescent rat offspring was evaluated following prenatal treatment with the immune stimulant, lipopolysaccharide, or the short chain fatty acid, PPA. Rat offspring were further administered a PPA regimen in the second week of life to see if a postnatal insult would further exacerbate any behavioural effects of prenatal treatment.

Prenatal LPS produced pronounced hyper-sensitivity to startle in males, but not females, while prenatal and postnatal PPA each produced transient effects on startle response in both males and females. A deficit in prepulse inhibition in female offspring prenatally exposed to PPA was present, while prenatal LPS and postnatal PPA did not alter prepulse inhibition. In addition, evidence supporting a double-hit hypothesis was apparent in females, showing that further environmental insults during development can exacerbate the effects of a prenatal insult. The combination of prenatal and postnatal PPA produced an increased, or sensitized, startle response over the first 10 startle trials, rather than habituation. Among all animals, there was little habituation to startle. Taken together, the present results indicate that prenatal LPS or PPA, postnatal PPA, and combined prenatal and postnatal PPA treatments can produce subtle and sexually dimorphic effects on sensory processing in adolescent rats. Effects on sensory processing were specific to each treatment and are discussed below.

3.4.1. Prenatal LPS treatment influenced acoustic startle response, not prepulse inhibition

Prenatal treatment with LPS on G15-16 produced significant effects on startle responses, with increased startle responses to initial stimuli in females and throughout the

startle testing session in males. Females acclimated to the acoustic pulse after Session 1, with hyper-sensitivity to the first trials disappearing. In male offspring, a pattern of hyper-sensitivity to initial trials did not develop until Sessions 3 and 4. Additionally, prenatal LPS affected the average peak startle response in the bulk of the testing session, with males hyper-sensitive to acoustic startle, regardless of test session.

These results suggest that with a low dose of prenatal LPS, startle behaviour is altered in a sex-specific manner in adolescent offspring; transiently in female offspring (to novel stimuli) and more permanently in male offspring. Interestingly, hyper-responding was non-significant in prenatal LPS males postnatally treated with PPA when it was hypothesized that an exacerbation with the 2 treatments might occur. However, valproic acid (VPA) and sodium butyrate (a SCFA) have been shown to decrease LPS-induced proinflammatory responses via their activity on gene expression as histone deacetylase inhibitors (Chen et al., 2007). PPA can also act as a histone deacetylase inhibitor (Nguyen et al., 2007) and may have similarly countered effects of LPS treatment.

There is limited information on the effects of maternal immune activation (MIA) on ASR in adolescent rats. Most startle response studies report prepulse inhibition and do not report ASR data. Where reported, MIA on G15 using the viral mimetic, poly I:C, has been found to produce no change in startle responses in adolescent male (Wolff et al., 2010) and adolescent male and female rats (Howland et al., 2012). The present findings of hyper-sensitivity to acoustic startle in male and, to a lesser extent, female adolescent rats with MIA using LPS are novel and suggest developmental differences in responses.

Female adolescent rats are hyper-responsive to an acoustic startle stimulus for a short time and then acclimate, while males showed more long-term effects. This pattern of immune activation during development affecting the behaviour of males and not females has been previously reported with postnatal LPS and adult rats (Tenk et al., 2008; Tenk et al., 2013). Sex differences in the innate immune response, with males more susceptible to immune stimulation and females better able to handle an immune insult, may be related to estrogen modulated cytokine gene expression (Dimayuga et al., 2005; Klein, 2012).

Hypersensitivity to startle with prenatal LPS administration has been shown in adult male rats. Fortier et al. (2004) report increased startle responses in adult males with LPS administered at the same dose as the present study (50 µg/kg) on G18-19, but did not find changes in males when a higher dose of LPS (100 µg/kg) was administered on G15-16 (Fortier et al., 2007). Injection later in gestation tends to increase risk of infant mortality and, as such, an increased sensitivity at G18-19 is observed. No change in ASR in adult males with prenatal LPS exposure at G15-16 suggests that adolescent rats may be more vulnerable to the effects of maternal LPS.

Repeated LPS administration for the duration of gestation produced mixed results on ASR in adult males and females. Increased startle responses were found in both male and female offspring (Basta-Kaim et al., 2011), while Borrell et al. (2002) found no change in ASR in males or females. Clearly, the effects of prenatal LPS appear to be influenced by the intensity and timing of immune system activation and, as the present study demonstrates, the age at which offspring are tested.

Contrary to previous results, prenatal LPS treatment did not alter prepulse inhibition in male and female adolescent offspring. Romero et al. (2010) found decreased prepulse inhibition in male and female adolescent offspring exposed to LPS throughout gestation. Similar effects are reported with poly I:C administered on G12 (Deslauriers et al., 2013) or G15 (Howland et al., 2012). This is consistent with PPI deficits observed in adult male and female offspring following MIA (Basta-Kaim et al., 2011; Fortier et al., 2007; Howland et al., 2012; Smith et al., 2007). LPS was administered at a low dose and a fixed time (G15-16) in the present study. Time of injection, dose, and immune stimulant affect the subsequent effects on offspring, and the present dose was not sufficient to produce changes in prepulse inhibition. It is interesting that prenatal LPS produced alterations in acoustic startle response in the absence of PPI deficits, reinforcing the idea that differing levels of severity of maternal immune insult at similar time points in development may produce different behavioural phenotypes.

In summary, maternal immune activation with the bacterial mimetic, LPS, altered sensory processing of acoustic startle, with intact prepulse inhibition, in adolescent rats. Effects of LPS were sexually dimorphic, as hyper-responding to acoustic startle was more permanent in male offspring and occurred only to novel stimuli in female offspring.

Importantly, these results demonstrate that a low dose of LPS can alter sensory processing in adolescence at a dose that has been shown to not alter adult startle responses. In light of the male prevalence in ASD, bacterial infection during gestation may influence sensory responsiveness to stimuli.

3.4.2. Prenatal PPA and postnatal PPA treatment alone each influenced acoustic startle response and prepulse inhibition

Prenatal PPA treatment produced hyper-sensitivity to the initial startle trials (first 2 trials of a session) in both male and female offspring on the last session, following repeated experience with the startle pulse. In contrast, postnatal PPA affected initial responses to startle stimuli only on Session 1. Male offspring were hypo-sensitive and female offspring were hyper-sensitive to acoustic startle. Unlike prenatal LPS treatment, the effects of prenatal and postnatal PPA were present only for the first trials of the startle test as results for the average peak response to acoustic startle in the bulk of the session (during prepulse inhibition trials) did not show an effect of prenatal PPA or postnatal PPA. It appears that animals treated with prenatal or postnatal PPA have altered sensitivity to sudden stimuli, but with repeated presentations within a session, are quickly able to adapt their responses.

Habituation measures showed that postnatal PPA enhanced startle in male offspring, in a non-specific manner, across trials 3-10 of a session and enhanced within session startle (sensitization) on Session 1, while no effects were observed in female offspring. Males habituated to startle within a session similarly to other groups on Sessions 2-4. This effect in postnatal PPA treated males could indicate a delay in habituation compared to other animals. Perry et al. (2007) report habituation to acoustic startle in adults with ASD; however, while the end result was habituation, it took longer for ASD patients to reach that habituation across trials.

Although this is, to date, the first study assessing the effects of prenatal and postnatal PPA on acoustic startle in rat offspring, comparisons to previous studies using environmental toxins as models for neurodevelopmental disorders can be made. One of the most widely used toxins in models of autism is valproate (VPA). Again, most work has been carried out with adult offspring and ASR is rarely reported. Hypo-sensitivity (Vorhees, 1987) and no change in ASR (Markram et al., 2008) has been reported in adult

male and female offspring prenatally exposed to VPA, while Dendrinios et al. (2011) report hypo-sensitivity in juvenile offspring following prenatal VPA, but did not separate males and females. A study using postnatal VPA in the first week of life also found hypo-sensitivity in both male and female adolescent rats (Reynolds et al., 2012). Postnatal PPA treated male adolescent rats also showed hypo-sensitivity to startle on the first 2 trials of Session 1.

Other toxins have also been administered prenatally in rats. As mentioned above, MIA produced hyper-sensitivity to startle. Zerrate et al. (2007) reported hypersensitivity to acoustic startle in female adolescent offspring following postnatal treatment (first week of life, P2-5) with terbutaline, a drug administered to arrest preterm labor in humans. Hypersensitivity in the current study suggests the effects of PPA may be similar to these toxins. It may also be the case that the ASR profile with PPA administered at various times in development has a distinct profile. Prenatal PPA and postnatal PPA in the second week of life produced hypersensitivity to startle across the first 10 trials in the current study, while postnatal PPA in the first week of life produced no change in startle across the first 10 trials (unpublished data).

Prenatal and postnatal PPA each produced a prepulse inhibition deficit in female adolescent offspring. Prenatal PPA did not alter prepulse inhibition in male offspring, but produced a decrease in prepulse inhibition in female offspring at both ISIs (60 and 100 ms). Both of these intervals are used in the human literature with ASD patients, while 100 ms is often used in behavioural rodent studies in order to draw comparisons with human literature. Although it is unclear why this decrease was only significant on later sessions and not on session 1, it does emphasize the utility of repeated testing. Similar to prenatal PPA, females treated with postnatal PPA showed a decrease in prepulse inhibition, but males showed an increase in prepulse inhibition. This increase in males could also be interpreted as a deficit, with fixation on prepulse processing resulting in an inability to respond appropriately to stimuli in the environment. A similar pattern was observed in previous work with postnatal PPA in the first week of life increasing prepulse inhibition in female, not male offspring (Foley et al., 2009).

A deficit in prepulse inhibition is consistent with previous animal work with VPA and MIA models. Decreases in prepulse inhibition have been reported following MIA in

both male and female treated adolescent offspring (Howland et al., 2012; Romero et al., 2010) and prenatal VPA in adolescent males and adult males and females (Markram et al., 2008; Schneider et al., 2005). Postnatal VPA also produced a prepulse inhibition deficit in both male and female adolescent rats (Reynolds et al., 2012).

The present results indicate that prenatal and postnatal PPA alone affects sensory processing in adolescent offspring. Subtle effects on acoustic startle responses in both male and female adolescent offspring, and sustained effects on prepulse inhibition were observed. Effects on prepulse inhibition were sexually dimorphic, with prenatal PPA producing a decrease in females and no change in males, and postnatal PPA producing a decrease in females and an increase in males.

3.4.3. Combined effects of prenatal and postnatal PPA: Evidence for the double hit hypothesis

The combination of prenatal and postnatal PPA produced augmented startle responses in female offspring, but not male offspring. Prenatal PPA in combination with postnatal PPA showed increased normalized startle across trials 3-10 for the first 2 sessions in female offspring, showing a sensitized startle response that was not observed in other animals. Females with this ‘double-hit’ of PPA require longer adjusting to the startle stimuli compared to other treatment groups. Increased startle responses return to a level similar to other animals as repeated sessions occur, suggesting a heightened sensitivity to repeated stimuli that gradually adapts. There was no evidence of a double hit effect in males or for prepulse inhibition in males and females. Coupled with the effects of prenatal PPA on prepulse inhibition in female offspring, it appears that female offspring were more susceptible to PPA induced alterations in behaviour. In this respect, anxiety and gut-derived illnesses are more prevalent in females over males (Donner and Lowry, 2013; Tang et al., 2012). That the main source of endogenous PPA is from metabolism by enteric bacteria could link the susceptibility of females to the developmental effects observed in the current study.

Combined PPA effects in adolescence could distinguish this treatment from that of prenatal LPS and MIA. Yee et al. (2011) found no evidence for a double hit hypothesis in adult rats using prenatal poly I:C (G15) and juvenile stress (P27-29), with no combined effects of treatment on ASR and prepulse inhibition, while evidence for a double hit

hypothesis has been reported in adult mice following combined prenatal poly I:C (G9) and adolescent stress (P30-40) (Giovanoli et al., 2013). Prenatal LPS combined with postnatal PPA did not produce enhanced effects. This may be a product of timing of administration, or the type of insult, as studies of postnatal LPS administration have shown augmented responses of rats with subsequent insults of LPS or stress in adulthood (Tenk et al., 2008; Walker et al., 2009). Previous work with postnatal PPA in the first week of life and adolescent PPA challenge increased habituation across the first 10 startle trials in male rats (unpublished data). In a more naturalistic setting, both LPS and PPA together would be produced by enteric bacteria, along with other SCFAs. Future investigation warrants combining LPS and PPA into one insult and/or combining these environmental toxins with a genetic animal model. Regardless, the effects of a ‘double hit’ of PPA suggest that multiple environmental insults, perhaps altering immune responses, can have sexually dimorphic effects on sensory behaviour in adolescent rats.

3.4.4. Lack of habituation and effects of vehicle injections on behaviour

The effect of trial indicated that normalized startle responses were significantly lower at trial 10 than at the beginning of the session, suggesting habituation to acoustic startle. However, when animals were divided by treatment group this habituation was no longer present. Methodology may account for the lack of progressively decreasing responses to startle in all treatment groups, including controls. A larger number of trials (e.g., 30) may be required to produce habituation of the startle response and/or constant inter-trial intervals facilitate habituation (Geyer et al., 2001; Schmid et al., 2011). Wolff and Bilkey (2008) also used a small number of trials at the beginning and end of their startle session to assess habituation and also found no change in startle in MIA treated or control animals.

It is also very interesting to note that there were some significant differences in offspring behaviour of those rats that were prenatally exposed to 2 or 5 vehicle control injections; specifically, that there were effects of postnatal PPA on acoustic startle and prepulse inhibition in 2VEH, but not 5VEH animals. Laboratory procedures can alter physiological and hormonal parameters in rodents. For example, repeated vehicle injections can alter baseline levels of plasma corticosterone (Balcombe et al., 2004; Drude et al., 2011; Ryabinin et al., 1999). Furthermore, with prenatal stress, the enzyme

11 β -hydroxy steroid dehydrogenase Type II (11 β -HSD2) that normally protects the fetus from maternal cortisol can be downregulated in animals and humans, exposing the developing animals and the fetus to cortisol (O'Donnell et al., 2009). Prenatal stress can result in anxiety-like behaviour and increased responding to novelty in offspring (Henry et al., 1994; Fride and Weinstock, 1988). In fact, prenatal handling and saline injection can produce hyper-sensitivity to acoustic startle on first presentation of the stimulus in male offspring (White and Birkle, 2001). A stress response associated with saline injections, although quite mild in comparison to maternal stress paradigms, may have been enough of a stressor to alter development and mask the effects of postnatal PPA.

3.4.5. Potential neurodevelopmental changes from LPS and PPA administration

Alterations in neurodevelopment in excitatory and/or inhibitory neurotransmission may have contributed to the effects of treatments on ASR. Glutamate is the major neurotransmitter mediating the acoustic startle response in the midbrain, with GABA receptor blockade shown to increase the ASR (Koch and Schnitzler, 1997). Decreased GABA could alter excitation/inhibition balance and lead to less inhibition of glutamatergic inputs in the startle pathway. Fear or anxiety to the startle stimuli could account for the subtle effects on startle responses. Inputs from the amygdala modulate startle and are involved in sensitization and fear-potential of startle (Koch and Schnitzler, 1997; Van Nobelen and Kokkinidis, 2006). In the case of prenatal LPS in females and prenatal and postnatal PPA in males and females, these are subtle alterations as offspring are able to adjust their behaviour over repeated startle sessions and respond to startle similar to other animals, perhaps through learning that the stimuli are not threatening. Associations have been made between sensory abnormalities and increased anxiety in children with ASD (Goldsmith et al., 2006; Pfeiffer et al., 2005).

Supporting the possibility of PPA induced developmental alterations in neurotransmission are reports of a PPA-laced diet administered throughout prenatal and postnatal life produced altered cortical migration, increased synaptic density, and reduced inhibitory interneurons in the cortex of rat offspring (Taylor et al., 2013) while orally administered PPA depleting GABA in young rat brains (El-Ansary et al., 2011). SCFAs and VPA may gain access to the developing fetus through active transport via monocarboxylate transporters in the placenta (Nagai et al., 2010; Ushigome et al., 2001).

It should be noted that the effects of PPA and SCFAs on biological systems are broad (MacFabe, 2012), and also, at many levels, may be beneficial (Al-Lahham et al., 2010).

Prenatal LPS has recently been shown to decrease the number of GABA neurons in adult offspring and alter expression of genes regulating the migration of GABAergic interneurons, important for proliferation, migration, and synaptogenesis in early development (Nouel et al., 2012; Oskvig et al., 2012; Owens and Kriegstein, 2002). This may contribute to an imbalance in excitation/inhibition, favouring excitability of neural circuits. Increases in the proinflammatory cytokines IL-1 β , IL-6, and TNF- α in the amniotic fluid and the fetal brain with LPS treatment may also contribute to adverse outcomes (Gayle et al., 2004; Ning et al., 2008; Oskvig et al., 2012).

Prepulse inhibition is modulated by prefrontal and mesolimbic dopamine inputs to the brain stem. Dopamine and serotonin antagonists, and norepinephrine agonists can decrease prepulse inhibition (Koch, 1999). PPA may alter gene expression of catecholamine synthesis. Butyrate (a SCFA) and VPA, both histone deacetylase inhibitors, both increase transcription of the tyrosine hydroxylase (TH) gene in PC12 cells, *in vitro* (DeCastro et al., 2005; D'Souza et al., 2009). PPA also acts as a histone deacetylase inhibitor (Nguyen et al., 2007; Phiel et al., 2001) and preliminary results have shown that central administration of PPA can alter gene expression in ASD associated genes (Nankova et al., 2012).

3.4.6. Relevance for autism spectrum disorders

An imbalance between excitation and inhibition within neural circuits may explain behavioural impairments observed in autism, with alterations in GABA suggested to be critical. A meta-analysis reports that multiple ASD mouse models share a decrease in GABA cells in the cortex (Gogolla et al., 2009). The cortex is organized into minicolumns, consisting of glutamatergic and GABAergic neurons. In autistic patients, minicolumns in the frontal and temporal cortex were narrower and less compact than controls (Casanova et al., 2002), while there was a 50% reduction in enzyme protein levels responsible for GABA synthesis in parietal and cerebellar areas (Fatemi et al., 2002). A decrease in inhibition may impair neural circuit maturation and/or leave neural circuits in a hyper-excitable state, resulting in either withdrawing or hyper-reacting to

environmental stimuli in order to cope (Markram et al., 2008; Rubenstein and Merzenich, 2003).

In reports of MIA or VPA animal models of neurodevelopmental disorders, prepulse inhibition is frequently used in behavioural test batteries, acoustic startle response is less frequently reported, and habituation to acoustic startle rarely investigated. Behavioural startle response studies often discard the first trials from analysis. Current results suggest that it may be useful to include habituation measures in acoustic startle testing. It may also be informative to use different modalities (e.g., tactile, heat) as multiple sensory abnormalities in ASD have been reported that do not include acoustic (Leekam et al., 2007).

Prepulse inhibition deficits have not been extensively investigated in the ASD population and very few reports have been published, with conflicting results (McAlonan et al., 2002; Oranje et al., 2013; Perry et al., 2007). Prepulse inhibition is governed by long term neural connections between prefrontal/striatal regions, midbrain, and brain stem. As ASD may involve impaired long-range neural connectivity (Shukla et al., 2011), including prepulse inhibition in a behavioural test battery has utility.

3.4.7. Concluding Remarks

In summary, this study is the first, to date, to assess the effects of prenatal and postnatal PPA, and one of a few assessing effects of prenatal LPS, on the acoustic startle response and prepulse inhibition in both male and female adolescent offspring. The results highlight the importance of using both male and female rats in developmental neuroscience and provide new information on adolescent rats. Prenatal LPS increased the acoustic startle response in adolescent males, while postnatal PPA altered initial startle responses in both male and female offspring. A greater effect on habituation to the startle response was observed in females who received a double hit of PPA, prenatal and postnatal. A decrease in prepulse inhibition in female offspring prenatally and postnatally exposed to PPA was present, postnatal PPA increased prepulse inhibition in males, and prenatal LPS did not alter prepulse inhibition. These results provide evidence that by-products of enteric bacteria metabolism can alter development and behaviour in ways that resemble sensory problems observed in ASD. Repeated infection or immune insult throughout gestation and early life may influence the gut microbiome and lead to

production of metabolic products that alter neurodevelopment in susceptible populations. That different developmental time points of PPA administration produced different behavioural phenotypes illustrates how one environmental insult may contribute to a range of disorders on the autism spectrum.

3.5. References

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Chapter 4

Prenatal exposure to the enteric bacterial metabolic product, propionic acid, and the bacterial mimetic, lipopolysaccharide, alters social behaviour in neonatal, adolescent and adult male and female rats

4.0. Summary

Emerging evidence suggests the gut microbiome plays an important role in immune functioning, behavioural regulation and neurodevelopment. Altered microbiome composition, including elevated short chain fatty acids, and/or immune system dysfunction may contribute to autism spectrum disorders (ASD) with some children with ASD exhibiting both abnormal gut bacterial composition and immune system dysfunction. This study describes the effects of prenatal propionic acid (PPA), a short chain fatty acid and metabolic fermentation product of antibiotic resistant enteric bacteria, and of prenatal lipopolysaccharide (LPS), a bacterial mimetic, on social behaviour in male and female neonatal, adolescent and adult rats. Pregnant Long-Evans rats were injected once a day with either a low level of PPA (500 mg/kg SC) on gestation days G12-16, LPS (50 µg/kg SC) on G12, or vehicle control on G12 or G12-16. Sex- and age-specific, subtle effects on behaviour were observed. Both male and female PPA treated pups were impaired in their nest seeking response test, suggesting impairment in olfactory recognition at a very young age. As well, adolescent males born to PPA treated dams approached a novel object more than control animals and showed increased levels of locomotor activity compared to prenatal PPA females. Prenatal LPS produced subtle impairment in social behaviour in male and female adults. These sex differences, with males affected more by treatments, are consistent with the male predominance in ASD. These findings raise the possibility that prenatal exposure to elevated levels of microbiome products, such as PPA, can subtly influence neonatal, adolescent and adult social behaviour.

4.1. Introduction

Attention has increasingly focused on how host gut microbial populations, known as the microbiome, influence health. Through communication with the central and peripheral nervous system, modification in the various components of the microbiome have the potential to contribute to gastrointestinal (GI), immune, and neuropsychiatric disease (Cryan and Dinan, 2012; Nicholson et al., 2012). Results of recent studies with germ-free mice have demonstrated that alterations in the GI microbiome are associated with changes in early gene expression, neurotransmitter turnover, stress response, as well as reduced social behaviour (e.g., Desbonnet et al., 2013; Foster and Neufeld, 2013; Heijtz et al., 2011).

There is also mounting evidence alterations in the composition of the microbiome may also contribute to the development and/or maintenance of autism spectrum disorders (ASD) in children. Autism spectrum disorders (ASD) are a broad range of neurodevelopmental disorders of unclear etiology. ASD are behaviourally diagnosed, with impairments in verbal and social communication, social behaviour, sensory functioning, and stereotyped and repetitive behaviour (Patterson, 2011). There are a number of comorbid traits in ASD, including a subset of patients that have gastrointestinal (GI) symptoms which can include increased permeability or inflammation of the intestinal tract. Indeed, the severity of autistic symptoms has been associated with severity of GI dysfunction in some patients (Adams et al., 2011; Horvath and Perman, 2002).

Abnormal levels of bacteria flora, including augmented *Clostridia*, *Bacteroidetes*, and *Desulfovibrio* subtypes, have been found in the GI tract of autistic children (Finegold et al., 2012; Parracho et al., 2005). Metabolic products of these include the short chain fatty acids (SCFA, from carbohydrate metabolism) (Finegold et al., 2010) which at normal levels are essential for normal and immune associated functions (Al-Lahham et al., 2010), but at higher levels may alter immune function and/or exacerbate ASD behaviours.

The SCFA, propionic acid (PPA), produced by enteric bacteria, has been proposed as a potential environmental factor in the development of ASD. Elevated levels of PPA characterize the neurodevelopmental metabolic disorder propionic acidemia

(Feliz et al., 2003), with Al-Owain et al. (2013) recently reporting a case study of propionic acidemia and ASD comorbidly. Central and peripheral administrations of PPA in male rats have produced brain and behavioural changes consistent with ASD (MacFabe et al., 2007; MacFabe et al., 2011; MacFabe, 2012; Shultz et al., 2008; Thomas et al., 2012). PPA may exert its effects through a variety of modes including immune dysregulation, inflammation, oxidative stress, mitochondrial dysfunction, and epigenetic actions through inhibition of histone deacetylase, all of which have been associated with ASD (Frye et al., 2013; MacFabe, 2012; Rossignol and Frye, 2012).

Several studies have linked maternal infections or inflammation during pregnancy to the development of ASD (reviewed by Patterson, 2011). An immune insult during critical periods, and the accompanied release of proinflammatory cytokines acting both peripherally and centrally, may have adverse consequences for neurodevelopmental processes, such as cell differentiation, migration, and synaptogenesis (Bilbo and Schwarz, 2012; Patterson, 2011).

Maternal immune activation (MIA) in rodents has been used to investigate the role of the immune system in various behavioural disorders including that of ASD using a number of agents to induce an inflammatory response (e.g., the viral mimetic, polyinosinic:polycytidylic acid (poly I:C) and the bacterial mimetic, lipopolysaccharide). Lipopolysaccharide (LPS) is not only the major component of the cell wall of Gram-negative bacteria but also is a by-product of metabolism of many enteric bacteria. Offspring of dams treated with these immune agents display behavioural deficits in exploratory behaviour and social interaction (Smith et al., 2007). Prenatal treatment with other environmental agents, such as valproate (VPA), an epilepsy treatment that increases the risk of ASD and a precursor of PPA, also produces alterations in social behaviour in adolescence and adulthood rats (Kim et al., 2013; Schneider and Przewlocki, 2005).

Impairments in social behaviour have been reported following acute and central administration of PPA in male adolescent and adult rats, both in social interaction and social approach (MacFabe et al., 2011; Shultz et al., 2008), but to date, there have been no investigations of the potential effects of prenatal PPA on social behaviour. Likewise, there are also relatively few reports on the effects of prenatal LPS on social behaviour in either adult or, in particular, adolescent male and female rats. LPS administered in early

or mid-late gestation resulted in decreased social play behaviour and interaction in male adolescent and adult rats (Taylor et al., 2012; Kirsten et al., 2010). MIA at G12.5 also decreased social approach in adult mice (Malkova et al., 2012; Smith et al., 2007).

The present study investigated the effects of prenatal treatment with either LPS or the microbiome metabolite, PPA, on social and related behaviour in male and female neonatal, adolescent, and adult rats. Low doses of LPS and PPA were used to examine if subtle changes in the components of the microbiome and its products can affect development. Following administration of prenatal LPS and prenatal PPA, a variety of social behaviour measures were assessed throughout the lifespan of male and female offspring. It was hypothesized that prenatal LPS and PPA would affect social behaviour in adolescent and adult rats.

4.2. Methods

4.2.1. Animals

Female Long-Evans rats (230-305 g) were mated with adult males (370-575 g, Charles River, Canada) for a total of 16 litters. Females were paired with a male the night before behavioural estrus. Sperm present on a vaginal smear (hematoxylin and eosin stain) the next morning indicated successful mating and this was designated as gestational day 0 (G0). Dams were housed individually in standard polypropylene cages (45 x 22 x 20 cm) with *ad libitum* access to both food (ProLab RMH 3000) and water. A 12/12 h light-dark cycle (lights on at 07:00 h) was maintained in a temperature controlled colony room ($21 \pm 2^\circ\text{C}$). Litters were born on G22 (designated as postnatal day 0 (P0)), toe-clipped for identification, and were weaned at P21. Prenatal treatments did not differ in pregnancy length (22 days) and there were no significant differences in litter size between treatment groups ($M = 13.06$ pups, $SD = 2.67$). On P21, pups were weaned and culled to 8 or 10 animals per litter. Weaned rats were housed in same-sex, same-drug groups of 2-3, in standard polypropylene cages under the same conditions as dams, unless otherwise stated. All behavioural testing took place during the light phase. Body weight was monitored weekly. All procedures were approved by the University of Western Ontario Animal Use Subcommittee and were in accordance with the Canadian Council of Animal Care (CCAC) guidelines.

4.2.2. Prenatal LPS and PPA administration

Sodium propionate (PPA, P1880, Sigma Chemical, St. Louis, MO, USA) was dissolved in 0.1 M phosphate buffered saline and administered at a dose of 500 mg/kg SC (250 mg/mL, pH corrected to 7.4 with concentrated HCl) once a day on G12-16 for a total of 5 injections. Injections started on G12 to mimic the VPA model of ASD (Schneider and Przewlocki, 2005). Multiple injections were administered given the short half-life of PPA (20 min, Brusque et al., 1999). Lipopolysaccharide (LPS from *E. coli* serotype 0111:B4, L2630, Sigma Chemical, St. Louis, MO, USA) was dissolved in 0.1 M phosphate buffered saline and administered SC at a dose of 50 µg/kg on G12 based on other studies of MIA (e.g., Smith et al., 2007). Phosphate buffered saline was injected SC as a vehicle control to yield two control groups, either on G12 (VEH) or on G12-16 (5VEH). All injections were between the shoulder blades.

4.2.3. Experimental procedures

4.2.3.1. Nest seeking behaviour (Olfactory discrimination), P9-11

All pups (n=209) underwent determinations of nest seeking response behaviour (VEH: Male n=21, Female n=31; LPS: Male n=23, Female n=28; 5VEH: Male n=26, Female n=23; PPA: Male n=33, Female n=24). On P9, 10, and 11, pups were individually placed in the centre of a plastic cage (28 cm x 17.5 cm x 13 cm) with either home or clean bedding placed on filter paper (Whatman No. 1, Whatman International Ltd., England) at each end of the cage, 10 cm from the centre. Bedding was 3 days old for all of the tests and the side of the cage that each bedding was placed on was counter-balanced across trials. The time (s) to reach the home bedding was measured, with a maximum score of 180 s. A choice was made when the rat pup's head emerged in the bedding or when all 4 feet were resting on the bedding. The apparatus was cleaned in between animals. This test is considered to reflect a nest-seeking response mediated by the olfactory system (Gregory and Pfaff, 1971).

4.2.3.2. Open-field activity and adolescent social interactions, P30-33

Litters were culled at weaning (n=126), and these animals were behaviourally tested in adolescence and adulthood (VEH: Male n=16, Female n=16; LPS: Male n=16, Female n=14; 5VEH: Male n=14, Female n=18; PPA: Male n=16, Female n=16). Behaviour was evaluated in a circular open-field arena (90 cm diameter, 40 cm high)

with Beta Chip bedding covering the floor. A CD camera was mounted to the ceiling above the centre of the arena and connected to a computer, allowing behaviour to be recorded using the *EthoVision 3.0.15 Behavioural Monitoring and Analysis System* (Noldus Information Technology) at a rate of 5.994 frames/s. The x-y coordinates of each animal in the arena are tracked and several variables quantified. The camera was also connected to a DVD-R, allowing recordings for experimenter manual scoring.

Habituation to the circular open-field arena took place from P30-32. Animals were placed individually into the centre of the open-field for 15 min and the following locomotor activity variables were monitored: total distance – total horizontal distance traveled in cm; movement time – time in seconds spent in horizontal movement; velocity – distance traveled per unit time (cm/s); vertical time – time in seconds an animal spent rearing; percent time spent in periphery – time in seconds spent in the outer third of the open-field.

On P33, animals were individually housed for 3 hours to encourage social interaction. Following this brief period of social isolation, animals were placed at opposite sides into the open-field in same-sex, same-prenatal drug pairs to assess social interaction. Members of a pair were from the same litter, but were not cage-mates since weaning. Total locomotor activity and social behaviour was recorded for 15 minutes. Immediately prior to being placed in the open-field (and the day before as a habituation), the dorsal surfaces of rats were colored with black, non-toxic marker so that the EthoVision system could identify individuals. One member of a pair had the entire dorsal surface colored and the second member had the natural black markings on the dorsal surface colored. Following this social behaviour test, animals were pair housed in their home cages with their original cage-mate. Analysis for social interaction used animal pairs as subjects (VEH: Male n=8, Female n=8; LPS: Male n=8, Female n=7; 5VEH: Male n=7, Female n=9; PPA: Male n=8, Female n=8).

Locomotor activity of each animal was measured (total distance, movement time, velocity, vertical time). Social behaviour was quantified using the automated measures of the average distance between pairs of animals in cm, and the time animals spent within 5 cm proximity of each other. Manual scoring of social behaviour for each animal was carried out according to previously described criteria (Pellis et al., 1997):

1. Frequency of social initiations: number of snout to nape contacts.
2. Probability of defense: the number of defenses performed by an animal (withdrawal of the nape from the partner's snout) divided by the number of social initiations performed by the partner towards that animal.
3. Type of defense: i) Probability of facing defense: the number of facing defenses (withdrawal of the nape from the partner's snout by turning to face the partner) divided by the total number of defenses times 100. ii) Probability of evasive defense: the number of evasive defenses (withdrawal of the nape from the partner's snout by either running or turning away from the partner) divided by the total number of defenses times 100.

A second experimenter manually scored a subset of the data in order to calculate inter-rater reliability using Pearson correlations (15 pairs, 30 animals).

4.2.3.3. Novel object vs. Novel rat choice test, P42

Rats were re-habituated to the open-field arena without any bedding on P39-41 for 5 min each. On P42, a novel object vs. novel rat directed behaviour test was conducted to assess social approach to a confined social animal (Choleris et al., 2009) and to determine if prenatal LPS or PPA treated offspring would direct behaviour to an inanimate object rather than a novel conspecific.

A novel sex, age, and weight-matched untreated stimulus rat was restrained in a small cage with a circular Plexiglas top and bottom and a wire mesh cylindrical wall (diameter, 18 cm; 1.0 cm wire mesh). The cage was large enough for the stimulus rats to be able to turn around freely and rear, with the stimulus rats habituated to the cage and open-field for 3 days prior to testing. No stimulus rat was used in more than 5 novel object vs. novel rat choice tests. A small plastic children's toy, approximately 5 cm x 7 cm x 8 cm, served as the novel object.

The caged novel rat and novel object were placed opposite each other in the circular arena approximately 10 cm from the wall. Experimental rats were placed at the centre of the arena midway between the novel object and the novel rat facing the wall. Rats were tested one at a time for 5 min, and the arena and object were cleaned with an alcohol-water solution after each rat. Each rat was tested once and the same object was used for all of the tests, with the novel object/rat positions counterbalanced within litters. The percent of time approaching the novel rat or the novel object (moving towards either

stimulus while within 35 cm of stimulus) and total duration within 10 cm proximity of the novel rat or the novel object were determined by the EthoVision software.

4.2.3.4. Novel object recognition, P43

In order to determine whether or not there was a general impairment in recognition, a novel object recognition test was employed on P43. Two novel objects (plastic children's toys, 5 cm x 7 cm x 8cm) were placed in the open-field at opposite ends, approximately 10 cm from the wall. Rats explored the open-field and the objects in this 5 min exploration phase before being removed from the arena for 2 min. During this time, one of the objects from the exploration phase was removed and replaced by a new novel object so that one object was now familiar and one was novel. Rats were then recorded for 5 min in this test phase. If recognition is intact, more time should be spent in contact with the novel object as opposed to the familiar object (Choleris et al., 2009). The same 3 objects were used for all of the rats, with object placement and novel object identity in the test phase counterbalanced between and within litters. Objects were cleaned with an alcohol-water solution after testing with each rat and between the exploration and test phases. Manual scoring of each animal was performed. The time spent in contact (s) with each of the 2 objects was measured in both the exploration and test phase.

4.2.3.5. Adult social interaction test, P70

Animals were housed individually overnight (24 hr) before social interaction testing took place in adulthood on P70. Animals were colored with a marker the day before and immediately prior to the 15 min test with a partner in the arena. Pairings were same-sex, same-prenatal drug, but as in adolescence, with a non-cage mate from the same litter. Similar to adolescence, behaviour was recorded with the EthoVision software and the same automated and manual behavioural measures were obtained.

4.2.4. Data analysis

All analyses were performed with IBM Statistics 20 (formerly Statistical Package for the Social Sciences). As groups of rat pups belong to litters, they are not independent samples and effects associated with being raised in a litter must be accounted for. To do this, linear mixed models were used for each of the dependent variables, with Litter used as a subject variable and litter size as a covariate. Fixed factors in most models were Sex

and Prenatal drug. For body weight, nest seeking response, and habituation to the open-field, Week or Day was included as a fixed factor. A fixed factor of Stimulus type was included in the models for the novel object vs. novel rat (2 levels: object and rat) and the novel object recognition test (2 levels: one for each object). LSD post-hocs were performed. Significance was set to $\alpha = 0.05$.

4.3. Results

4.3.1. Body weight across lifespan

A significant Week x Sex interaction, $F(10,1623) = 752.0, p < 0.001$, showed that for the first 3 weeks of life, males and females were not significantly different (Figure 4.1A-B). From P28 on, males weighed significantly more than females, $ps < 0.001$. Additionally, there was a Week x Prenatal drug interaction, $F(30,1623) = 4.62, p < 0.001$. There were no differences in body weight between prenatal groups for the first 5 weeks of life. On P42 and P49, prenatal 5VEH treated animals weighed significantly more than prenatal VEH treated animals, $ps < 0.05$. This was found for females on P42 ($p = 0.041$) and males and females on P49 ($ps < 0.05$). On P49 (males) and P63 (males and females), prenatal LPS treated animals weighed significantly more than prenatal VEH treated animals, $ps < 0.05$. The last 2 weeks (P63, P70), prenatal PPA and 5VEH treated animals weighed significantly more than prenatal VEH animals ($ps < 0.01$) in both males ($ps < 0.05$) and females ($ps < 0.05$).

4.3.2. Nest seeking behaviour (olfactory discrimination), P9-11

Latency to reach the home bedding was recorded across P9-11. There was a significant effect of Day, $F(2,602) = 15.60, p < 0.001$, with all pups improving from P9 to P10 and taking significantly less time to reach their home bedding. There was no effect of Sex and a significant effect of Prenatal drug, $F(3,602) = 4.17, p = 0.006$. Overall, prenatal PPA treated offspring took significantly longer to approach and reach their home bedding than either prenatal LPS or VEH, $ps < 0.01$ (Figure 4.1C). On P9, prenatal PPA treated offspring took significantly longer to reach home bedding than all other prenatal groups, $ps < 0.05$. Prenatal LPS treated offspring did not significantly differ from their prenatal control treated offspring in nest seeking behaviour.

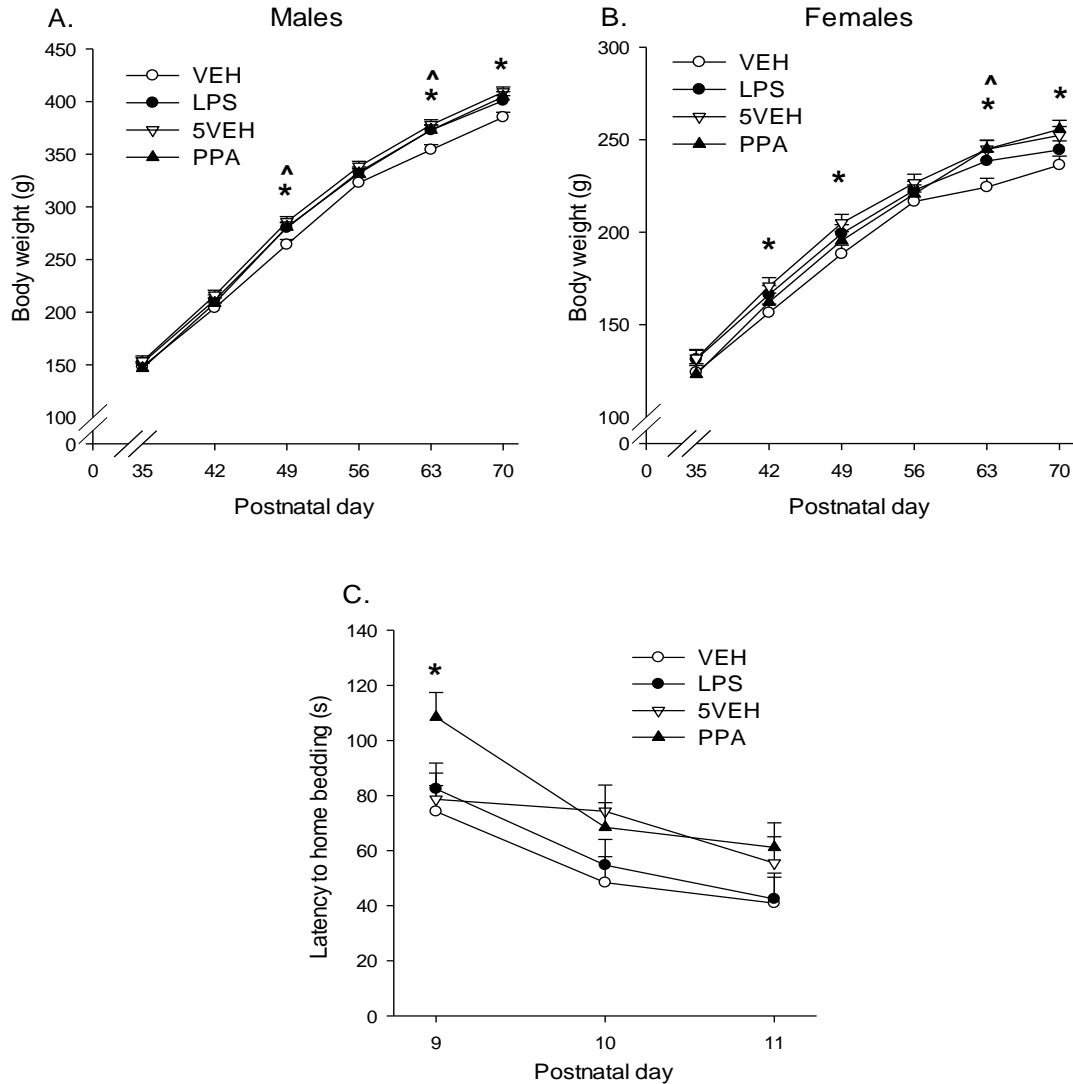


Figure 4.1. Body weight and nest seeking response.

Rats were prenatally exposed to either lipopolysaccharide (LPS), propionic acid (PPA), or their respective vehicle controls (VEH and 5VEH). Body weight was monitored weekly. A: Males and B: Females. * $p < 0.05$: Prenatal 5VEH animals weighed significantly greater than prenatal VEH animals; additionally, prenatal PPA animals weighed greater on P63, 70. ^ $p < 0.05$: Prenatal LPS animals weighed significantly greater than prenatal VEH.

C: Nest seeking response. Home and clean bedding were placed at opposite ends of a small chamber. The time to reach home bedding by individual pups was measured (max. score 180 s). Prenatal PPA treated offspring, regardless of sex, took significantly more time to reach home bedding on P9 than all other prenatal treatment groups. * $p < 0.05$. Error bars represent S.E.M. Further details of the prenatal treatments, sample sizes, and statistical analysis are provided in the text.

4.3.3. Open-field behaviour, P30-32

Total distance traveled and velocity showed similar patterns with both measures decreasing across days, $F(2,348) = 103.18, p < .001$ and $F(2,348) = 103.45, p < 0.001$ (Figure 4.2A-B). Significant Sex x Prenatal drug interactions were found for total distance $F(3,349) = 4.51, p = 0.004$, and velocity $F(3,349) = 4.52, p = 0.004$. While there were no significant differences between prenatal treatments, there were sex differences within treatments. In VEH treated animals, females traveled a significantly greater total distance ($p = 0.018$) and with a greater velocity than males ($p = 0.017$), while in PPA treated offspring, males traveled a significantly greater total distance ($p = 0.018$) and with a greater velocity than females ($p = .018$). Movement time also showed a pattern of decreasing activity across days, $F(2,348) = 68.06, p < 0.001$, with a significant Sex x Prenatal drug interaction, $F(3,349) = 2.96, p = 0.032$. Again, PPA treated males spent significantly more time moving than PPA treated females ($p = 0.007$), and LPS treated males compared to females neared significance ($p = 0.051$, Figure 4.2C).

Vertical movements, or rearing, did not show any significant effects of prenatal drug. Rearing on P30 was significantly greater than on P31 and 32, $F(2,348) = 11.46, p < 0.001$ (data not shown), and females reared significantly more than males, $F(1,352) = 6.43, p = 0.012$ (Figure 4.2D). Lastly, a significant effect of Day was found for percent time in the centre of the open-field, $F(2,348) = 13.02, p < 0.001$. Animals spent significantly more time in the centre on P30 than on P31 and 32. This likely reflects that the starting position of the rats was the centre of the open-field (data not shown).

4.3.4. Social interaction test – Adolescence P33 and Adulthood P70

4.3.4.1. Adolescent social behaviour

On P33, there were no significant effects of prenatal drug on the distance between pairs of animals or on time rats spent within 5 cm proximity of each other during the social interaction test. There was however an effect of sex, as males spent significantly more time within 5 cm of each other than did females, $F(1,43) = 6.09, p = 0.018$ (Figure 4.3A) and initiated interactions significantly more often than did females, $F(1,107) = 16.39, p < 0.001$ (Figure 4.3B). There were no significant effects of prenatal drug for initiations, or significant effects of sex or prenatal drug for probability of defense, and probability of facing and evasive defense (Figure 4.3C-D). There was

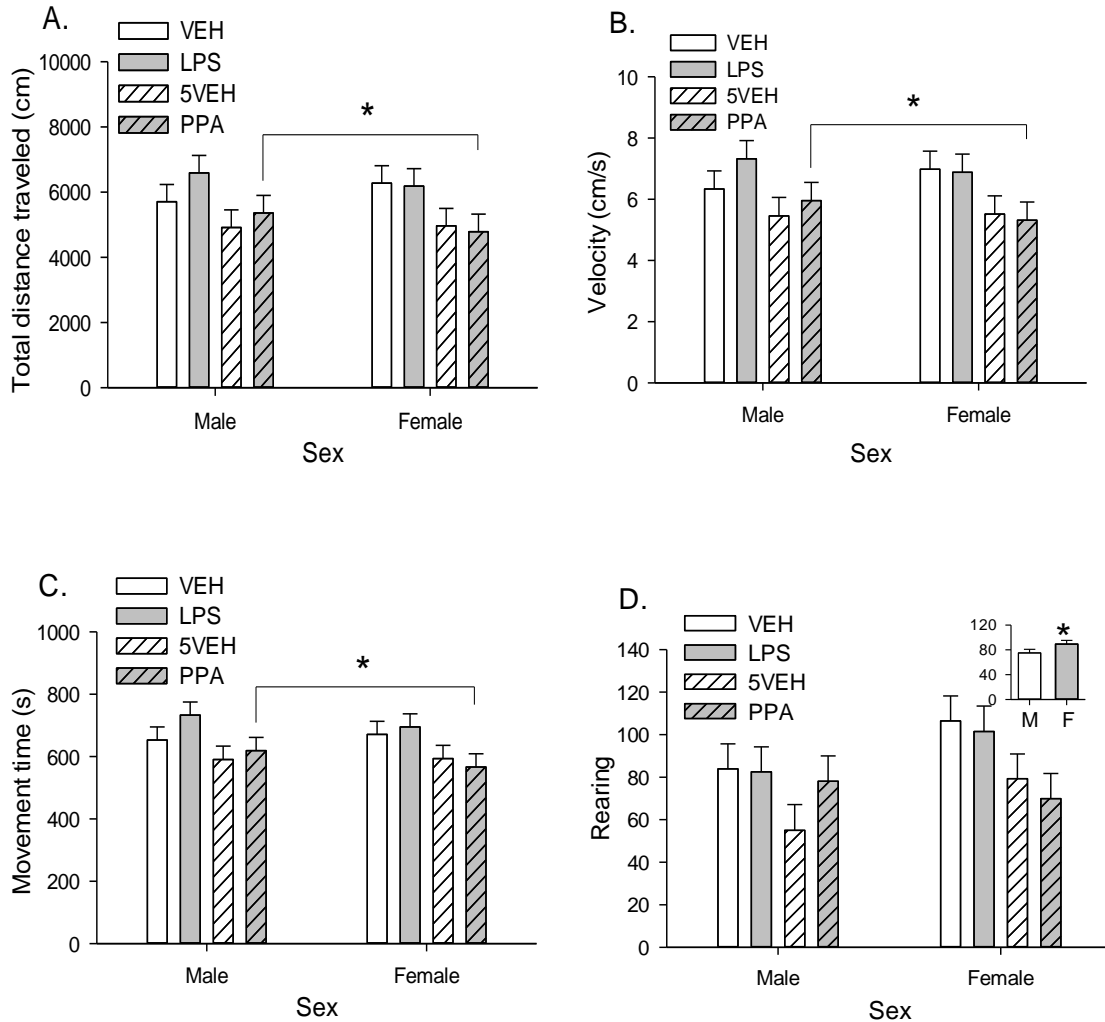


Figure 4.2. Locomotor activity in the novel open-field collapsed across days (P30-32) in male and female rats.

A: Total distance traveled (cm), B: Velocity, and C: Movement time (s). Rats were prenatally exposed to either lipopolysaccharide (LPS), propionic acid (PPA), or their respective vehicle controls (VEH and 5VEH). Prenatal PPA treated male offspring were significantly more active than prenatal PPA treated female offspring for all 3 measures, while prenatal VEH treated female offspring traveled a greater distance than prenatal VEH treated male offspring, $p < 0.05$. D: Rearing. Female offspring reared significantly more than male offspring, $p < 0.05$. Error bars represent S.E.M. Further details of the prenatal treatments and sample sizes are provided in the text.

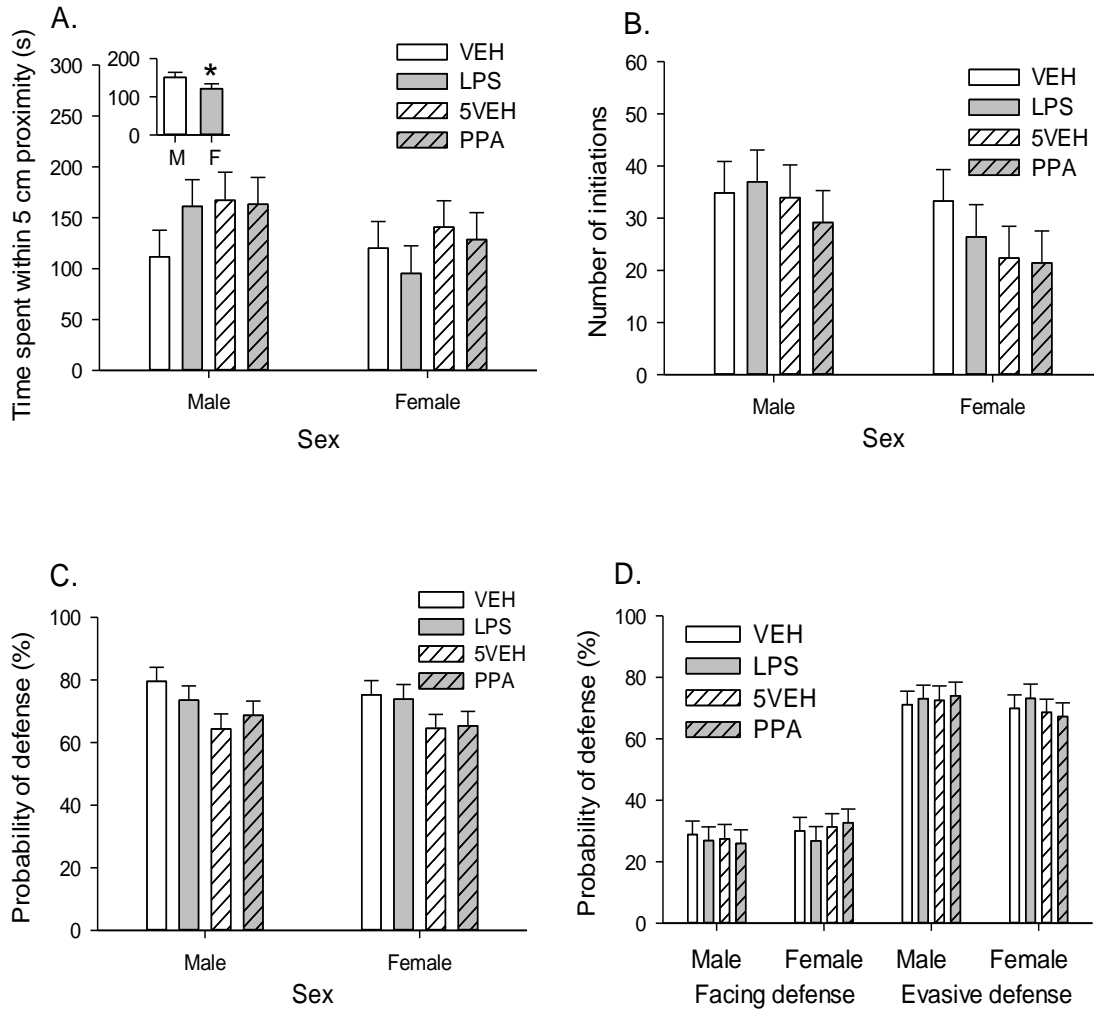


Figure 4.3. Adolescent social behaviour on P33.

Rats were prenatally exposed to either lipopolysaccharide (LPS), propionic acid (PPA), or their respective vehicle controls (VEH and 5VEH). A: Time animals spent within 5 cm proximity to each other. Male offspring spent significantly more time within 5 cm proximity to each other than female offspring, $p < 0.05$. B: Number of initiations, C: Probability of defense once contact is initiated, and D: Type of defense: Facing or evasive. Error bars represent S.E.M. Details of the prenatal treatments and sample sizes are provided in the text.

significant inter-rater reliability for manual scoring of adolescent social behaviour (initiations $r(28) = 0.88$, $p < 0.001$; facing defense $r(28) = 0.54$, $p < 0.01$; evasive defense $r(28) = 0.81$, $p < 0.001$).

4.3.4.2. Adult social behaviour

On P70, there were also no significant effects of prenatal drug or sex on the distance between pairs of animals or on time the rats spent within 5 cm proximity of each other (Figure 4.4A). Males again initiated more interaction than females, but the effect was not significant, $F(1,99) = 3.77$, $p = 0.055$ (Figure 4.4B). A significant effect of Prenatal drug for probability of defense, $F(3,99) = 3.13$, $p = 0.029$, showed that prenatal PPA and prenatal LPS treated animals engaged in defense significantly less than prenatal VEH treated animals, $ps < 0.05$ (Figure 4.4C). There were no significant differences between prenatal PPA and 5VEH ($p = 0.330$) or VEH and 5VEH ($p = 0.128$). Probability of facing and evasive defense did not show any significant sex or prenatal drug differences (Figure 4.4D). There was significant inter-rater reliability for manual scoring of adulthood social behaviour (initiations $r(28) = 0.89$, $p < 0.001$; facing defense $r(28) = 0.63$, $p < 0.01$; evasive defense $r(28) = 0.76$, $p < 0.001$).

4.3.4.3. Locomotor activity during social interaction

During the social interaction test on P33, there were no significant effects of prenatal drug and no sex differences in the total distance traveled and the number of vertical movements. There was no effect of prenatal drug on movement time, but a significant effect of Sex, $F(1,107) = 7.60$, $p = 0.007$, with male adolescent offspring spending significantly more time moving than females. On P70, there were significant effects of sex for total distance traveled, $F(1,100) = 13.64$, $p < 0.001$, and movement time, $F(1,109) = 46.67$, $p < 0.001$, with females moving significantly more than males. A significant Sex x Prenatal drug interaction for number of vertical movements, $F(3,100) = 2.77$, $p = 0.045$, showed that female rats performed significantly more vertical movements in the prenatal PPA, VEH, and 5VEH groups ($ps < 0.05$), but not in the prenatal LPS group (data not shown).

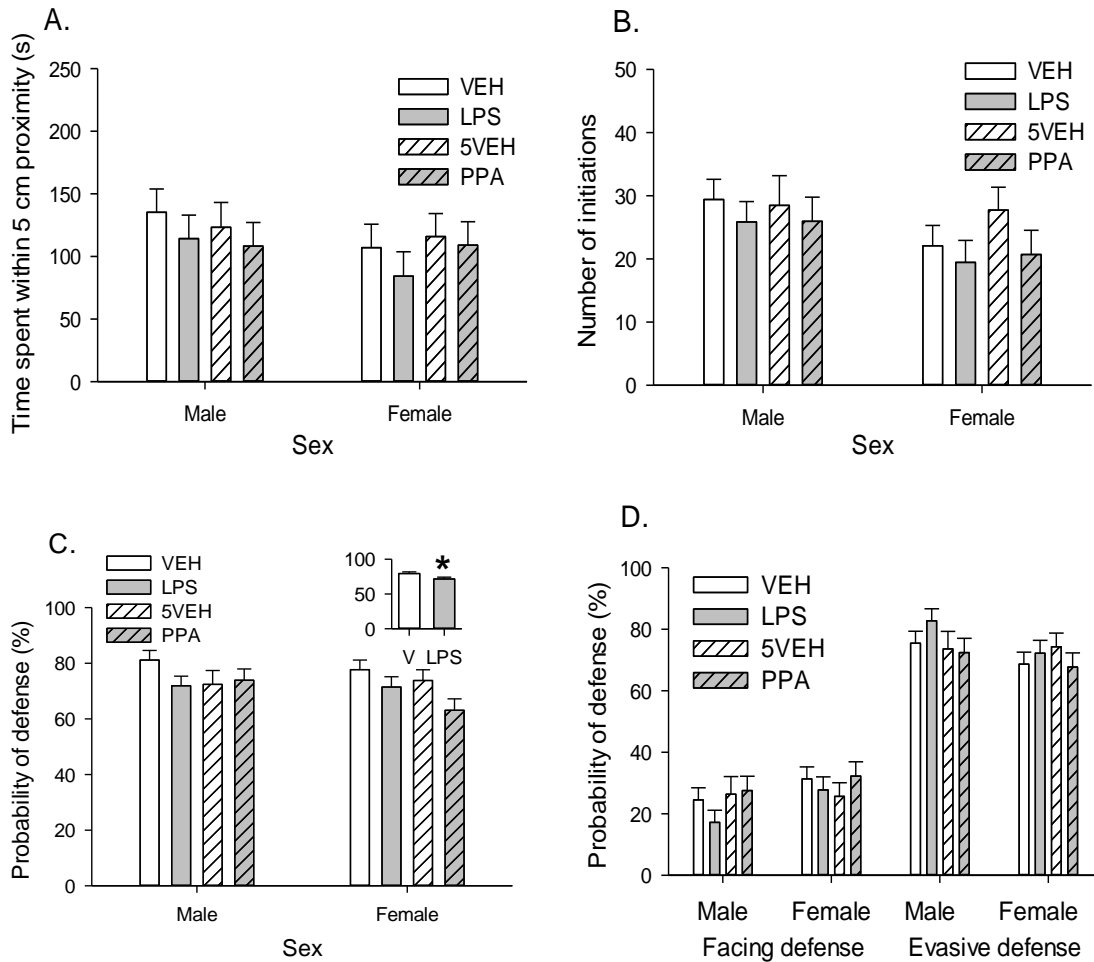


Figure 4.4. Adult social behaviour on P70.

Rats were prenatally exposed to either lipopolysaccharide (LPS), propionic acid (PPA), or their respective vehicle controls (VEH and 5VEH). A: Time animals spent within 5 cm proximity to each other, B: Number of initiations, C: Probability of defense once contact is initiated, and D: Type of defense: Facing or evasive. Prenatal LPS treated animals, collapsed across sex, engaged in defensive behaviour significantly less than prenatal VEH treated animals, $p < 0.05$. Error bars represent S.E.M. Details of the prenatal treatments and sample sizes are provided in the text.

4.3.5. Novel object vs. Novel rat choice – P42

There was a significant effect of Stimulus type, $F(1,239) = 6626$, $p < 0.001$, as all of the animals spent significantly more time within 10 cm of the novel rat compared to the novel object. Females in the prenatal 5VEH treated group spent significantly less time within 10 cm of the novel rats than did the prenatal PPA treated females ($p = 0.023$) and prenatal 5VEH treated males ($p < 0.001$), Stimulus type x Sex x Prenatal drug $F(3,239) = 3.33$, $p = 0.020$ (Figure 4.5A).

Percent time approaching the novel rat or novel object also revealed that all of the animals spent significantly more time approaching the novel rat, Stimulus type $F(1,239) = 2898$, $p < 0.001$. A significant Stimulus type x Sex x Prenatal drug interaction, $F(3,239) = 2.87$, $p = 0.037$, showed that prenatal PPA treated males spent significantly more time approaching the novel object compared to prenatal 5VEH treated males, $p = 0.009$ (Figure 4.5B). A sex difference in the prenatal 5VEH group showed that 5VEH treated females spent significantly more time approaching the novel object than did the males, $p = 0.021$.

4.3.6. Novel object recognition – P43

There was no significant sex or prenatal drug effect on the initial exploration of the objects. The rats made a similar number of visits to, and spent a similar time exploring, the objects. In the novel object recognition test, animals spent significantly more time in contact with the novel object compared to the familiar object, $F(1,224) = 69.26$, $p < 0.001$, regardless of prenatal drug. However, females made significantly more visits to the objects ($F(1,225) = 7.65$, $p = 0.006$) and spent significantly more time in contact with the objects than did males, $F(1,225) = 6.52$, $p = 0.011$ (Table 4.1).

4.4. Discussion

These results demonstrate that prenatal exposure to low levels of either PPA or LPS has selective and sexually dimorphic effects on neonatal, adolescent, and adult behaviour. Prenatal PPA treated male and female rats displayed delayed olfactory mediated nest seeking behaviour. Male adolescent PPA treated rats displayed increased approach to a novel object and enhanced novel open-field activity, without any changes in social interaction. There were also no evident or significant effects of PPA on adult

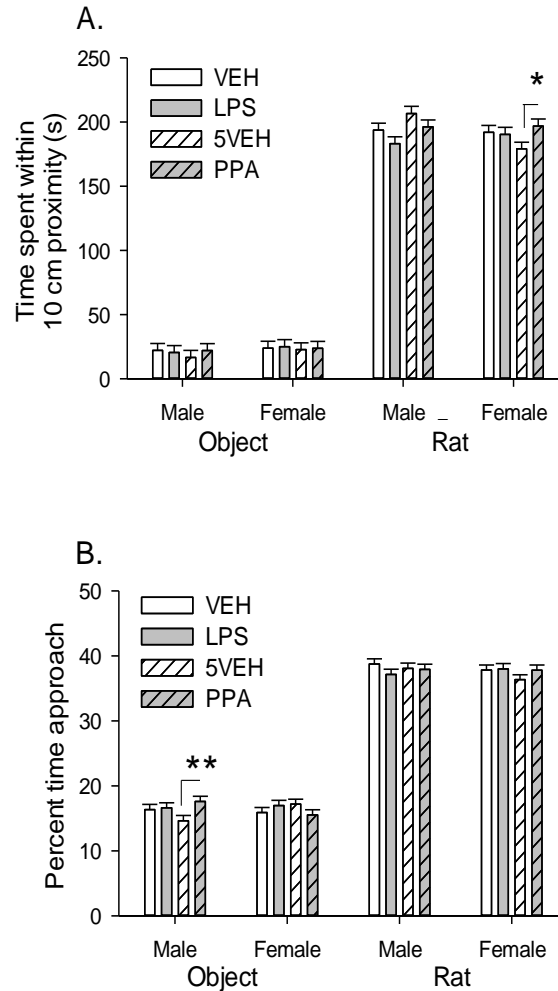


Figure 4.5. Novel Object vs. Novel Rat choice test on P42.

Rats were prenatally exposed to either lipopolysaccharide (LPS), propionic acid (PPA), or their respective vehicle controls (VEH and 5VEH). A. Time spent within 10 cm proximity to the stimuli, and B. Percentage of time spent moving towards either stimulus. All animals spent more time near and more time approaching the novel rat. Prenatal 5VEH treated female offspring spent significantly less time near the novel rat than prenatal PPA treated females. Prenatal PPA treated male offspring spent significantly more time approaching the novel object than prenatal 5VEH treated males. * $p < 0.05$, ** $p < 0.01$ Error bars represent S.E.M. Further details of the prenatal treatments and sample sizes are provided in the text.

Table 4.1. Time spent at, and number of visits to, objects in the novel object recognition test on P43 by male offspring (M) and female offspring (F) prenatally exposed to either lipopolysaccharide (LPS), propionic acid (PPA), or their respective vehicle controls (VEH and 5VEH). Further details of the prenatal treatments, sample sizes, and statistical analysis are provided in the text.

		Prenatal treatment			
		PPA	5VEH	LPS	VEH
Time-Familiar	(M)	5.12 ± .77 s	6.85 ± .94 s	6.22 ± .81 s	4.13 ± .52 s
	(F)	6.44 ± .74 s	6.35 ± .86 s	5.59 ± 1.26 s	5.83 ± .96 s
Time-Novel	(M)	9.40 ± 1.58 s	8.85 ± 1.18 s	8.84 ± 1.08 s	7.29 ± .78 s
	(F)	11.31 ± 1.19 s	11.89 ± 1.32 s	9.90 ± 1.38 s	10.22 ± 1.25 s
# Visits-Familiar	(M)	6.50 ± .98	6.27 ± .52	7.80 ± .83	6.69 ± .65
	(F)	6.94 ± .70	6.83 ± .72	6.86 ± 1.04	9.0 ± .92
# Visits-Novel	(M)	5.44 ± .88	6.60 ± .72	7.13 ± .82	7.06 ± .67
	(F)	7.75 ± .62	7.67 ± .62	7.50 ± .86	9.63 ± .75

social behaviour. In contrast, although prenatal LPS did not influence social behaviour in neonatal and adolescent rats, in adulthood, it decreased the probability that male and female rats engaged in defensive behaviour. Taken together, these results suggest that prenatal exposure to low levels of PPA and LPS produces subtle and sexually dimorphic alterations in social and related behaviour with prenatal PPA having a greater impact on neonatal and adolescent behaviour.

4.4.1. Nest seeking behaviour (olfactory discrimination)

As maternal separation has adverse consequences for rats, it is necessary for pups to be able to recognize and respond appropriately to the olfactory cues of the home nest. Consistent with this, and with the results of previous studies (Roullet et al., 2010; Schneider and Przewlocki, 2005), vehicle treated male and female rats showed a rapid discrimination and approach to maternal nest bedding.

Prenatal PPA increased the latency of male and female pups to reach home bedding in the nest seeking response test, consistent with findings from animals receiving prenatal VPA showing an increased time to find the home bedding (Roullet et al., 2010; Schneider and Przewlocki, 2005). The present results may reflect decreased interest in the social odors of the maternal nest and/or increased perseverance towards the clean odor. Interestingly, adult male rats that received central PPA persevered at a location where soiled bedding from the home cage of an unfamiliar rat had been placed and did not investigate novel bedding (MacFabe, 2012; Meeking et al., unpublished). This, coupled with nest seeking behaviour, may be interpreted as PPA-induced impairment, either in the recognition, or utilization, of socially relevant olfactory cues. However, it cannot be ruled out that prenatal PPA treatment may have influenced development of the olfactory system.

Prenatal LPS had no significant effect on the latency to reach home bedding in the test. This is contrary to a previous report reporting increased latency in nest seeking response with prenatal LPS (Baharnoori et al., 2012). This discrepancy likely reflects differences in timing and dose of LPS, as LPS exposure in this study is earlier in gestation, and of a lower dose (50 vs. 100 µg) than that used in Baharnoori et al. (2012).

The alteration in nest seeking response behaviour of pups receiving prenatal PPA was not associated with any significant consistent changes in growth. During one week

for males and two weeks for females, prenatal LPS treated animals were heavier. Results of other studies have reported decreased, or no change in, body weight (see Patterson, 2011). This likely reflects dose and strain effects. However, the number of injections received in gestation affected growth, suggesting that stress associated with injections may have a developmental effect. Repeated vehicle injections can alter baseline levels of plasma corticosterone (Drude et al., 2011), similar to chronic stress protocols (Weinstock, 2001). Rats born to dams treated with prenatal PPA or 5VEH control were heavier as adolescents and adults than those born to dams treated with one injection of VEH. Prenatal stress has been shown to result in heavier adult weights in mice (Mueller and Bale, 2006) and decrease propensity for social interactions (Weinstock, 2001). Effects associated with number of prenatal injections have also been seen in open-field activity and an acoustic startle paradigm (Chapter 2, 3). The stress response associated with saline injections, although mild in comparison to maternal stress paradigms, may have limited the detection of some subtle effects of PPA.

4.4.2. Social behaviour and the effects of prenatal PPA and LPS

There were sex-specific changes in basal locomotor activity during habituation to the open-field. Previous work has shown that adolescent and adult female rats generally are more active than male rats (Lynn and Brown, 2009). However, in prenatal PPA animals, adolescent males were more active than females, suggesting that prenatal PPA may have increased activity in males, or possibly decreased activity in females. Previously, central administration of PPA has induced hyperactivity in adult male rats (MacFabe et al., 2007; Thomas et al., 2012), but female activity has not been investigated. In contrast, prenatal LPS did not have any evident effects on adolescent basal activity.

Consistent with the results of previous studies in adolescent rats, males engaged in more social behaviour than females, as measured by time animals spent in proximity to each other and in the number of social initiations (Olioff and Stewart, 1978). The higher incidence of social play in males may also account for the sex difference in adolescent locomotor activity during behavioural interaction. In adulthood, there was no sex difference in social interaction, although sex differences in social initiations approached significance. Social play activity peaks in adolescence (Thor and Holloway Jr., 1984),

making differences in adulthood less likely, although overall males are still generally more likely to engage in social interaction. This may also be reflected in the greater locomotor activity displayed by females.

Prenatal LPS and PPA did not significantly affect social interactions in adolescence as measured in a paired interaction test of social play. Results of previous MIA studies using the viral mimetic, poly I:C, and LPS have reported reduced social play in adolescent males (see Patterson, 2011). This difference may again be attributed to the relatively low doses of LPS and PPA as well as differences in the timing of administration.

Evidence of subtle alterations in adolescent social behaviour with prenatal PPA was however present in the novel object vs. novel rat choice test. Prenatal PPA adolescent males spent significantly more time approaching the novel object than corresponding vehicle treated males, suggesting a subtle alteration and decrease in social preference. This increased interest in the novel object is similar to the decrease in social approach to an unfamiliar mouse relative to a non-social object in a 3 chamber apparatus seen in VPA and MIA mice (Kim et al., 2013; Smith et al., 2007). Taken together with the delay in nest seeking response in prenatal PPA offspring, it is possible that odor associated with the novel rat may not have been as salient of a social cue leading to changes in social responses. Interestingly, in a similar task, adolescent males receiving central PPA spent less time in proximity to, and less approach behaviour towards, the novel rat (MacFabe et al., 2011). This effect in prenatal PPA males is also consistent with the male predominance seen in ASD.

In the novel object recognition task, all adolescent animals spent more time investigating the novel object, with female rats spending more time than males in total contact with the objects, consistent with prior studies (Howland et al., 2012). As well, there was no effect of prenatal treatment, again consistent with prior MIA studies (Howland et al., 2012; Mychasiuk et al., 2012). While novel object recognition confirmed intact recognition abilities for non-social items, it does not necessarily rule out the possibility of an alteration in the recognition of novel conspecifics.

In adulthood, once social interactions were initiated, although prenatal LPS treated male and female rats spent a similar amount of time near each other as control

rats, they showed a decrease in the probability to engage in defensive behaviour (effect collapsed by sex). Decreased defensive behaviour could be interpreted as an indifference of the animals towards the social partner. This change in defensive behaviour may be associated with prenatal LPS-induced alterations in dopaminergic activity which has been shown to influence defensive behaviour (Baharnoori et al., 2013).

Prenatal PPA did not significantly alter social interaction between pairs of animals in adulthood (P70). It should be noted that prenatal PPA treated rats displayed a significant decrease in defensive behaviour relative to the VEH group, similar to LPS. However, repeated VEH injections (5VEH) also non-significantly decreased defensive behaviour, potentially attenuating or masking the effects of PPA. Previous work in adult male rats has demonstrated that central PPA can rapidly decrease social behaviour (Shultz et al., 2008). However, the effects of acute PPA treatment and prenatal PPA are not necessarily comparable.

Previous MIA studies used higher doses of immune stimulants while the current study used a low dose of LPS (50 µg vs. 100 µg). Studies of neonatal LPS using a similar dose as this study produce a number of sexually dimorphic behaviours, including increased anxiety-like behaviour and altered responses to adult immune challenges (Tenk et al., 2008; Walker et al., 2009). Although milder effects of prenatal LPS on social behaviour were seen here, this does not necessarily exclude alterations in neurodevelopment. Manipulations in development may leave animals susceptible to later environmental insults. Neonatal LPS enhanced hypoactivity to an immune challenge in adulthood, while neonatal LPS combined with restraint/isolation stress in adulthood decreased locomotor activity and increased anxiety-like behaviour (Tenk et al., 2008; Walker et al., 2009). Prenatal immune activation may act to predispose individuals to a number of neuropsychiatric conditions, or act on pre-existing genetic predispositions (Patterson, 2011). While the current dose of LPS did not directly influence social behaviour in adolescent rats and minimally affected adult behaviour, it is possible that a second environmental stressor is required for changes in social and other behaviour to be expressed.

Similar to LPS, a low dose of PPA was administered in this study to represent subchronic changes in the gut microbiome. PPA is endogenous and very quickly

metabolized, and this may have contributed to the subtle alterations in behaviour. It is possible that prenatal PPA treated rats are susceptible to further environmental trauma. An additional insult in adolescence or adulthood may have allowed for further behavioural changes to manifest. A combination of prenatal and postnatal PPA sensitized acoustic startle responses in female adolescent rats (Chapter 3), consistent with prenatal immune activation predisposing rats to further environmental insults.

4.4.3. Relation to autism spectrum disorders

ASD include altered neural synapse maturation and connectivity, leading to a possible imbalance between inhibition and excitation (MacFabe, 2012). Immune dysregulation in patients with ASD suggests the presence of an inflammatory state and maternal infection as a risk factor. Prenatal LPS, as mentioned, alters dopaminergic functioning in offspring and has also been shown to decrease the number of GABAergic neurons in adult animals (Nouel et al., 2012). These and other changes in neurotransmission may occur via increases in proinflammatory cytokines altering gene expression involved in neural migration of inhibitory interneurons or the developmental processes itself (Garbett et al., 2012; MacFabe, 2012).

PPA may alter neurotransmission via epigenetic developmental changes in gene expression through histone deacetylation of ASD implicated genes (Nguyen et al., 2007), similar to that suggested for valproic acid (D'Souza et al., 2009; Phiel et al., 2001). As well, PPA has specific G-protein coupled receptors and can have adverse effects on oxidative stress and decrease neurotransmitter levels such as GABA, serotonin, and dopamine (MacFabe et al., 2007; MacFabe, 2012). PPA and other SCFAs are part of gut functioning and under normal conditions serve a variety of physiological and immune functions. However, shifts in the composition of the microbiome may lead to augmented and inappropriate levels of PPA that could have adverse effects (Al-Lahham et al., 2010).

In conclusion, prenatal exposure to low levels of PPA subtly altered social behaviour in neonatal and adolescent rats in a sex-specific manner, while a low dose of LPS altered social behaviour in adult male and female rats. Repeated infection or immune insult throughout gestation and early life may influence the gut microbiome and provide an enteric environment that promotes the overgrowth of certain bacteria, leading to

production of metabolic products, such as PPA or LPS, which may adversely alter neurodevelopment.

4.5. References

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Chapter 5

General Discussion

The effects of prenatal and postnatal administration of the microbiome bacterial metabolic products, PPA and LPS, on development and behaviour of male and female rats were examined. Additionally, PPA was administered in the second week of life to determine if a postnatal insult would exacerbate any behavioural effects. The relatively subtle, sex-specific, and treatment-specific alterations in behaviour found are summarized in Table 5.1. The developmental delay, altered locomotor activity, sensory responses, and social behaviour found in adolescent male and female rats resembled previous animal models and features of human ASD, suggesting metabolic products of enteric bacteria may contribute to the development of ASD.

5.1. Developmental milestones

Assessment of milestones and body weight provided a gross measure to determine if prenatal and postnatal drug treatments affected neurodevelopment and general health. Minimal consistent effects on body weight were observed. Developmental delay in eye opening (Chapter 2) in both males and females receiving prenatal PPA or LPS and delay in acquisition of odor mediated nest finding (Chapter 4) in males and females receiving prenatal PPA was observed in the first 2 weeks of neonatal life. The developmental delays see here point to an impact of early infection on socially related factors. Additionally, the free fall righting reflex of female pups was impaired with a combination of prenatal LPS and postnatal PPA. A delay in eye opening and motor reflexes have been reported in the VPA animal model of ASD (Roullet et al., 2010; Schneider and Przewlocki, 2005; Wagner et al., 2006). Developmental delay is observed within the first 12-24 months of life in infants with ASD or high risk siblings and may include delays in gesturing or language (Filipek et al., 1999; Mitchell et al., 2011). Infants may also display postural instability, head lag, hypotonia and/or delay in walking (see Mitchell et al., 2011 for review).

5.2. Locomotor activity and anxiety-like behaviour

Minimal changes in total locomotor activity were seen after prenatal treatment with PPA or LPS. However, males receiving prenatal PPA were more active than females receiving PPA (Chapter 4), opposite to what was observed in vehicle control animals. Repetitive behaviour in females was observed with the combination of pre- and postnatal PPA, consistent with the double-hit hypothesis (Chapter 2). These results suggest, again,

Table 5.1. Summary of significant effects of either prenatal LPS, pre-, or postnatal PPA on subsequent offspring behaviour

		Body Weight			Eye	Milestones		O/F	O/F Centre	O/F Centre		
		Neonatal	Adolescent	Adult	Opening	Physical	Reflex	Activity	Time	Activity		
Pre-PPA	M				-				-	+		
	F				-				-	+		
Pre-LPS	M		+	+	-							
	F			+	-							
Post-PPA	M	+	+	ND								
	F	-		ND								

		EPM	EPM	ASR			%PPI	Nest Seeking	Social interaction		Social		
		Open	Closed	Initial	Habituation	Average		Latency	Adolescent	Adult	Approach	NOR	
Pre-PPA	M			+				+					-
	F	-	+	+			-	+					
Pre-LPS	M			+		+							-
	F			+									-
Post-PPA	M			-	-		+	ND	ND	ND	ND	ND	ND
	F		+	+			-	ND	ND	ND	ND	ND	ND

M- Males, F- Females. + denotes increase in behaviour, - denotes decrease in behaviour compared to vehicle control offspring.

Empty space denotes no effect of drug, ND = not determined.

Pre-PPA: PPA administered on G12-16, 500 mg/kg SC; Pre-LPS: LPS, 50 µg/kg SC, administered on G12-16, or G12 (social measures, last 5 columns); Post-PPA: PPA administered 2x/day, every other day, P10-18, 500 mg/kg SC

Physical Milestones: top and bottom incisor eruption and pinna detachment. Reflex Milestones: righting reflex, negative geotaxis, free-fall righting reflex

O/F- open-field, EPM- elevated plus maze open and closed arm time, ASR- acoustic startle response, %PPI- percent prepulse inhibition, NOR- novel object recognition

that PPA produces subtle and sexually dimorphic changes in activity.

Both hypo- and hyperactivity are reported in ASD. Hyperactivity and a range of repetitive or restricted behaviours are observed throughout childhood. MIA rodent studies report restricted behaviours, with decreased exploratory behaviour in an open-field (Malkova et al., 2012; Smith et al., 2007), while VPA studies report decreased exploration and hyperactivity, although this is not always the case (Schneider and Przewlocki, 2005; Mychasiuk et al., 2012). Decreased exploratory behaviour was observed in both male and female rats receiving prenatal PPA (decreased open-field centre time), but there was limited evidence supporting hyper- or hypoactivity. Prenatal PPA, LPS, and postnatal PPA alone did not alter locomotor activity in a small open-field (Chapter 2). In Chapter 4, a large open-field arena was used and males receiving prenatal PPA were more active than females receiving prenatal PPA, opposite to what was observed in vehicle controls. Lastly, increased repetitive behaviour (number of revolutions) in females receiving a double hit of prenatal and postnatal PPA provided evidence for a double hit hypothesis (Chapter 2). Increased repetitive behaviour specific to females has been observed in the VPA rodent model (Schneider et al., 2008). This indicates that locomotory effects are dependent on the measures used, suggesting interactions with other environmental/experiential factors.

Both male and female adolescents prenatally exposed to PPA showed an increase in anxiety-like behaviour in the open field and pre- and postnatal PPA produced an increase in anxiety in females in the EPM (Chapter 2). Anxiety is one of the most common psychiatric disorders that occurs comorbidly with ASD (Skokauskas and Gallagher, 2010). Additionally, anxiety and depression are commonly seen with GI dysfunction (Donner and Lowry, 2013). Previous studies with MIA report increased anxiety-like behaviour in adult offspring, with limited and conflicting reports in adolescence (Enayati et al., 2012; Schwendener et al., 2009; Smith et al., 2007). The current results support a role for prenatal and postnatal PPA in contributing to anxiety-like phenotypes. Enhanced behaviour to threatening situations may be analogous to atypical affective responses seen in children with ASD (e.g., inappropriate emotional response, increased neutral expressions) (Mitchell et al., 2011; Zwaigenbaum et al., 2005) and as such consistent with the present results.

5.3. Sensitivity to acoustic stimuli and sensorimotor gating (Chapter 3)

Hyper- and hypo-sensitivity to environmental stimuli and difficulties with habituating to repeated stimuli have been reported in patients with ASD, with some evidence of decreased prepulse inhibition (Leekam et al., 2007; Ornitz et al., 1993; Perry et al., 2007). Consistent with these observations in humans, prenatal PPA, prenatal LPS, and postnatal PPA altered acoustic startle response behaviour in male and female adolescent rats. Effects were subtle and short-lived, as animals displayed abnormal responses to the initial startle pulse and then adjusted responding.

Prenatal LPS did not alter prepulse inhibition. This is contrary to previous MIA studies in adolescent and adult rats (Howland et al., 2012; Fortier et al., 2007). Robust hypersensitivity to startle in males was seen over the course of a startle session similar to Fortier et al. (2004), providing a dissociation of aberrant startle responses and sensorimotor gating with a low dose of LPS. Prenatal LPS did not affect habituation to the acoustic stimuli. On the other hand, postnatal PPA in males, and the combination of prenatal and postnatal PPA in females, produced sensitization (hypersensitivity) rather than habituation to the startle pulses over the first 2 sessions. With repeated exposure over days, all animals habituated in a similar manner to control animals. In one report, adults with ASD took longer to reach the same level of habituation to acoustic stimuli as other adults and (Perry et al., 2007), with evidence of decreased habituation to auditory sounds in at risk infants and males with Fragile X syndrome (Guiraud et al., 2011; Van der Molen et al., 2012). Similar to decreases in prepulse inhibition observed in MIA and VPA studies (Howland et al., 2012; Schneider and Przewlocki, 2005), decreases in prepulse inhibition were observed in female rats receiving prenatal PPA and in male and female rats receiving postnatal PPA. The current results indicate that PPA and LPS administration in development can alter sensory processing in ways that may resemble behaviours seen in ASD.

5.4. Social and related behaviours

Altered social behaviour is one of the core behavioural deficits in ASD (DiCiccio-Bloom et al., 2006; Zwaigenbaum et al., 2005). Impaired social behaviour has been observed in rodents in social interaction tests and social approach tests in both MIA and VPA models. Chapter 4 assessed social behaviour in rat offspring following prenatal PPA

or prenatal LPS using a social interaction test and social approach to a novel object versus to that of a novel rat. In addition, olfactory mediated nest seeking behaviour in neonatal rats was measured and prenatal PPA treated male and female neonatal rats were delayed in reaching home bedding. This is consistent with VPA studies (Roullet et al., 2010; Schneider and Przewlocki, 2005) and may suggest a social impairment in recognizing or using socially relevant cues in very young animals.

Prenatal PPA did not alter paired social interaction in adolescent or adult rats. However, evidence of alterations in social approach was seen in adolescent male rats, with more time spent approaching the novel object than vehicle controls. This subtle effect resembles that seen in MIA and VPA studies using the 3 chamber apparatus where treated animals spend more time in a chamber containing a non-social object compared to a chamber containing a conspecific, or more time in an empty chamber compared to controls (Dufour-Rainfray et al., 2010; Malkova et al., 2012; Smith et al., 2007). The current study provides some evidence supporting prenatal PPA producing behaviours that resemble ASD. The absence of PPA effects in social interaction may be due to drug administration (discussed below) or alternatively, a 3 chamber apparatus or test of novel social recognition may more effectively parse out any effects of PPA.

Prenatal LPS did not alter social behaviour in neonatal or adolescent rats; however, in adulthood, decreased social behaviour (decreased probability to engage in defensive behaviour) was observed. Previous MIA studies with the viral mimetic, poly I:C, have shown decreased social behaviour in mice (Smith et al., 2007) and Taylor et al. (2012) report LPS-induced decreases in social interaction and play behaviour in adolescent rats. The results observed in the current study may be explained by the earlier administration of LPS and the lower dose of LPS used here compared to other studies (Oskvig et al., 2012; Taylor et al., 2012). Overall, changes in social behaviour are consistent with decreases observed in previous animal studies and in ASD.

5.5. Evidence for the double-hit hypothesis

PPA was administered during the second postnatal week to act as a double ‘hit’. The double hit hypothesis describes that a genetic predisposition or environmental insult early in life may confer vulnerability to an environmental trigger later in life that results in emergence of adverse behaviour or neuropathology (Bayer et al., 1999). In humans,

adverse childhood experiences have been associated with an increased susceptibility to psychopathology such as mood disorders and altered stress responses (Heim and Nemeroff, 2001; McGowan et al., 2009). Evidence for the double-hit hypothesis was found in female rats, but not male rats. The combination of prenatal and postnatal PPA altered behaviour in females where individual drug treatments alone did not. Repetitive behaviour and sensitization to acoustic startle was present in female rats, but not males. The free-fall righting reflex was also impaired in females receiving a combination of prenatal LPS and postnatal PPA. This is similar to a previous MIA study where a stress protocol in adolescence manifested MIA-induced alterations in behaviour similar to that seen in schizophrenia and autism (Giovannoli et al., 2013). Children with ASD were found to have had a greater number of ear infections and to use more antibiotics than typically developing children (Niehus and Lord, 2006), suggesting a circumstantial link to repeated early life insults and immune activation.

5.6. Sex differences: Females may be more susceptible to the effects of PPA

The prevalence of ASD favors males, with 4 males to every 1 female diagnosed with ASD. Prenatal PPA influenced the approach to a novel object and prenatal LPS produced hyper-sensitivity to acoustic startle in male, not female, adolescents, coinciding with the idea of male predominance in ASD. However, prenatal and postnatal PPA and prenatal LPS influenced male and female offspring behaviour in similar ways for many behaviours measured. Furthermore, female offspring were more sensitive to the effects of prenatal and postnatal PPA as both treatments combined produced sensitization to acoustic startle and increased repetitive movements. Additionally, pre- and postnatal PPA decreased sensorimotor gating and increased anxiety on the EPM in females that was not observed in males.

These behavioural results in females may relate to phenomena observed in human ASD. Females may be under-diagnosed due to differences in normal coping behaviour and social communication skills compared to males. Girls that display high scores on autistic traits similar to that of boys are nevertheless diagnosed less frequently than boys (Russell et al., 2011). When females are diagnosed with ASD, they are more severely affected and often intellectual disability brings attention to the behavioural symptoms (Fombonne, 2009; Russell et al., 2011). Females are likely to show more repetitive

interests, sensory symptoms, and display emotional symptoms such as anxiety or depression (Lai et al., 2011; Mandy et al., 2012). Acquired metabolic dysfunction in mitochondria has been suggested to contribute to ASD symptoms; a more balanced male to female ratio and presence of gastrointestinal abnormalities were significantly greater in children with ASD and mitochondrial disease (MD) as opposed to ASD or MD alone (Rossignol and Frye, 2012). PPA may interfere with mitochondrial metabolism, inducing oxidative stress and disrupting fatty acid oxidation (Frye et al., 2013; MacFabe et al., 2008).

It is possible then, that the sex ratio observed in studies may reflect the nature of the environmental toxin, with a certain sex more vulnerable to particular toxins. VPA studies have shown male-specific behavioural effects, while MIA studies have not shown many sex differences. There is, however, evidence that adult females may be more vulnerable to the effects of drug abuse (Fattore et al., 2008), suggesting sex-specific alterations in neurochemical systems. Adult female rats are more sensitive to the reinforcing effects of stimulants (Lynch, 2006). Neonatal LPS increased the development of dopaminergic locomotor sensitization in female, but not male, rats (Tenk et al., 2007), while altering neophobia in both males and females (Tenk et al., 2013). As such, it is possible that SCFAs and PPA may affect males and females similarly for some behaviour, with either a greater female or male susceptibility for other behaviours. The behavioural data of the current studies tentatively support this hypothesis, though further investigation is needed.

5.7. Potential mechanisms for alterations in neurodevelopment

Short-term LPS and PPA administration during prenatal and early life may have the capacity to alter neurodevelopmental processes leading to changes in brain and behaviour later in life. SCFAs may gain access to the developing fetus through active transport via monocarboxylate transporters in the placenta (Nagai et al., 2010), while immune stimulants may exert effects via cytokines; for example, through IL-6 activation of intracellular pathways in the placenta (Hsiao and Patterson, 2011; Shi et al., 2005).

An ongoing innate neuroinflammatory state may contribute to ASD as post-mortem study of brains of ASD patients show increased astrocytes, activated microglia, and cytokines (Li et al., 2009; Vargas et al., 2005). Microglia are present in rodent brain

from about G13-14 and have been shown to play a role in neurodevelopmental processes such as cell differentiation and synapse pruning. Cytokine release from microglia could perpetuate a chronic inflammatory response throughout life, with sensitized responding to environmental insults (see Bilbo and Schwarz, 2012; Deverman and Patterson, 2009). Increases in proinflammatory cytokines have been detected in the amniotic fluid and the fetal brain following LPS treatment (Gayle et al., 2004; Ning et al., 2008) with some evidence suggesting that PPA may also be capable of inducing a neuroinflammatory response and cytokine release (El-Ansary et al., 2011; Foley et al., 2008; MacFabe et al., 2007). Elevated IL-6 has been hypothesized to contribute to the excitatory/inhibitory imbalance in neuronal circuits that might contribute to ASD (Rubenstein and Merzenich, 2003; Wei et al., 2013).

Epigenetic mechanisms present a plausible mechanism for how environmental toxins or insults in early fetal or neonatal life could lead to altered neurotransmission and/or circuitry and delayed manifestation of behavioural impairments as children develop. Epigenetic involvement, that is, changes to chromatin structure without altering the genetic sequence, is being recognized as contributing to ASD. Modifications to genes results in increased or decreased gene expression. For example, a recent post-mortem brain analysis of ASD patients identified novel methylation sites in the temporal cortex and cerebellum that were modified compared to non-ASD brains. These sites may be involved in immune and mitochondrial functions (Ladd-Acosta et al., 2013). Patients with known epigenetic disorders, such as Rett syndrome or Fragile X syndrome, often have autistic features and can be diagnosed on the autism spectrum (Grafodatskaya et al., 2010).

VPA and PPA are both histone deacetylase inhibitors, placing them in a position to effect epigenetic change through alterations in gene expression (Nguyen et al., 2007; Phiel et al., 2001). Thus far, studies have shown that VPA can interfere with neural migration and cell differentiation via the Wnt signalling pathway (Go et al., 2012; Wiltse, 2005). Preliminary results have shown that central administration of PPA can alter gene expression in ASD associated genes (Nankova et al., 2012). Prenatal LPS administration has also been shown to alter expression of genes involved in neural migration and neurotransmission (Oskvig et al., 2012).

5.8. Relation to PPA rodent model of ASD

As discussed in Chapter 1, results of previous studies have shown central and peripheral administration of PPA produce changes in behaviour, including hyperactivity and decreased social interaction, and neuropathological and metabolic changes in adult and adolescent male rats similar to that seen in ASD (reviewed in (MacFabe, 2012)). The current results add to the face validity of this model by demonstrating that PPA administered during development can produce changes in the behaviour of rat offspring in ways that resemble ASD. It remains to be seen if PPA administered early in development induces neuropathological or metabolic changes similar to those seen following either central PPA or in ASD.

I hypothesized that PPA would alter behaviour in rat offspring in similar ways to that observed in other developmental models (VPA, MIA). Changes in behaviour with prenatal PPA, prenatal LPS, and postnatal PPA were subtle and even transient in some cases. Given that PPA is endogenous, metabolized fairly quickly, and was administered in healthy animals, the load may not have been sufficient to cause large functional changes in behaviour. It is unknown what amount of PPA is reaching the offspring in utero. Likewise, many behavioural results of prenatal LPS administration were not similar to past MIA studies. This may be due to the low level of LPS used in the present studies. It was important to first characterize the effect of prenatal administration of PPA and LPS before extending the work to multiple prenatal insults. There was limited evidence for the double hit hypothesis, with postnatal PPA combined with prenatal PPA or prenatal LPS altering behaviour in a couple of instances not otherwise seen with each treatment alone. Again, creating an experimental situation that is more naturalistic, such as combining SCFA or inducing colitis may alter the behavioural results observed.

5.9. Conclusions

In summary, my thesis provides a broad overview of the effects of pre- and postnatal PPA, and prenatal LPS administration on the behaviour of male and female rats. The emphasis of these studies on adolescent rat behaviour in both males and females add to the literature that exists on the topic and is important given the developmental and sexually dimorphic nature of ASD. Subtle and sex-specific effects on locomotor, anxiety, sensory, and social behaviour were observed, providing evidence that metabolic products

of enteric bacteria may alter development in ways resembling ASD. The different behavioural phenotypes for the different administrations of PPA and LPS illustrate how environmental insults may produce a range of symptoms on the autism spectrum, depending on developmental timing. Furthermore, the effects of PPA and LPS may not be limited to modelling ASD symptoms. Risk factors and behaviours associated with neurodevelopmental disorders such as schizophrenia and autism overlap, and it has been suggested that prenatal infection may be a general risk factor that confers vulnerability to multiple disorders, with the specific disorder determined by genetic or other environmental factors (Harvey and Boksa, 2012; Meyer, 2013).

There are many effects of PPA and SCFAs on biological systems that are favourable, including maintenance of normal immune function (Al-Lahham et al., 2010; Brestoff and Artis, 2013). However, shifts in the microbiome composition, as with alterations in diet or antibiotic use, may lead to increased levels of PPA, LPS, and other enteric metabolites that could induce inflammatory responses, alter neurodevelopment, and influence behaviour. Indeed, impaired carbohydrate digestion and increased behavioural symptoms in children with ASD following carbohydrate consumption have been reported (Adams et al., 2011; Jyonouchi, 2009). Colonization of the gut by microbes occurs throughout early development and perturbations in this process leading to gut dysbiosis may contribute to the development of neuropsychiatric disorders, including autism.

5.10. References

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Appendix A: Ethics



AUP Number: 2008-063

PI Name: Macfabe, Derrick

AUP Title: Neurobiology Of Enteric Short Chain Fatty Acids In Health And Disease

Approval Date: 02/11/2013

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Neurobiology Of Enteric Short Chain Fatty Acids In Health And Disease" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2008-063::5

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

Curriculum Vitae

Name: Kelly Foley

Post-secondary Education and Degrees:

The University of Western Ontario
London, Ontario, Canada
2009-2013 Ph.D. Neuroscience

The University of Western Ontario
London, Ontario, Canada
2004-2006 M.Sc. Neuroscience

The University of Western Ontario
London, Ontario, Canada
2000-2004 B.Sc. Physiology and Psychology

Honours and Awards:

Ontario Graduate Scholarship
2005-2006, 2011-2013

Ontario Graduate Scholarship: Science and Technology
2010-2011

Natural Sciences and Engineering Council of Canada (NSERC)
Undergraduate Research Award
2003, 2004

Research Experience:

Animal Care Technician (Part-time)
Department of Psychology, The University of Western Ontario
2009-2013

Research Associate
The Kilee Patchell-Evans Autism Research Group
Department of Psychology, The University of Western Ontario
2006-2009

Teaching Experience:

Graduate Teaching Assistant
The University of Western Ontario
2004-2006, 2009-2010, 2012-2013

Courses: Research in Behavioural and Cognitive Neuroscience, Research Methods in Psychology, Neuroscience of Motivation and Emotion, Perspectives in Neuroscience

Nominated: Teaching Assistant Award, 2004-2005, 2009-2010

Instructor (Part-time), Psychology of Addictions
 School of Language and Liberal Studies, Fanshawe College
 2010

Publications:

Shultz S.R., MacFabe D.F., **Foley K.A.**, Taylor R., Cain D.P. (2012) Sub-concussive brain injury in the Long-Evans rat induces acute neuroinflammation in the absence of behavioural impairments. Behavioural Brain Research. **229: 145-152.**

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