The development and validation of a maternal immune activation (mIA) animal model relevant to neurodevelopmental disorders: A focus towards Autism Spectrum Disorder (ASD)

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

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Abstract of thesis

Autism spectrum disorder (ASD) is a pervasive neurodevelopmental disorder (NDD) which, despite much research, is not well understood. The heterogeneity of ASD patient cohorts supports the hypothesis of complex interactions of genetic and environmental mechanisms in its aetiology and severity. The presence of gastrointestinal (GI) disturbances in ASD patients that disrupt the bi-directional signalling of the gut-brain axis, suggest that this may play a role in the manifestation of ASD phenotypes. There is also accumulating evidence for the role of neuroinflammatory processes, in both pregnant mother and affected offspring, in the aetiology of NDDs. The increased risk of ASD following infection during pregnancy highlights maternal immune activation (mIA) as a key animal model for NDDs (notably for schizophrenia and ASD) where relevant behavioural phenotypes manifest in the offspring of mIA dams. ASD remains a poorly managed NDD, with no evidence-based therapies for prevention or treatment currently available. Validating new mIA models and refining current methods is vital to the development of new therapeutic strategies.

This thesis has explored the development of an mIA model to test the maternal infection hypothesis using exposure to the viral mimetic polynosinic-polycytidylic acid (poly (I:C)) administered mid-way through pregnancy (gestational day (GD) 12.5) in rats. First, the acute effects of poly (I:C) in non-pregnant female rats were investigated, providing a validation of the experimental methods (strain, route of administration and dose). Separate cohorts of Wistar rats were then used in the GD12.5 mIA model and the physiological responses to 10 mg/kg i.p. poly (I:C) measured. Various measures including elevation of plasma IL-6 and changes in core body temperature were found to be variable between treated dams. These results confirmed the need to standardise analysis of mIA in dams, to correlate maternal inflammation with subsequent offspring outcomes such as phenotype and neuronal development changes. Poly (I:C) batch differences were also thought to contribute to these variable results and refinement of mIA methods are proposed.

Longitudinal effects of mIA on offspring of both sexes were investigated including their developmental trajectory measuring specific gene expression, morphology at GD21 and postnatal day (PD) 21, and behavioural phenotyping at adolescence and adulthood. Gestational and early postnatal changes in offspring following mIA have yet to be systematically explored. Gene expression analysis at GD21 and PD21 in offspring shows that this mIA model is useful for the identification of early developmental changes relevant to NDDs. Moreover, analysis of gene expression changes related to synaptic function, glial cells and blood-brain barrier integrity demonstrated that changes were dependent on both sex and brain region of interest. This confirms the need to include both sexes of offspring and inclusion of different brain regions for analysis which is a common limitation of previous preclinical mIA studies. The investigation of protein level changes is required to determine functional relevance, the results obtained provide evidence for subsequent targeted protein analysis in this model. Changes to the development of the gut microbiota were investigated at PD21. mIA produced a non-significant reduction in the diversity of the faecal microbiota, however at a more specific level, significant upregulation of Clostridiales was found. Further validation of microbial changes at the genus level will determine the biological relevance of these findings. This is a critical area for further studies in this model in order to understand the interaction between the gut and brain in ASD, since changes to the gut microbiota are common in ASD patients. A robust analysis was conducted to target key behavioural symptoms of ASD at adolescence and into adulthood in the mIA model with identification of an anxiety-like phenotype in male offspring at adolescence.

In conclusion, these studies have demonstrated previously unknown effects of mIA through the investigation of the immune response in pregnant dams following poly (I:C) exposure alongside an in depth, developmental analysis of brain, behaviour and gut in both male and female offspring. The results suggest that 10 mg/kg i.p. poly (I:C) in Wistar rats is a useful model to study the mechanisms of mIA in relation to ASD, including the role of gut dysbiosis in anxiety-like behaviour relevant to specific ASD phenotypes in the clinical setting.
Declaration

Submitted by Joanna M Oladipo for the degree of Doctor of Philosophy and entitled: The development and validation of a maternal immune activation (mIA) animal model relevant to neurodevelopmental disorders: A focus towards Autism Spectrum Disorder (ASD), 2017

I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other University or institute of learning.

Joanna M Oladipo. 22nd September 2017

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**List of abbreviations**

Autism spectrum disorder (ASD)
Antiepileptic drug (AED)
Autonomic nervous system (ANS)
Animal Welfare and Ethical Review Body (AWERB)
Blood-brain barrier (BBB)
Body weight (BW)
Brain weight (BrW)
Cerebellum (CB)
Central nervous system (CNS)
Core body temperature (CBT)
Copy number variations (CNVs)
Cycle threshold (CT)
Deoxyribonucleotide triphosphate (dNTPs)
Dithiothreitol (DTT)
Discs large homolog 4 (DLG4)
Dizygotic (DZ)
Dorsal prefrontal cortex (DPFC)
Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5)
Double-stranded RNA (dsRNA)
Elevated plus maze (EPM)
Enteric nervous system (ENS)
Enzyme-linked immunosorbent assay (ELISA)
False discovery rate (FDR)
Fetal growth restriction (FGR)
Frontal cortex (FC)
γ-Aminobutyric acid (GABA)
Germ free (GF)
Gastrointestinal (GI)
Gestational day (GD)
Geometric mean (GM)
Genome wide association studies (GWAS)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
Glial fibrillary acidic protein (GFAP)
Glutamate decarboxylase (GAD1)
Hour(s) (hr)
Housekeeping (HK)
Hypothalamic–pituitary–adrenal (HPA)
Immunohistochemistry (IHC)
Interleukin-6 (IL-6)
Interferon gamma (IFN-γ)
Intraperitoneal (i.p.)
Knockin (KI)
Knockout (KO)
Lipopolysaccharide (LPS)
Magnetic resonance imaging (MRI)
Major facilitator superfamily domain containing protein 2a (MFSD2a)
Maternal immune activation (mIA)
Medial prefrontal cortex (M-PFC)
Melting point (Tm)
Minute(s) (min)
Monozygotic (MZ)
Myocele-specific enhancer factor 2c (MEF2C)
Neural crest cells (NCCs)
Neurodevelopmental disorder(s) (NDD(s))
N-methyl-D-aspartate receptor (NMDAR)
Non-metric multidimensional scaling (NMDS)
Olfactomedin-like protein (OLFML3)
Open field test (OFT)
Operational taxonomic unit (OTU)
Positron emission tomography (PET)
Polymerase chain reaction (PCR)
Placenta weight (PW)
Polyinosinic–polycytidylic acid potassium salt (Poly I:C)
Postnatal day (PD)
Prepulse inhibition (PPI)
Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)
Quantitative Insights into Microbial Ecology (QIIME).
Ribonucleic acid (RNA)
Room temperature (RT)
Rostral-lateral prefrontal cortex (RL-PFC)
Seconds (sec)
Semaphorin 3a (SEMA3A)
Serotonin receptor 2a (HTR2A)
SH3 and multiple ankyrin repeat domains 3 (SHANK3)
Standard deviation (SD)
Standard error of the mean (SEM)
Succinate dehydrogenase complex, subunit A (SDHA)
Social interaction test (SIT)
Sodium dodecyl sulfate (SDS)
Synaptosomal-associated protein 25 (SNAP-25)
Toll-like receptor 3 (TLR3)
Tris-acetate EDTA (TAE)
Trophoblast inclusions (TIs)
Ultrasonic vocalisation (USV)
Ultraviolet (UV)
Valproate (VPA)
Acknowledgments

I would first like to thank Professor Jo Neill and Dr Jaleel Miyan for their support throughout this PhD, for their guidance in the planning and writing-up of these experiments and their trust, optimism and encouragement when I needed it. This PhD was made by the people I was able to befriend, work, laugh and cry with. So I would like to thank the entire team for keeping me going and having fun as we went along: Katie, Vicki, Michelle, Chlöe, Giovanni, William, Josh, Nazanin, Lisa, Daniela, Andy, Antonio, Marianne, Matt, Maurizio, Bushra, Mike and Ben. Thank you also to b-neuro and the Neuroscience Research Institute (NRI) for funding me through this research.

More specifically I would like to thank Katie for putting up with me in first year and letting me learn hands on from the first day – you taught me a lot of what I know but I won’t miss our 12 hr stints in the BSF! Vicki and Michelle, my poly (I:C) buddies, I wouldn’t have been able to see this project through without your helping hands or thinking brains. I am so thankful for you both and the fun and friendship you brought me. And to Chlöe, our then resident rat whisperer, you saved me from many a crisis and I cherish our friendship that started over a set-shifting experiment 3 years ago. I hope all of us will be friends for many years to come! A massive thank you also goes to Gavin Humphreys, for all of the help in performing the microbiota analysis, dealing with me asking stupid questions and saving the day when ‘R’ refused to work.

A big thank you also goes to all of my friends and housemates over the past 3 years who have seen me at my worst and been there for me when it wasn’t easy. To my dear friends Will, Rachael, Emily and Thomas, for welcoming me into their family, for their love, support and prayers through the joys and the sorrows of these past 3 years. Also to Lydia, for being such a loyal friend and offering her ears to me whenever I needed them. To my church family who made me feel at home from my very first week in Manchester and for the friends that I have made there, particularly the “All of the fun” crew. Of special mention is my dearest Penny (Pen Pen), a massive thank you for loving and looking after me for the past 21 years and for agreeing to proof read my thesis! (No doubt you will remain the only non-scientist to read this cover to cover!)

Finally, I would like to say a special thank you to my family, which has grown since I started this PhD! First, to my parents who have always supported and prayed for me in whatever I have done and have willingly sacrificed so much over the years that I might get to this point. To my sister and best friend Catherine, for her love and encouragement and for always answering the phone when I was in floods of tears. And to my new family, the Oladipos, for welcoming me, teaching me how to dance and just being great fun!
I wish to dedicate this thesis to my husband and dearest friend, Emmanuel. First for asking me to marry him in the middle of this PhD and for his unceasing love and support to me throughout these past 3 years. Without his prayers and help to keep trusting and persevering, I would not have made it to this point.

“No to him who is able to keep you from stumbling and to present you blameless before the presence of his glory with great joy, to the only God, our Saviour, through Jesus Christ our Lord, be glory, majesty, dominion, and authority, before all time and now and forever.”

Amen.
Preface

I. Conferences where presentations have been made on work in this thesis
British Neuroscience Association (BNA): Festival of Neuroscience, April 2015
British Association of Psychopharmacology (BAP): Summer meeting, July 2015
BNA: Festival of Neuroscience, April 2017
BAP: Summer meeting, July 2017
Society for Neuroscience (SfN): Neuroscience meeting, November 2017

II. Prizes and Awards
University of Manchester President’s Doctoral Scholar (PDS) award: Awarded September 2014 for 3 years. PDS provided a stipend supplement alongside advanced training sessions in areas such as leadership, self-awareness, collaboration, public engagement.

University of Manchester – Pharmacy and Optometry Showcase 2016: Award for best second year PhD student poster

BPS, December 2016 – Winter conference: award for best poster presented at the meeting

BNA, April 2017 – Bursary for attendance at the festival of Neuroscience

Hannah Steinberg BAP Conference Bursary, July 2017 – This was awarded to me as a postgraduate training BAP member to support my attendance at the BAP Summer Meeting

BAP Non-clinical training workshop, March 2016: certificate achieved in Non-Clinical Psychopharmacology- (Royal Society of Biology approved) 108 CPD credits

Laboratory Animal Science Association (LASA) Travel Grant, September 2017 - £1000 towards attendance of Society for Neuroscience Meeting 2017

Guarantors of Brain Travel Grant, October 2017 - £1000 towards attendance of Society for Neuroscience Meeting 2017
III. Conference abstract publications


See Appendix C, for additional conference abstracts as co-author and copies of conference posters.
Chapter 1: General Introduction
1.1. **Autism spectrum disorder**

Autism spectrum disorder (ASD) is a highly prevalent neurodevelopmental disorder (NDD) that affects central and peripheral neural development, neurobiology, and behaviour (Masi et al., 2017; Varghese et al., 2017; Yenkoyan et al., 2017). First described by Kanner in 1943 this pervasive condition is characterised by the appearance of restricted and repetitive behaviours as well as deficits in social behaviour and communication.

ASD describes a group of heterogeneous disorders including subtypes of NDD, which can be defined using similar criteria; these include atypical autism, Rett Syndrome and childhood disintegrative disorder. Cases of regressive autism also exist. Regressive ASD sees apparent neurotypical development followed by subsequent relapse in previously acquired skills (language and social), typically around the ages of 18 and 24 months of age (Goldberg et al., 2003).

ASD prevalence has been estimated at around 1 in 100 in the U.K. (Brugha et al., 2012). In the U.S., the incidence of ASD has been reported to be as high as 1 in 68 children (average age 8 years), compared to 1 in 150 in the years 2000-2002 (U.S. Department of Health and Human Services, 2014). A more recent community report from the Disease Control and Prevention corroborates this high incidence rate where males remain four times more likely to be identified with ASD than females (Christensen et al., 2016). The increased diagnosis of ASD in recent years is considered a true reflection of the changing prevalence of this NDD. However, changes and improvements to diagnostic procedures should also be considered (Hansen et al., 2015).

1.1.1. **Clinical manifestation**

The diagnosis of ASD is solely dependent on the appearance of three characteristic behavioural abnormalities as described in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5); impairments in social behaviour, stereotypic or repetitive behaviours and deficits in communication (DSM-5, American Psychiatric Association). Typical diagnosis occurs between the ages of one and three as behaviours manifest in early life. Clinical evidence supports the appearance of repetitive movements as reliable early-symptoms in ASD children (Kim and Lord, 2010) and such symptoms have been considered as reliable markers of the severity of ASD cases in later development (Lord
et al., 2006). Furthermore, deficits in core social communication are found to be more severe in children with ASD compared to other developmental disorders, and are reliably evident at 18-24 months (Wetherby et al., 2007).

1.1.2. Treatment status and comorbidity

Research surrounding ASD and related NDDs has aided the understanding of ASD aetiology. The advanced understanding of the causes and underlying pathophysiological mechanisms may enable the development of appropriate preventative treatments for the heterogeneous population of ASD patients. There are currently no validated biomarkers for ASD leaving clinical diagnosis reliant on the appearance of characteristic changes in behaviour. Whilst the manifestation of symptoms occurs early in life, most individuals sustain a disability that requires life-long care, highlighting the reach and significance of this condition. Furthermore, the large economic burden of ASD on society, due to the special education required during childhood and support throughout adulthood poses a significant issue (Buescher et al., 2014).

Alongside the behavioural symptoms of ASD a number of comorbid conditions are commonly reported (Gorrindo et al., 2012; Kohane et al., 2012; Masi et al., 2017). These include gastrointestinal (GI) disorders, immune system dysfunction, sleep disorders and cognitive disorders such as intellectual disability (Matson and Shoemaker, 2009). In addition to, and largely resulting from the lack of biomarkers for early diagnosis, there are no approved drug therapies currently available. Instead, ASD and related co-morbidities are typically controlled through behavioural therapies and lifestyle changes such as diet.

Owing to the growing realisation that the gut-brain axis is a key regulator of both brain and behaviour within neurodevelopmental and neuropsychiatric disorders, an emerging focus has been placed on the incidence of GI disturbance within ASD (Dinan and Cryan, 2016; Kennedy et al., 2016).
1.1.3. **A focus on comorbid gut dysfunction**

### 1.1.3.1. GI tract development and control

The GI system is the peripheral organ responsible for the processing, passage and digestion of food as well as the expulsion of waste. Unlike other peripheral organs, the GI system contains its own intrinsic nervous system. This “second brain” can determine gut function in the absence of the central nervous system (CNS). Comparable to the CNS, the development of the enteric nervous system (ENS) is tightly controlled and any disruption to developmental course can have detrimental effects on system functionality.

Formed of both extrinsic and intrinsic parts, the ENS is derived from neural crest cells (NCC). The migration of these NCCs and the interaction with the GI environment is critical to the spatiotemporal development of the GI tract. This has been traced effectively through neurotrophin receptor labelling in humans. Development of the GI system begins around week four of gestation where NCCs enter the foregut and advance to the hindgut by week seven (Wallace and Burns, 2005). The rostrocaudal progression of gut development continues as myenteric and submucosal plexi develop respectively. By week 14, the layers of smooth, circular and longitudinal muscle are formed and become innervated by neural cells to form the mature foetal gut. Considering the neurodevelopmental nature of ASD, the GI disturbances reported by patients may result from a developmental delay in the ENS that causes abnormalities in gut function. It is possible that changes to NCC migration may be involved in this process.

Whilst the GI system is able to perform peristaltic movements independently from the CNS (Bayliss and Starling, 1899), extensive connections do exist between the CNS and ENS. Thus, modulation and homeostatic maintenance of GI function occurs through local and sympathetic ganglion controlled reflexes (Browning and Travagli, 2014) as well as spinal and central pathways ([Figure 1](#figure1)). Interactions also occur between the gut, endocrine and immune systems (Furness, 2012).
1.1.3.2. Gut microbiota

The human gut is host to trillions ($1 \times 10^{13} - 1 \times 10^{14}$) of microorganisms, controlled by the gut microbiome (Cryan and Dinan, 2012). It is well established that microbiota are highly regulated and hence are critical for the maintenance of a healthy gut. Furthermore, the composition of the gut microbiota is changeable and dependent on environmental factors such as, diet, use of probiotics and antibiotics (Flint et al., 2012). Metagenetic analysis has revealed that the human gut harbours over 1000 bacterial species but the composition varies vastly between the infant and adult microbiome (Kurokawa et al., 2007; Qin et al., 2010). The infant microbiome is largely undifferentiated compared to the adult, demonstrating the colonisation of bacterial
species that occurs throughout development post-parturition (Figure 2). Whilst there are gaps in our understanding of the development of the human gut microbiota, it is known that the early gut microbiome is important for establishing and regulating the immune system (Groer et al., 2014).

The formation of the infant microbiota is largely dependent on the delivery method of a new-born (Dominguez-Bello et al., 2010). Thus exposure to the vaginal environment is vital for the initial development of an appropriate gut microbiota. Babies born via caesarean section (C-section) often develop a gut microbiome that resembles that of the human skin microbiota, and this may influence the susceptibility of new-borns to infection. Interestingly, caesarean birth has been linked to an increased risk for ASD due to the changes in the gut microbiota that have been recorded (Curran et al., 2014). Furthermore, children born via C-section show differences in their gut microbiota compared to vaginal delivery that persist for months if not longer after birth (Gronlund et al., 1999; Wampach et al., 2017), highlighting a critical time of susceptibility to subsequent neurodevelopmental insults.

Multiple mechanisms exist for communication between the gut microbiota and the brain forming the so-called gut-brain axis (Cryan and Dinan, 2012). The contribution of gut microbiota to GI disturbances has been examined in disorders such as irritable bowel syndrome (IBS) and other related GI disorders. Within these prominent GI disorders, microbiota dysbiosis has been highlighted as a critical factor in the regulation of gut permeability, immune function and ENS connections (Round and Mazmanian, 2009; Haar et al., 2016).
Figure 2: Timeline of the development of the GI microbiota and brain including the onset of neurodevelopmental disorders. (Figure taken from Borre et al. (2014)).
1.1.3.3. Gut abnormalities in ASD

In recent years the presence of GI comorbidity in ASD patients has received significant attention. However, the true prevalence of such gut disorders remains a contentious issue within the field. A variety of GI symptoms have been reported by ASD patients. These include both functional gut problems and microbial changes in the GI tract. Chronic abdominal pain and diarrhoea as well as constipation are among the most commonly reported symptoms (Buie et al., 2010; Hsiao, 2014). However, controversy resides over the reported prevalence of GI disturbances in ASD and whether this is a natural occurrence within the age-matched, healthy population. Reports of GI symptom manifestation have ranged from as low as 9% to as high as 91% in ASD patients (Coury et al., 2012; Chaidez et al., 2014), which may be a result of differential methodology and data source.

Evidence from a number of studies has shown an association between disruption of the gut microbiota and ASD symptoms in children (Song et al., 2004; Parracho et al., 2005; Finegold et al., 2010; Kang et al., 2013). As a result, the focus for underlying mechanisms has been placed on changes in the release of soluble factors (cytokines, toxic metabolites etc.) following changes to gut microorganisms (Wang et al., 2012). Changes in the neural connections that stimulate the microbiota responses within the GI system have not been explored or documented.

It is difficult to assess the exact composition of microbiota as a result of ASD pathology given the many confounding lifestyle changes that exist (such as altered diet, antibiotic use) in patient cohorts since these factors largely influence the bacteria present within the gut. However, evidence suggests that the microbiota of ASD patients is abnormal relative to neurotypical controls. For example, altered expression of the main bacterial phyla including Firmicutes, Bacteroidetes, Fusobacteria and Verrucomicrobia has been reported in ASD patients (Finegold et al., 2002; Williams et al., 2012; Kang et al., 2013). Not only the microbial composition, but also the levels of bacterial metabolites show disruption in ASD cases. By measuring the level of short chain fatty acids (SCFA) present in faecal samples, disruption to the fermentation process in the GI system of ASD patients has been demonstrated (Wang et al., 2012). One particular genus of the Firmicutes phylum shown to be altered within the microbiota of ASD patients is Clostridium (Song et al., 2004; Parracho et al., 2005; Martirosian et al., 2011). The
*Clostridium* species can produce harmful neurotoxins, thus their specificity within ASD faecal samples may be linked to gut dysbiosis in ASD. Interestingly, the differences in *Clostridium* species between ASD and healthy unrelated controls were not found to be significantly different to their healthy siblings (Parracho et al., 2005) supporting a complex interaction between genetic and environmental factors in the aetiology of ASD.

Findings in human patients have been further supported by the study of the gut microbiota in a rodent model of NDDs known as maternal immune activation (mIA). Notably this area of gut research in the mIA model is in its infancy. Not only was the composition of the gut microbiota altered in adult mice but exclusive analysis of the *Clostridia* and *Bacteroidia* classes revealed that these were responsible for the changes in microbiota compared to controls (Hsiao et al., 2013). Following analysis of the microbiota dysbiosis seen in mIA offspring, treatment with *B. fragilis* (a commensal bacteria) at weaning was shown to produce improvements in GI dysfunction at eight weeks of age, and reduced the inflammatory response seen (namely IL-6 levels in the colon). Furthermore, the amelioration of ASD-like behaviours following *B. fragilis* treatment provides key evidence for the gut-brain interaction (Hsiao et al., 2013). Although these findings in adulthood are useful in displaying a long-term alteration to the microbial system, further studies are required to assess the effects in early development to increase translation of findings to ASD children. These findings stimulate the need for further investigation using the mIA rodent model in order to further understand ASD and the related GI comorbidities. Critically, there is a requirement to validate this model in rats owing to their favourability with regard to the social behaviour (e.g. complex behaviour repertoire including specific play behaviours during adolescence), as well as increased brain size (desirable for neuroimaging).

The short-term relief of the behavioural symptoms of ASD has been shown via the use of oral antibiotics. This is suggestive of the presence of a causal relationship between gut-brain interaction and ASD behavioural symptoms that is yet to be elucidated fully. Vancomycin (an antibiotic for bacterial infections) administered to 11 patients suffering with regressive autism produced a short-term improvement in ASD behaviours that was particularly striking given the minimal absorption of Vancomycin and its effect on the gut microbiota. This suggests that the positive behavioural changes resulted from the effects on intestinal-tract microflora (Sandler et al., 2000). This small-scale, cohort specific study cannot be used as an example of a causative link. However, it is
suggestive of how nervous stimulation within an abnormal gut environment can produce deviant behaviours in ASD (Kraneveld et al., 2014). Further evidence of the influence that the microbiota exert on brain and behaviour is shown in mice through the study of social behaviour in germ free (GF) mice (Desbonnet et al., 2014). The application of GF rearing conditions produced deficits in social behaviour within the mice, which was stronger in males (akin to ASD-like behaviours). On bacterial colonisation of the gut, the social deficits were reversed, thus revealing the critical role of microbiota on the programming of specific behaviours (Desbonnet et al., 2014).

In an attempt to assess the reported prevalence of GI disturbance within ASD, Table 1 provides examples of key clinical studies published to date. These examples reflect a trend towards increased incidence of GI abnormalities in ASD patients. McElhanon et al., 2014 support this hypothesis in their rigorous meta-analysis of publications from the past 32 years surrounding GI disturbance in ASD. The results show a few examples where no significant difference was found in the gut disturbance between ASD and control subjects (Ibrahim et al., 2009; Dalton et al., 2014; Jiang et al., 2017). Most commonly it is concluded that there is insufficient clinical evidence to propose the aetiological association of these observations. This is largely due to the limitations with regard to study methodology, lack of standardised diagnostic criteria, and inclusion of appropriate control groups (non-affected siblings of ASD patients), which would strengthen clinical findings. Furthermore, numerous studies employ criteria bias within their selection of subjects and recruit low numbers of ASD patients. In 2005 Erickson et al. stated that there was insufficient published data to determine whether GI disturbances are to be considered a key defining feature of ASD and more than 10 years on this conclusion remains appropriate. Further clinical and preclinical studies are required to refine these findings.
Table 1: Prevalence of gut comorbidities recorded in the literature

<table>
<thead>
<tr>
<th>Paper</th>
<th>Conclusion</th>
<th>Study details (Patient cohort assessment of GI status)</th>
<th>Control group (Absent/present, details)</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>D’Eufemia et al., 1996</td>
<td>Gut mucosal damage found in 43% ASD patients but not in controls</td>
<td>21 ASD (15 male, 6 female) Sugar intestinal permeability test (IPT) used to assess small intestine mucosal damage</td>
<td>Present 40, healthy age-matched controls</td>
<td>Limited analysis of GI abnormalities present</td>
</tr>
<tr>
<td>Horvath et al., 1999</td>
<td>70% prevalence of GI disturbance in ASD patient’s recruited GI problems contribute to some of the behavioural problems seen in ASD</td>
<td>33 male children (mean age 5.7 years) GI assessment included: presence of reflux esophagitis, inflammation of the gastric mucosa, decreased activity of gut enzymes</td>
<td>Absent No comparison with healthy population digestive enzymes were compared to normal specimens</td>
<td>Patient cohort recruited based on prevalence of GI symptoms Only a subset of patients on a gluten-free diet at time of analysis</td>
</tr>
<tr>
<td>Molloy and Manning-Courtney, 2003</td>
<td>24% prevalence of GI disturbances in ASD patients Lower GI symptoms more common</td>
<td>139 children all 24-96 months. GI assessment from patient medical record Patients recruited from a medical clinic specialising in ASD.</td>
<td>Absent Prevalence of GI within a general population of ASD cases only</td>
<td>Control absent. Definition of GI symptom: “present at any point through medical history” (no definition of a duration of symptom)</td>
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<tr>
<td>Parracho et al., 2005</td>
<td>91.4% of ASD cases had GI disturbance, 25% in sibling group, 0% in healthy controls</td>
<td>58 ASD patients (48 males, 10 females) Assessment of GI disturbance by questionnaire. GI problems associated with high levels of clostridia bacteria in faecal flora</td>
<td>Present Non-ASD sibling group (12), unrelated healthy control group (10).</td>
<td>ASD subjects had all taken a variety of antibiotics for GI disorders before the study</td>
</tr>
<tr>
<td>Study</td>
<td>Findings</td>
<td>Methodology</td>
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<tr>
<td>Valicenti-McDermott et al., 2006</td>
<td>70% significant prevalence of GI symptoms in ASD children compared to 28% in neurotypical and 42% with other NDDs. Abnormal stool patterns found in 18% ASD children.</td>
<td>Present Two control groups one typical development (50) and one other developmental disorders (50), matched for age, sex, ethnicity. Retrospective study. Control group from a different population of children (i.e. not non-ASD affected siblings) many other factors influencing their development (e.g. developmental environment).</td>
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<tr>
<td>Ibrahim et al., 2009</td>
<td>No significant difference in gut disturbance between ASD and control subjects. Only differences in the cumulative incidence by age of constipation, feeding issues and food selectivity.</td>
<td>121 (92 male, 29 female, mean age patients from medical centre. All GI diagnoses for patients were recorded in a medical index. Patients followed until ~18yrs – average age of patients at ASD diagnosis ~6yrs. Present 242 controls, 2 selected per ASD case (matched by age, gender, year of first registration and duration of follow-up). Retrospective study</td>
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<tr>
<td>Finegold et al., 2010</td>
<td>Significantly higher diversity of bacteria found in the faeces of ASD subjects compared to controls Bacteroidetes increased but Firmicutes decreased in ASD.</td>
<td>33 ASD subjects with GI dysfunction (ages 2-13; 24 male, 9 female) Bacterial 16S rDNA from faecal DNA samples analysed. 7 non-ASD siblings, 8 other controls. No analysis of immune status</td>
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<tr>
<td>Adams et al., 2011</td>
<td>Strong correlation of severity of ASD symptoms with presence and severity of GI disturbance. Children with ASD had reduced levels of beneficial GI bacteria.</td>
<td>58 ASD/Asperger’s patients (50 male, 8 female) GI disturbances evaluated based on a modified GI severity index (constipation, diarrhoea, stool consistency, stool smell, flatulence, abdominal pain) Present Control group (39, 18 male, 21 female) recruited from a population of non-related subjects. Participants recruited based on an ASD or Asperger’s diagnosis. ASD children had more probiotic usage which would affect gut microbiota.</td>
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<tr>
<td>Study</td>
<td>Description</td>
<td>Methodology</td>
<td>Findings</td>
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<td>Wang et al., 2011</td>
<td>GI abnormalities significantly increased in ASD children (42%) compared with non-affected siblings (12%). Constipation and chronic diarrhoea were the most common</td>
<td>589 ASD subjects recruited from a family registry of patients with idiopathic ASD. Assessment of GI disturbances by parent report. Present Control group of unaffected siblings (163) (same maternal environment).</td>
<td>Retrospective study. Parental questionnaire on GI symptoms only. Lack of uniform definitions and duration of GI symptoms.</td>
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<tr>
<td>Kang et al., 2013</td>
<td>ASD cases with GI symptoms show distinct and less diverse gut microbial compositions (lower levels of Prevotella, Coprococcus)</td>
<td>20 ASD subjects with GI dysfunction (ages 3-16, 18 male, 2 female). Bacterial 16S rDNA from faecal DNA samples analysed. Assessment of special diets/antibiotics.</td>
<td>Low number of participants. No analysis of immune status.</td>
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<td>Chaidez et al., 2014</td>
<td>Parental report of GI abnormalities significantly increased in ASD patients. Patients with NDD were more likely to suffer a frequent GI symptom. GI symptoms were correlated with social withdrawal, irritability and stereotypic behaviours</td>
<td>499 ASD patients. ASD cases confirmed using Autism diagnostic interview. Assessment of GI disturbances by questionnaire. Children with ASD three times more likely to experience GI symptoms compared to neurotypical controls.</td>
<td>Results solely based on parental questionnaires. Lack of standardised definitions for GI symptoms.</td>
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</tr>
<tr>
<td>Dalton et al., 2014</td>
<td>No evidence for increased small intestine permeability within ASD compared to control children despite the presence of GI symptoms</td>
<td>103 (98 male, 14 female) ASD and 30 control children (aged 10-14 years). Gut permeability assessed via urine lactulose/mannitol ratio after oral administration.</td>
<td>Control group (30, 26 males, 4 females) was cohort of children with special needs/learning difficulties but without ASD. Limited numbers of children included in control group. Absence of NT control group. Effects on gut permeability attested in later childhood presence of changes in early life absent.</td>
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</tbody>
</table>
Marie et al., 2017

Jiang et al., 2017

Prevalence of GI symptoms higher in ASD participants but not significant compared to control groups

112 (60 male, 52 female) participants (age 17-37 months)

Parent interview and child observations

Four groups of participants (28 in each; ASD and GI issues, ASD without GI issues, not ASD but atypical development and GI issue, atypical development and no GI issue)

Low patient numbers
Assessment made only performed in toddlers

Despite the lack of conclusive findings from clinical investigation, alongside pre-clinical data there is convincing evidence to explore further the potential role of a gut-brain interaction within ASD. It is likely that the GI abnormalities seen in ASD patients are caused by a variety of factors including susceptibility from both genetics and the environment. The extensive connections between the gut and brain forming the complex gut-brain axis are tightly controlled showing that GI functionality can be influenced by both ANS and ENS. The expansive bacterial environment of the gut is also influential. Recent evidence suggests that alterations to the microbiota in rodent ASD models can play a role in ASD pathophysiology and may hold the potential for novel gut-directed therapies (deTheije et al., 2014b).
1.2. The ASD brain

1.2.1. Typical neurodevelopment

Early brain development in humans involves a highly orchestrated timeline of events (Figure 3). In contrast to human brain development, the majority of rat brain development occurs postnatally. In the human foetus, synapses start to form during the 17th week of pregnancy and continue multiplying until 3-4 months after birth (Illes, 2006). Neurogenesis in specific areas of the CNS begins at approximately gestational day (GD) GD9.5 in the rat (Rice and Barone, 2000). Subsequently, the processes of neuronal proliferation and migration GD9.5–15, synaptogenesis postnatal day (PD) 10-30, and apoptosis occur. Synaptic pruning occurs after synaptogenesis in early postnatal life of the rat. Myelination begins at PD10 and continues through adolescence until PD45, after which point only small changes are seen (Babikian et al., 2011). Hindbrain structures in the rat (pons, medulla and cerebellum) develop during the embryonic period in the rat (GD10-16). The hypothalamic and thalamic nuclei develop in the late embryonic period in the rat.
Figure 3: The timeline of neurodevelopment in the human. The steps of brain, immune system and gut development from gestation to early postnatal life which are highly orchestrated (taken from (Estes and McAllister, 2016)).
1.2.2. **Anatomical findings in ASD**

Despite the lack of standardised neurological biomarkers for this disorder, considerable effort has been placed on exploring the anatomical abnormalities found within the brains of ASD patients. A multitude of anatomical abnormalities have been reported implicating large areas of grey matter, white matter, and cortical brain structures through the use of magnetic resonance imaging (MRI). As a result, these findings have driven different mechanistic theories of ASD involving various brain regions. These include the “amygdala theory of autism” where this area of the social brain is implicated in the underlying pathology (Schumann et al., 2009). Bilateral amygdala volume has been shown to be increased in ASD toddlers and young children with some correlation with social behaviour deficits (Schumann et al., 2009; Kim et al., 2010). Preclinical work in non-human primates, alongside evidence from human studies, supports the role of amygdala in affective processing and the presence of anatomical and functional amygdala abnormalities in ASD (Schumann et al., 2011). However, total congenital absence of the amygdala has been shown to cause only minimal deficits in social behaviour and thus, effects of amygdala defects may be a downstream target in ASD aetiology (Wang et al., 2014a). Added to this, volumetric reduction in specific areas of the corpus callosum (for example, the genu), as well as a reduction in the synchronisation between frontal and parietal brain areas has driven the theory of “under connectivity” for ASD (Just et al., 2007).

Gross anatomical brain changes have been found in ASD patients through the study of cortical thickness or volume (Hazlett et al., 2005; Raznahan et al., 2010; Wallace et al., 2010; Ecker et al., 2015). Increased brain size as measured by cortical thickness (CT) was recorded in an imaging study involving 84 males diagnosed with ASD and their age-matched controls (Ecker et al., 2013). The frontal lobe increase in CT was found across the medial (M-), dorsal (D-) and rostral lateral (RL-) prefrontal cortex (PFC), and also in the superior frontal gyrus. In the anterior temporal lobe and parahippocampal cortex (PHC), decreases in CT were found. Significant regional differences were also recorded in surface area in ASD vs. control, which were paralleled by proportional changes in cortical volume (CV) (Ecker et al., 2013). Interestingly, these changes in neuroanatomy were correlated with symptom severity in some cases, highlighting a relationship between structural change and functional output.
Changes in brain anatomy have also been shown in white matter (Hazlett et al., 2005) as well as within the limbic system, where both the hippocampus (Groen et al., 2010) and amygdala (Bellani et al., 2013) are implicated. These findings are not ubiquitous and the significance of brain volume changes in ASD patients remains a contentious issue. A large age-dependency for structural brain changes exists, where the reduction in CV and CT in young patients was reversed at adulthood (Raznahan et al., 2010). The exact time window for these structural changes in early development is not known, however overgrowth in the infant and subsequent decline of the adult ASD brain has been documented by other extensive analyses of cross-sectional and longitudinal MRI scans (Courchesne et al., 2011a). This is a likely explanation for the contrasting findings with anatomical changes reported. Evidently subject age is a critical factor for consideration when assessing the structural brain changes found in ASD subjects.

A recent study performed an extensive analysis of MRI data from ASD patients obtained from the Autism Brain Imaging data exchange. This study critically analysed the contrasting data for structural changes in ASD patients (aged 6-35 years old) and concluded that compared to the large within-group variability, the anatomical changes shown in ASD patients were unconvincing and provide only weak clinical significance for ASD (Haar et al., 2016). Given the heterogeneity of patient groups it would be impossible to use these anatomical changes as biomarkers for the disorder. However, the classification of ASD phenotypes by genetic influence or the appearance of clinical comorbidity may allow for subgroup-specific, neuroanatomical changes to be identified.

1.2.3. Neural connectivity

Added to the gross anatomical changes recorded in ASD brains, evidence exists at the cellular level to support the theory of impaired connectivity. Indeed, changes at the cellular level have been reported through small-scale post mortem analysis of young (2-16 years) ASD brains. In male ASD subjects, the mean neuron number in the D-PFC and M-PFC was increased compared to controls (Courchesne et al., 2011b). This results in disruption of the shaping and tuning of neural circuits which is critical in early development to enable subsequent effective connectivity between long-distance brain regions (Courchesne et al., 2007). The lack of necessary synaptic pruning to remove non-functional neurons may affect the process of lateralisation which is involved in language function, hence a potential mechanism for the behaviour manifestation in ASD
(Eyler et al., 2012). In addition, neural migration has been implicated in ASD aetiology. Failure of typical neural migration may contribute to the increased cortical thickness that has been previously discussed. A focus of interest is the protein Reelin, given the major role it holds in neural migration during the embryonic period as well as in axonal growth in the hippocampus (Eastwood et al., 2003; D’Arcangelo, 2014). Indeed, mutations in the protein Reelin have been associated with increased ASD risk (Wang et al., 2014b; Lammert and Howell, 2016).

1.2.4. Microglia

The role of microglia, as the resident immune cells of the CNS, is particularly interesting as these cells mediate CNS inflammation (Aguzzi et al., 2013). Whilst initially considered only as responders to pathological insult, the central role of microglia in typical neurodevelopment (including neurogenesis) and postnatal apoptosis and phagocytosis has become more pertinent in recent years. Added to this, abnormal microglia have been implicated in ASD aetiology where the global innate immune response has been altered (Ashwood et al., 2011; Onore et al., 2012; Goines and Ashwood, 2013). Evidence for these microglial abnormalities has been found in both post-mortem brain analysis and in animal models (Morgan et al., 2010; Derecki et al., 2012; Suzuki et al., 2013).

Although based on low sample size, a significant increase in the density of microglia in grey matter has been shown alongside an altered cytokine profile in the brain tissue and cerebrospinal fluid of ASD patients. A cytokine protein array was used to show an increased level of pro-inflammatory cytokines in ASD brain tissue with the most prominent upregulation being observed in the levels of IL-6 where a fold increase of 6.10, and 31.4 (compared to control) in the middle frontal gyrus and the anterior cingulate gyrus were found respectively. Furthermore, histological analysis revealed an increase in the number of activated microglia and astrocytes in ASD brains highlighting the likely presence of neuroinflammation (Vargas et al., 2005). More recently, confirmation of this inflammatory state within ASD patients has been achieved through the use of improved antibody and staining methods (Morgan et al., 2012).

Alongside brain tissue analysis for microglial activation, advances in the applications of positron emission tomography (PET) have enabled the monitoring of neuroglia activation via neuroimaging. A study of 20 ASD males and their age- and IQ-matched
controls using a radiotracer for microglia revealed significantly increased activation of microglia across multiple brains (Suzuki et al., 2013). This *in vivo* analysis of microglial activation supports the findings in post-mortem brain analyses and taken together these findings demonstrate that elevated microglial activation may be critically involved in ASD, at least in certain patient cohorts. In addition, molecular evidence has been provided using comparison of mRNA expression levels of specific astrocyte- and microglial-markers (Gupta et al., 2014). ASD brain samples showed significantly increased expression of neuroglial markers in the PFC and the cerebellum. Both of these findings were coupled with a significant reduction in the expression of neuron-specific markers in both brain regions (Edmonson et al., 2014). Further work is required to understand the mechanisms behind and integrate the findings of abnormal microglia and impaired neuronal connectivity.

1.2.5. Cerebellum

The cerebellum accounts for only 10% of human brain volume. However, it contains over half of the neurons in the whole brain (Herculano-Houzel, 2010). Although originally considered only for its role in motor function, it is now accepted that the cerebellum plays a role in cognitive function (Rogers et al., 2013; Hampson and Blatt, 2015). Furthermore, anatomical and structural abnormalities of the cerebellum are amongst the most commonly reported brain defects in ASD patients (Palmen et al., 2004; Courchesne et al., 2005). The most common of these are reduced Purkinje cell numbers alongside more general cell loss (Bauman, 1991; DiCicco-Bloom et al., 2006). MRI imaging has also been applied to measure the difference in cerebellar vermal volumes. Children (aged 3-4 years) with ASD showed significantly reduced cerebral vermal volume when controlling for age, although these abnormalities were not correlated with symptom severity. Thus, the relationship between structure and symptoms from the cerebellum are unclear (Webb et al., 2009). Further meta-analyses support these findings with ASD children showing significant reductions in the vermal lobes IV-VII (Stanfield et al., 2008). In addition, hypoplasia in the vermis of adult ASD patients has been shown in specific vermal regions (Wang et al., 2014a). Evidence to the contrary of these findings suggests that care should be taken when interpreting these cerebellar anatomical changes as specific to the ASD phenotype. More work is required to determine whether these defects are subtype specific (Scott et al., 2009). Interestingly, the behavioural abnormalities resulting from these cerebellar defects have
been successfully modelled in animals. Cerebellar lesions, (splitting of the inferior cerebellar vermis), in juvenile rats resulted in a reduction in social behaviour and vocalisation in the early post-surgical phase (Al-Afif et al., 2013). Thus the cerebellum is predicted to play a key role in the development of social behaviour.

Alongside anatomical findings, disruption in specific genes have been associated with an increased risk of ASD, including Retinoic acid-related orphan receptor alpha (RORα), Engrailed 2 (EN2) and Ca2+-dependent activator (CAPS2) (Rogers et al., 2013). All of these genes are implicated in the genetic drive of typical cerebellar development with RORα involved in Purkinje cell differentiation (Boukhtouche et al., 2010) and CAPS2 controlling the release of neurotrophic factors (Sadakata and Furuichi, 2009). Owing to the complexity of ASD aetiology, it is predicted that a combination of genes contribute to ASD susceptibility and these are not unique to the cerebellum (Rogers et al., 2013).
1.3. **Aetiology**

Current aetiological understanding of ASD is limited, although the heterogeneity of patient cohorts supports a complex interaction of genetic and environmental mechanisms. Early research was focused on underlying genetic causes owing to high heritability rates. However, increasing epidemiological evidence to date supports a role for multiple environmental risk factors in ASD susceptibility. Environmental perturbations (e.g. drug exposure or maternal infection) occurring during gestation can have significant effects on the developing foetus. It is currently accepted that a disruption during early gestation (mid-late first trimester) increases the risk for ASD development whereas insults during the second trimester may give rise to phenotypes similar to schizophrenia (Knuesel et al., 2014).

1.3.1. **Typical pregnancy**

Human pregnancy is divided into three stages, known as trimesters. These stages span gestational weeks 1-12 (first trimester), weeks 13-28 (second trimester) and weeks 29-40 (third trimester). Although it was first believed that immunosuppression was critical for the maintenance of a successful pregnancy, it is now accepted that pregnancy requires a dynamic and responsive immune system (Mor et al., 2017). It is now known that a variety of immune cells are present at the maternal-fetal interface during the early stages of pregnancy including natural killer cells, macrophages and, in lower numbers, T and B cells (Moffett-King, 2002; Muzzio et al., 2014). As shown in Figure 4, the stages of pregnancy have different immunological profiles which are required for maintaining the pregnancy as well as protecting the foetus from environmental insults.
Figure 4: Immunological stages of pregnancy. The first trimester of pregnancy shows as inflammatory profile that is required for implantation. Similarly, the third trimester shows an inflammatory state which is required for labour. The second trimester, which involves fetal growth, is anti-inflammatory and sustained inflammation during this period is associated with pregnancy complications such as miscarriage. (Figure taken from Mor et al. (2017)).
The placenta plays a critical role in protecting the developing foetus by providing nutrients for development as well as protection from infection (Jonakait, 2007). The placenta provides a direct interface between mother and foetus allowing immune interactions (via various cytokines) to occur (Zaretsky et al., 2004; Colucci et al., 2011). Cytokines are cell-signalling proteins that are involved in the immune response modulation and programming throughout development (Bauer et al., 2007; Hsu and Nanan, 2014). Although critical for preventing rejection of the foetus in the event of severe maternal immune activation it is possible that excessive exposure to maternal cytokines may harm the growing foetus (Reisinger et al., 2015).

1.3.2. Genetics of ASD

In the early years of ASD research, a large focus was given the genetic component of aetiology given the high concordance rate of ASD within monozygotic (MZ) compared to dizygotic (DZ) twins (Cook, 1998). Concordance rates, (the probability of the appearance of a specific characteristic in a pair of subjects) have been reported as high as ~60% for ASD and 90% for related cognitive abnormalities in MZ twins (Smalley et al., 1988; Bailey et al., 1995). MZ twin studies provide a natural opportunity to study the contribution of genetics to disease as both individuals share all of their alleles having originated from a single zygote. Therefore, the high concordance rate for ASD phenotypes in MZ vs. DZ twins supports a genetic aetiology. The assumption of these studies is that the concordance rate for a disorder in MZ twins is driven by genetics alone. The role of the shared prenatal environment is often overlooked, and the influence of environmental similarity of MZ twins is also a key determinant of the concordance of disease traits. Indeed, no MZ twin study has revealed a 100% concordance rate highlighting that the heritability of ASD is contingent on other factors such as environmental risk. A more recent large-scale population-based twin study reported that the susceptibility for ASD was better explained by shared environmental components (a rate of 55%) than the heritability component (a rate of 37%). Thus, while genetics plays an important role, its influence may have been originally overestimated (Hallmayer, 2011).

The genetic component of ASD is heterogeneous where multiple genes and chromosomal regions have been implicated across the phenotypic spectrum of this condition (Banerjee et al., 2014; Fakhoury, 2018). Approximately 10-25% of ASD
cases are associated with an identifiable Mendelian condition or single gene disorder. Common examples include as Fragile X Syndrome, Rett syndrome and, Tuberous sclerosis complex (Devlin and Scherer, 2012). Added to this, molecular genetics studies including linkage studies and genome wide association studies (GWAS) have been used to better understand the complex genetic basis of ASD. Such studies have revealed numerous single nucleotide polymorphisms (SNPs), as well as copy number variations (CNVs) within families of ASD patients. It is believed that for some cases of ASD at least, a strong genetic component exists.

In order to further outline the complexity of the genetic basis of ASD an online database (https://gene.sfari.org/) has been produced (Simons Foundation Autism Research Initiative) (Basu et al., 2009). At the time of writing, 881 genes were listed in this database for association with ASD. The database also provides a scoring system enabling the validity of this association to be considered based on the replication of findings across the literature (including independent replication), degree of statistical testing performed, and the genome-wide significance. Of the genes identified, 23 were given a score of “1”, defined as recurrent and convincing mutations that are likely to be functional within the pathophysiology of ASD (Table 2).

Critical to our understanding of the genetics involved in ASD has been the generation of various animal models. Manipulation of genes that encode for neural connectivity and architecture are relatively simple to achieve, particularly with rodent models, especially in the mouse (Banerjee et al., 2014). The majority of genetic modelling has been performed in the mouse (Moy et al., 2006; Provenzano et al., 2012). However, in recent years, advances have been made in the generation of knockout models in the rat. Moreover, some of the first rat models to be released were focused on ASD (Fmr1, Nlgn3 etc.). Considering the favourable characteristics of the rat model over that of the mouse (e.g. increased social repertoire, complex communication system) this is a key area for future development.

The contribution of CNVs to neurodevelopmental disorders is becoming increasingly recognised as the disruption of DNA structure results in potential developmental delay. The scope of this section does not include an exhaustive list of the CNV deletions and duplications associated with ASD, although duplications in 15q13 and microdeletions in 16p11.2 loci have been reported (Marshall et al., 2008; Miller et al., 2009). A more
comprehensive list of the known CNVs within ASD can be found elsewhere (Schanen, 2006). Genes associated with these CNVs have been implicated in the regulation of synaptogenesis, highlighting the potential importance of synapse formation and regulation in the aetiology of ASD.

Despite the large genetic research emphasis, no coherent theory of genetic-ASD pathophysiology has been defined, although important progress has been made in the identification of genetic mutations that predispose to ASD, at least in specific patient cohorts. More recently, the focus has turned to environmental factors that may give rise to ASD. This does not however detract from the importance of genetics underlying aetiology. On the contrary, there is most likely a strong genetic influence over, and an interaction with, such environmental factors. Indeed, a multidisciplinary approach is required that considers the contribution and interaction of both genetic and environmental factors.
Table 2: Identified genes associated with ASD pathophysiology and their function where determined

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADNP</td>
<td>Activity-dependent neuroprotector homeobox</td>
<td>Neuroprotective factor, transcription factor</td>
</tr>
<tr>
<td>ANK2</td>
<td>Ankyrin 2, neuronal</td>
<td>Cytoskeletal, membrane binding protein</td>
</tr>
<tr>
<td>ARID1B</td>
<td>AT rich interactive domain 1B (SWI1-like)</td>
<td>Neural progenitor-specific chromatin remodelling complex</td>
</tr>
<tr>
<td>ASH1L</td>
<td>Ash1 (absent, small, or homeotic)-like (Drosophila)</td>
<td>Transcriptional activator, functions as a histone methyltransferase</td>
</tr>
<tr>
<td>ASXL3</td>
<td>Additional sex combs-like 3 (Drosophila)</td>
<td>Multiprotein complex (required for maintenance homeotic genes in development)</td>
</tr>
<tr>
<td>CHD8</td>
<td>Chromodomain helicase DNA binding protein 8</td>
<td>A DNA helicase, transcription repressor</td>
</tr>
<tr>
<td>CUL3</td>
<td>Cullin 3</td>
<td>Scaffold protein for ubiquitin ligases</td>
</tr>
<tr>
<td>DSCAM</td>
<td>Down syndrome cell adhesion molecule</td>
<td>Transmembrane protein</td>
</tr>
<tr>
<td>DYRK1A</td>
<td>Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A</td>
<td>Protein kinase for cell signalling</td>
</tr>
<tr>
<td>GRIN2B</td>
<td>Glutamate receptor, inotropic, N-methyl D-apartate 2B</td>
<td>NMDAR subtype of glutamate-gated ion channels</td>
</tr>
<tr>
<td>KATNAL2</td>
<td>Katanin p60 subunit A-like 2</td>
<td>Severs microtubules in ATP-dependent manner</td>
</tr>
<tr>
<td>KMT5B</td>
<td>Lysine methyltransferase 5B</td>
<td>Undetermined</td>
</tr>
<tr>
<td>MYT11</td>
<td>Myeline transcription factor 1-like</td>
<td>Transcription factor involved in repressing non-neuronal gene during neuron differentiation</td>
</tr>
<tr>
<td>NAA15</td>
<td>N(alpha)-acetyltransferase 15, NatA auxiliary subunitÂ</td>
<td>May be involved in neuronal growth and development</td>
</tr>
<tr>
<td>POGZ</td>
<td>Pogo transposable element with ZNF domain</td>
<td>Zinc finger protein</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
<td>Phosphatase involved in tumour suppression</td>
</tr>
<tr>
<td>RELN</td>
<td>Reelin</td>
<td>Regulates neural migration</td>
</tr>
<tr>
<td>SCN2A</td>
<td>Sodium channel, voltage-gated, type II, alpha subunit</td>
<td>Voltage-gated ion channel</td>
</tr>
<tr>
<td>SETD5</td>
<td>SET domain containing 5</td>
<td>This gene is predicted to encode a methyltransferase and resides within the critical interval for the 3p25 microdeletion syndrome.</td>
</tr>
<tr>
<td>SHANK3</td>
<td>SH3 and multiple Ankyrin repeat domains 3</td>
<td>Postsynaptic scaffold protein</td>
</tr>
<tr>
<td>SYNGAP1</td>
<td>Synaptic Ras GTPase activating protein 1</td>
<td>NMDAR associated protein</td>
</tr>
<tr>
<td>TBR1</td>
<td>T-box, brain, 1</td>
<td>Transcriptional regulator involved in development</td>
</tr>
<tr>
<td>TRIP12</td>
<td>Thyroid hormone receptor interactor</td>
<td>Binds with Golgi apparatus and microtubules</td>
</tr>
</tbody>
</table>
1.3.3. Immune system in ASD

Dysfunction of the immune system has long been recognised as a key feature of ASD (Onore et al., 2012). It has been highlighted that the immune system may be critical to the aetiology of ASD in certain subgroups of patients. Furthermore, whilst findings of neurobiological abnormalities within ASD are typically inconsistent, the report of neuroinflammation is reasonably consistent between publications (for review, see Goyal and Miyan, 2014).

1.3.3.1. Clinical evidence of immune abnormalities in ASD

A number of familial studies have been conducted showing an increased prevalence of autoimmune conditions in the families of children with ASD compared to control populations. In a study of 61 ASD and 46 healthy control families, it was reported that the mean number of autoimmune disorders was higher in families of ASD patients compared to healthy controls. 46% of the ASD patient families reported more than two members of the family who suffered with an autoimmune disease, with type 1 diabetes, adult rheumatoid arthritis and hypothyroidism being the most common (Comi et al., 1999). Whilst not all data supports this significant relationship between ASD and familial autoimmune disorders (Micali et al., 2004), a subsequent and larger study did show a significant increase in the number of autoimmune disorders in ASD families even when compared to the families of children with a known autoimmune disorder (Sweeten et al., 2003). Evidence has also been provided for the increased prevalence of familial autoimmune disorders in the families of children with regressive ASD, compared to those without a history of regression (Molloy et al., 2006). Although limited by a dependence on self-report questionnaires for data collection, these familial studies have provided critical information on the association of autoimmune disorders with the onset of ASD. Thus, evidence for the role of immune dysfunction in this NDD is provided.

Many studies have documented immune dysfunction in ASD patients through the measurement of different inflammatory markers such as T cells, monocytes and immunoglobulins (Ashwood et al., 2006). This data has been collected from blood samples, on post-mortem analysis, as well as through neuroimaging of ASD patients. Evidence exists for the up-regulation of both inflammatory cytokines in the brain and serum (Naviaux et al., 2014), as well as microglial activation (Morgan et al., 2010).
However, care must be taken with regard to validity of these studies in consideration of the control groups, numbers of recruited patients, and replicability of results. The large number of reports showing statistically significant alterations in pro-inflammatory markers encourages both the argument for a role of the immune system in ASD and a call for replication of these studies to confirm the claimed effects (Cryan and O’Mahony, 2011; Bruce-keller et al., 2017).

Exploratory analysis on the cytokine profile of ASD patients has also been performed through the use of blood samples. These are easy to collect from patients and allow for a study of a wide variety of pro-inflammatory cytokines. Interleukin 6 (IL-6), interleukin 1 beta (IL-1β), interferon gamma (IFN-γ), and Tumour necrosis factor alpha (TNF-α) are amongst the most commonly studied cytokines in the profiling of ASD patients. For a more extensive review of cytokine profiles studied see Table 1 from Rose and Ashwood, 2014. In a reasonably large scale clinical study by Abdallah et al., (2012), evidence was provided for a hypoactive neonatal immune system in ASD children when a total of 16 different cytokine analytes were measured. Neonatal blood samples from 359 ASD cases revealed that IL-1β and IL-10 cytokine levels were significantly reduced compared to controls (Abdallah et al. 2012). Although the precise immunological pathways implicated in ASD are not fully elucidated, evidence to date provides a convincing argument particularly with regard to the role of cytokines.

The presence of an inflammatory profile in many ASD patients highlights this as an area for further research in order to identify specific subpopulations of patients. The evidence to date demonstrates that immune dysregulation may present a biologically meaningful immune-related pathophysiology for certain patient cohorts. In light of the current and broad diagnostic criteria, immune-markers might provide an additional diagnostic tool, alongside the behavioural phenotype.

1.3.3.2. The neuro-immune axis: a mechanism for immune dysfunction

Both the autonomic nervous system (ANS) and the hypothalamic–pituitary–adrenal (HPA) axis form bi-directional connections, providing a means for extensive communication between the immune system, brain and gut (Kraneveld et al., 2014). Kraneveld et al. (2014) demonstrate that both the afferent and efferent pathways may be involved in the immunomodulatory control of CNS disorders and implicated in disorder such as ASD where immune dysregulation is reported (Figure 5). The afferent nerve
pathway sees interaction of the immune system activation and CNS activity through both vagal and sympathetic nerve endings. The chronic release of immune markers such as cytokines and chemokines following immune system activation produces sickness behaviours. The efferent pathway enables the stress-triggered CNS response to have effects on peripheral immune function, highlighting an alternative route for neuroimmune system control. The HPA axis, responsible for the control and neuroendocrine adaptation to the stress response, is also susceptible to the action of proinflammatory cytokines. This adds additional lines of communication to the neuroimmune axis with the action of cytokines resulting in the hypothalamic release of corticotrophin-releasing factor (CRF) and adrenocorticotropic hormone (ACTH) from the pituitary. The subsequent glucocorticoid response can further influence the CNS. The close interactions between the immune, brain and gut systems, alongside the immune dysfunction typically seen in ASD, together support a potential neuroimmune hypothesis in its aetiology. The bi-directional communication between the brain and gut provides a potential mechanism for the GI abnormalities commonly seen within CNS disorders such as ASD.
Figure 5: **Neuro-immune-gut axis.** The bi-directional interaction of the gut microbiota with the CNS has been well documented. The disruption of the gut homeostasis may result in the manifestation of GI symptoms such as those seen in ASD. Gut-brain interactions are made up from both autonomic and enteric nervous systems (ANS and ENS respectively), as well as hypothalamic-pituitary-adrenal (HPA) axis connections. Vagal afferents represent a major pathway for communication from the gut environment to the brain which as a result can affect behaviour. Altered bacterial environment (release of toxins e.g. short chain fatty acids (SCFAs)) within the gut can affect intestinal permeability and immune responses via the release of substances such as toxic metabolites.
1.3.4. Environmental risk factors for ASD

A myriad of factors including environmental chemicals, heavy metals, pesticides, drug exposure, Vitamin D levels etc., have been implicated as risk factors for the development of ASD (Dietert et al., 2011; Lyall et al., 2014). It is likely that critical windows of exposure will exist in early development where the nervous system of the foetus is particularly vulnerable to changes in the intrauterine environment. Maternal stress has received a lot of attention as an environmental risk factor for various NDDs. Indeed, there is evidence that exposure to stressful life events including natural disasters, financial or family conflict or disaster, as well as maternal infection are associated with an increased risk of ASD (Beversdorf et al., 2005; Kinney et al., 2008; Class et al., 2014; Boyle et al., 2017). The focus of this discussion is given to the role of maternal infection.

1.3.4.1. Maternal infection

It has long been established that maternal infection during pregnancy can have adverse effects on the development of the foetus in utero and, specifically, increase the incidence of neurodevelopmental disorders (Gilmore and Jarskog, 1997; Patterson, 2009; Parker-Athill and Tan, 2010).

Observation of a link between maternal infection and behavioural disturbances in the offspring was made following the rubella epidemic in the U.S. the mid-1960s (Chess, 1971). Of the 243 children assessed for behavioural disturbances 10 were diagnosed as having ASD and 8 further children showed a partial ASD phenotype. In follow up reports, the incidence of ASD in these children from mothers with congenital rubella syndrome was estimated at an increased rate of 905 in 10,000 (Meltzer and Van de Water, 2017). In a recent report, it has been proposed that the rubella virus may affect up to 5% of pregnant mothers globally, so rubella may remain a key risk factor for ASD in certain cohorts of pregnant mothers (Hutton, 2016). Investigation into other viral and bacterial infections during pregnancy has shown that this maternal risk factor for ASD is not limited to the rubella virus. Indeed, mumps (Ciaramello and Ciaramello, 1995), urinary polyomavirus (Gentile et al., 2013), Herpes simplex virus (Libbey et al., 2005), influenza (Zhang et al., 2010; Atladottir et al., 2012; Brown, 2012) as well as bacterial infections (Zerbo et al., 2015) during or prior to pregnancy have been associated with an increased risk for ASD. A recent meta-analysis of both case-control and cohort studies
highlighted that infection during pregnancy is associated with a 12% increased risk of ASD which may have a large population impact given the commonality of infections in pregnant women (Jiang et al., 2016).

The diversity of infections that have been associated with the risk of NDDs supports the hypothesis that it is likely to be the activation of the immune system rather than the nature of the infection that is important. The common feature of all these infections is the effect they have on the maternal immune system. In support of this, animal models have been used to show that the effects of maternal infection on offspring can be produced in the absence of a live viral or bacterial infection. Specific focus here is given to the maternal immune activation (mIA) model (see section 1.4.1.2, p.61) using the viral mimetic polyinosinic–polycytidylic acid (poly (I:C)) or bacterial immune agent lipopolysaccharide (LPS). Shi et al., 2003, conducted a study whereby pregnant mice were exposed to either the live human influenza virus or to poly (I:C). They found that the behavioural deficits in prepulse inhibition (PPI) following the highest dose of poly (I:C) (20 mg/kg) mirrored those from the mice in the virally infected group. It was concluded that the anti-viral response alone from poly (I:C) exposure was enough to produce the behavioural changes in offspring. In addition, no detectable level of the virus was found in offspring brain samples suggesting that the virus did not have a direct effect on the fetal compartment (Shi et al., 2003, 2005). In non-human primates similar evidence has been presented with gestational exposure to influenza. Upregulation of maternal immunoglobulin G (IgG) was recorded in offspring alongside cortical brain defects, in the absence of the virus (Short et al., 2010). Although the exact mechanism of developmental disruption following maternal infection remains to be elucidated it is suggested that maternal immune affects the foetus without direct viral transfer, perhaps through elevation of cytokines and altered placentation (Hsiao and Patterson, 2011; Patterson, 2011a).

1.3.4.2. Mechanisms of mIA

Cytokines are essential immunomodulating agents implicated in the maternal immune response to infection and the subsequent onset of NDDs such as ASD. Rather than direct transmission of the infection, it has been shown that mIA causes an increase in maternal serum cytokine levels that are paralleled in the amniotic fluid, placenta and fetal brain (Urakubo et al., 2001; Beloosesky et al., 2006; Koga and Mor, 2008) and can
influence foetal development. The mechanism of this may be through direct action of cytokines on the neurons and glia of the fetal brain (Bauer et al., 2007). However, studies in vitro on delivered term placentas have yielded conflicting results with regard to placental transfer of cytokines. Whilst one study concluded no transfer of cytokines (TNFα, IL-6 and IL-1β) across the placenta (Aaltonen et al., 2005), a similar study using perfusion techniques reported a bi-directional transfer of IL-6 between mother and foetus but not TNFα or IL-1α (Zaretsky et al., 2004). Alternatively, increased cytokine levels in the placenta may lead to altered placental function and contribute to disrupted fetal development. Evidence for this hypothesis has been shown with changes to placental enzymes and peptides involved in the support of typical fetal development following exposure to LPS during pregnancy (Xu et al., 2006; Zhang et al., 2007). In addition, the placenta is capable of producing its own array of cytokines in response to inflammation and this may be the mechanism of production of inflammatory markers within the fetal compartment (Schwarz and Bilbo, 2011). Moreover, the transfer of maternal IgG is a well-known phenomenon that provides passive immunity to the newborn offspring. In addition, the placenta has been implicated in the transfer of maternal antibodies against fetal brain proteins (Dalton et al., 2003; Braunschweig et al., 2013). Due to the developing and vulnerable blood-brain barrier (BBB) during gestation, passage of the maternal IgG may occur and this may further underpin the developmental disruption resulting from mIA (Knuesel et al., 2014).

As a key driver of the downstream effects of poly (I:C) exposure, IL-6 has received particular attention. Indeed, it has been shown that direct IL-6 exposure during pregnancy can lead to similar behavioural deficits in adult offspring as seen in the poly (I:C) model. As a result of IL-6 exposure, high levels of IL-6 are measurable in the maternal serum, placenta and fetal brain (Smith et al., 2007). In the same study, Smith et al., 2007, used IL-6 KO mice to show the absence of behavioural deficits in offspring following poly (I:C) exposure at GD12.5. In addition to IL-6, work with poly (I:C) outside of the mIA model has been shown to induce upregulation of TNFα and IL-1β 6 hr post-injection regardless of neurodegeneration (Field et al., 2010). mIA using LPS has implicated TNFα and IL-1β in the immune response alongside IL-6. Levels of mRNA expression were elevated in the brain of both foetus and dam following administration of LPS and this effect was dose-dependent (Cai et al., 2000). In a similar study, Rousset et al., 2006, show this upregulation of inflammatory cytokine gene
expression in offspring brains at PD1 when LPS exposure was performed three days earlier in the pregnant dam. Cytokines such as TNFα can inhibit neural growth for both neurotransmitter systems (e.g. serotonin) (Jarskog et al., 1997) cortical (Marx et al., 2001) and hippocampal areas (Neumann et al., 2002). Thus, exposure to these proteins during vulnerable periods of development may disrupt critical neurodevelopmental processes.

The placenta is not only implicated in the effects of maternal infection with regard to the transfer and production of cytokines but also to its own development. An alteration in the integrity or function of the placenta may vastly impact the health of both mother and foetus (Beall et al., 2005). Simply, the failure of sufficient nutrient transfer to the foetus could impact growth, and growth restriction has been associated with significant developmental deficits (Leonard et al., 2007). Evidence also exists for a link between altered placental morphology and ASD. This is in relation to placental trophoblast inclusions (TIs) where children who are subsequently diagnosed with ASD show increased TIs (Anderson et al., 2007; Walker et al., 2013). Trophoblast cells form the outer layer of a blastocyst and are involved in providing nutrients to a developing embryo and subsequently develop into the placenta. TIs occur with abnormal growth or folding of the placenta and have been linked to the onset of mild abnormal gestation and pregnancy loss (Walker et al., 2013). Not only does this evidence provide a potential early screen for ASD diagnosis in specific cases, it also highlights that altered placental pathology is present in ASD cases and may result in deficient maternal-foetal regulatory processes. Further research is required to determine the mechanism by which TIs are associated with ASD risk. It has been proposed that further investigation into developmental pathways regulating cell proliferation as well as hormone secretion will aid our understanding (Walker et al., 2013).
1.4. Animal models

Animal models are an integral tool for preclinical research in ASD with the goal of producing a phenotype in an animal that is accurately translatable to the symptoms and/or pathology seen in the clinic. In achieving this, it may be possible to perform retrospective analysis of changes to brain chemistry and neural connections, thus furthering our understanding of a disorder and developing new drug treatments.

Commonly, rodents (typically mice) have been employed for disease modelling given their low cost and availability. In the area of ASD research, some groups have successfully developed animal models using non-human primates (Bauman et al., 2014; Machado et al., 2015). The use of such advanced mammals, particularly with regard to assessing social behaviours, is arguably more beneficial and translatable to the human. In a recent longitudinal study in the rhesus monkey, elevated cytokine levels were recorded in the first and fourth year of life alongside stereotyped behaviours highlighting the clinical relevance of such NDD models (Rose et al., 2017). However, these models are far more time consuming, and both ethically and financially unfavourable. As a result, it is unlikely that the wide-scale application of such models would be feasible on a large-scale.

The complex and multifactorial nature of ASD dictates that one single animal model will never reproduce the entire spectrum of behavioural and neuropathological features of this disorder. Thus, multiple animal models have been developed and validated. Those currently in use can be broadly divided into genetic and environmental models (Gadad et al., 2013). As already discussed, the genetic focus in ASD research has resulted in the production of many animal models for ASD based on genetic alterations (e.g. chromosomal deletions, specific human gene knockout (KO)/knockin (KI)). A vast array of genetic KO models are available and are well validated, particularly in mice (Silverman et al., 2010; Provenzano et al., 2012) but also more recently in rats (Hamilton et al., 2014). These have provided significant insight into the role of specific ASD-related genes and the underlying molecular mechanisms.
1.4.1. Environmental models of ASD

1.4.1.1. Drug exposure

Environmental models have developed from clinical evidence suggesting a link between specific environmental factors that can influence the maternal environment and result in a risk for a NDD such as ASD. One such model that has been used extensively for ASD modelling in both rats and mice involves the exposure to the anti-epileptic drug (AED) valproate (VPA) during early pregnancy (Schneider et al., 2005; Yang et al., 2011; Favre et al., 2013; Roullet et al., 2013; deTheije et al., 2014a, 2014b; Lucchina and Depino, 2014; Sabers et al., 2014; Vinikoor-Imler et al., 2014; Hara et al., 2015). Evidence for the risk of VPA exposure and subsequent neural tube defects, developmental delay as well as other congenital malformations came from following birth outcomes of mothers prescribed the AED for seizure control that was maintained during pregnancy (Shallcross et al., 2011; Tomson and Battino, 2011).

The most commonly used VPA model involves a single injection of VPA (typically a high dose of 600-800 mg/kg) at GD12.5 and has been shown to induced ASD-like behaviours which show face validity to the human condition (Schneider et al., 2005). These behavioural changes include disruption to social interaction and recognition as well as the appearance of repetitive or stereotypic behaviours (i.e. increased marble burying and line crossings in the open field test OFT) (Schneider et al., 2005; Choi et al., 2016a). Evidence shows that the timing of VPA administration within pregnancy is critical, with reduced effects on social behaviour with late administration as well as increased teratogenic effects with early exposure around GD9 (Kim et al., 2011). VPA exposure has been shown to produce anatomical and molecular changes relevant to the pathophysiology of ASD such as increased cortical thickness in rats (Sabers et al., 2014), changes to γ-Aminobutyric acid (GABA) levels (Deidda et al., 2014) and dysregulation of gene expression (Jergil et al., 2009). Whilst the exact mechanism of action of this AED remains to be determined it is likely that the teratogenic properties of VPA leading to developmental neuronal toxicity will have detrimental effects on the developing foetus. It has been suggested that folic acid deficiency, oxidative stress, and inhibition of histone deacetylases may all contribute to the underlying mechanism (Ornoy, 2009).
1.4.1.2. Maternal immune activation

As previously discussed, epidemiological studies show that infection during pregnancy can increase the risk of NDDs in the offspring. It is this hypothesis that has led to the development of the mIA model in rodents and non-human primates. Indeed, work on mIA modelling began with a focus on schizophrenia and subsequently moved to include ASD (Knuesel et al., 2014). The focus of this discussion is given to a similarly well-known environmental model for NDDs such as ASD.

mIA involves the administration of an immune challenge to a dam (the pregnant female) at a specific gestational time point. The first of these studies saw the administration of the human influenza virus to mice. Offspring from treated dams showed deficits in PPI and social behaviour (Shi et al., 2003) highlighting the relevance of this insult to traits of NDDs. The findings from this pioneering study have subsequently been supported with exposure to a mouse-adapted influenza virus at GD9 that caused neurochemical changes, brainstem inflammation and behaviours relevant to ASD (Miller et al., 2013). More commonly, mIA is achieved through the use of immune activating agents such as lipopolysaccharide (LPS) (Borrell et al., 2002; Jonakait, 2007; Patterson, 2009) or the viral mimetic poly (I:C) (Onore et al., 2014; Meehan et al., 2017).

1.4.1.3. LPS and poly (I:C)

Administration of these different immune stimulating agents have yielded similar and overlapping results (Patterson, 2009). However, the administration of LPS or poly(I:C), not only provides increased biosafety over active viruses, but also a controlled and limited immune response (between 24-48 hr) (Meyer et al., 2009). As a synthetic double-stranded RNA (dsRNA) analogue, poly (I:C) acts as a ligand at the toll-like receptor 3 (TLR3). TLR3 plays a critical role in the innate immune response and activates pathways that result in the secretion of inflammatory markers (Alexopoulou et al., 2001; McCartney and Colonna, 2009) (Figure 6). The action of poly (I:C) at the TLR3 is dependent on both the molecular weight of the poly (I:C) and the cell type (Zhou et al., 2013). In contrast, LPS produces an innate immune response that is akin to gram negative bacterial infection and acts via TLR2 and 4 (Takeuchi and Akira, 2007) (Figure 6). Both of these compounds successfully elicit the controlled secretion of immune markers such as IL-6, IL-1β, TNFα, IFNγ which can be dependent upon the strain of animal used and GD of mIA exposure (Connor et al., 2012; Babri et al., 2014).
Although this is a significant advantage in enabling pre-clinical mIA work, the specific cytokine secretion following LPS or poly (I:C) is also a limitation of these models. Infections such as influenza elicit an extensive innate and adaptive immune response and, as such, the translatability of mIA using LPS or poly (I:C) is arguably limited. In spite of this, both LPS and poly (I:C) have been validated for use in a model of mIA producing phenotypes relevant to ASD (Taylor et al., 2012; Kirsten et al., 2015; Reisinger et al., 2015). For this project focus was given to investigating the effects of a viral rather than a bacterial infection and therefore the poly (I:C) model was chosen.

Figure 6: Innate response to immune activation elicited by LPS (gram negative bacteria) and poly (I:C) (dsRNA mimic). The downstream immune response elicits a number of inflammatory cytokines and other immune markers. Key: CD14 = cluster of differentiation 14, TRIF/TRAM = Toll-like receptor adaptor molecules, TIRAP = Toll-interleukin 1 receptor (TIR) domain containing adaptor protein, MyD88 = Myeloid differentiation primary response gene 88, IRAK = interleukin-1 receptor-associated kinase, TRAF = TNF receptor-associated factor, JNK = c-Jun N-terminal kinase, TKB1 = a tyrosine kinase, AP1 = activator protein 1 transcription factor, NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells transcription factor, IRF3 = interferon regulatory factor 3 transcription factor. (Figure taken from Schwarz and Bilbo, 2011)
1.4.1.4. mIA induction

In order to assess the effects of mIA in the dams, the peripheral inflammatory response (e.g. inflammatory cytokine release) can be monitored alongside any obvious sickness behaviour (e.g. fever) or weight loss that ensues following acute exposure (Fortier et al., 2004; Cunningham et al., 2007). As previously discussed IL-6 has been highlighted as critical for the induction of mIA in rodents. As a result, IL-6 has been used as a determinant of the success of mIA induction. However, the more recent publications neglect this measurement preventing the comparison of cytokine upregulation between different mIA models including those using a different gestational timing or strain/species of rodent. In some mIA models, the weight loss following mIA is considered a critical inclusion factor for a dam in the study (Bronson et al., 2011). Elsewhere body weight data is omitted from publication, if recorded at all. Notably, it has been shown that despite the transient effect on weight, poly (I:C) does not affect gestation period or litter size (Zuckerman and Weiner, 2005). It is clear that there is a considerable lack of standardisation between models of mIA.

The dose of the immune activating agent chosen in mIA models ranges between 5-20 mg/kg. Interestingly, even with a reasonably low dose of poly (I:C) (750 µg/kg) a fivefold increase in plasma IL-6 concentration was seen at 2 hour (hr) post injection and a similar trend seen for the expression of TNF-α (Fortier et al., 2004). The dose range of poly (I:C) is critical since a sufficient immune response must be achieved without using an excessively high or repeated dose that will cause death of the pups (Gilmore et al., 2005). Furthermore, differences in the route of dosing can affect the chosen concentration with both intravenous (i.v.) and intraperitoneal (i.p.) routes of administration commonly used in both mice and rats. In addition to different dose routes, mIA has been characterised at a number of gestational time-points. Broadly speaking, these capture early/middle and late gestation periods (GD9-12 and GD15-19 respectively). These gestational time points in the rodent are considered to roughly correspond to the end of the first trimester and middle-to-late second trimester of human pregnancy (Clancy et al., 2001; Workman et al., 2013). The NDD in focus is used to determine the GD that is chosen for mIA induction typically GD9-12 in relation to ASD and GD15-19 for schizophrenia. Following on from work in the valproate model of ASD (see Roullet et al., 2013 for review), GD12.5 has been commonly chosen for modelling ASD-like behaviours in mice (Ito et al., 2010; Garay et al., 2013; Hsiao et al.,
2013; Schwartzer et al., 2013; Lombardo et al., 2017). However, to date, no mIA models have been established at this time-point in rats. Considerable overlap can be seen in behavioural and pathophysiological phenotypes induced at different gestational points and, coupled with this, no mIA model will successfully capture the entire phenotype of a specific disorder. As a result, definitions of which gestational mIA model corresponds to a specific disorder should be made tentatively.

1.4.1.5. Behavioural testing in offspring

A number of behavioural tests are used with the mIA model to determine the validity of comparison to human NDDs (Patterson, 2011b). For ASD these centre on the three core behavioural abnormalities recorded in patients (social behaviour, communication and repetitive behaviour). Table 3 outlines the tests typically used for mice and rats across various models of mIA.

There are now a wealth of publications that explore the mIA and the effects on the pups post-partum. Given the manifestation of ASD symptoms in humans within early development it is critical that we reflect this in the testing that is performed on the pups within rodent studies. Unfortunately, this is an aspect that is lacking from a number of publications where testing is only performed in adulthood (Meyer et al., 2005; Zuckerman and Weiner, 2005). However, this does provide evidence of the lasting behavioural effects of an acute gestational insult, thus demonstrating the strong neurodevelopmental effect.

An elegant study performed by Malkova et al., 2012 explored the effect of repeated early gestational exposure to poly (I:C). Mice were challenged three times throughout early pregnancy (GD10.5, 12.5, 14.5) with a 5 mg/kg dose of poly (I:C). A robust behavioural test battery was applied highlighting the three specific symptom domains implicated in ASD (social behaviour and communication as well as repetitive/stereotyped behaviours), although only male mice were tested. This is a limitation found in a number of preclinical studies for ASD. A significant reduction in the number and duration of USVs produced during the isolation test, alongside decreased social preference in the three-chamber social interaction test demonstrated a disruption to typical sociability in these animals. Mice also exhibited increased levels of repetitive/stereotypic behaviours measured in marble burying and self-grooming tests (Malkova et al., 2012). Similar test batteries have been applied across a number of
studies supporting the finding of ASD-like behavioural deficits following mIA (Hsiao et al., 2012; Garay et al., 2013; Giulivi et al., 2013; Schwartzer et al., 2013; Wu et al., 2015; Kirsten and Bernardi, 2017). Notably a very recent study of mIA in mice shows a rare example of a robust behavioural test battery with sufficient $n$ numbers and the use of both male and female offspring for determination of an ASD-like phenotype (Pendyala et al., 2017). Specifically, offspring showed a significant disruption to social interaction in the three chamber approach task, reduced social communication as measured by USVs in addition to increased stereotyped behaviour in the marble buying test (Pendyala et al., 2017).
Table 3: A general description of the behavioural tests applied in the characterisation of different mIA animal models for ASD. References are listed where these have been used to highlight ASD-like phenotypes in offspring.

<table>
<thead>
<tr>
<th>Neurological domain</th>
<th>Experimental test</th>
<th>ASD symptom</th>
<th>Typical test description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anxiety behaviour</strong></td>
<td>Open field test (OFT)</td>
<td>Increased presence of anxiety disorders</td>
<td>Exploratory behaviour of animal is measured in a novel arena</td>
<td>(Shi et al., 2003) (Meyer, 2006)</td>
</tr>
<tr>
<td></td>
<td>Elevated plus maze (EPM)</td>
<td></td>
<td>Exploratory behaviour of the animal is measured in two armed maze one with open and one with closed arms</td>
<td>(Crawley, 2012) (Dachtler et al., 2014) (van Steensel et al., 2011)</td>
</tr>
<tr>
<td><strong>Stereotypy and preservative behaviour</strong></td>
<td>Repetitive digging, burrowing and/or grooming</td>
<td>Restricted repetitive behaviour</td>
<td>Spontaneous digging, burrowing or grooming measured in the animal (sometime during OFT)</td>
<td>(Weir et al., 2015) (Schwartz et al., 2013)</td>
</tr>
<tr>
<td><strong>Sensorimotor gating</strong></td>
<td>Prepulse inhibition (PPI)</td>
<td>Impaired</td>
<td>Measurement of the startle response in the animals. A weak pre-stimulus is used to transiently inhibit the response to a closely following strong sensory stimulus (e.g. auditory stimulus)</td>
<td>(Perry et al., 2007) (Smith et al., 2007) (Meehan et al., 2017) (Giovanoli et al., 2016)</td>
</tr>
<tr>
<td><strong>Social behaviour</strong></td>
<td>Social interaction test (SIT)</td>
<td>Reduced/impaired</td>
<td>Social interaction or recognition tested in three chamber social approach test</td>
<td>(Moy et al., 2006) (Crawley, 2012) (Foley et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>Social communication (e.g. ultrasonic vocalisations)</td>
<td></td>
<td>Production of USVs measured in isolation test from dam in early development or during social interaction test</td>
<td>(Hsiao et al., 2013) (Malkova et al., 2012) (Bauman et al., 2014)</td>
</tr>
</tbody>
</table>
1.5. General summary and objectives

It is evident that ASD is a complex and heterogeneous disorder with a high incidence and significant comorbid conditions. It is likely that environmental risk factors in combination with genetic or epigenetic susceptibilities are critical for the development of abnormalities leading to ASD. In addition, the type, timing and intensity of an environmental insult may determine the extent of disruption during development and how many physiological systems that are affected as a result. Indeed, both neurological (central and peripheral) and immunological systems may be perturbed in ASD patients resulting in the variety and spectrum of phenotypes that are observed. As such, a “variable insult hypothesis” (Goyal and Miyan, 2014) has been proposed to underlie ASD aetiology. The indication of an inflammatory state found in ASD patients and the influence of mIA on subsequent development of the condition promotes this line of investigation within ASD aetiology. The flow diagram in Figure 7 outlines this hypothesis in relation to the immune insult in pregnancy and shows how this might result in a condition such as ASD including the effects on the GI system.

In order to determine the relevance of this hypothesis, adequate preclinical and clinical studies are required with the inclusion of adequate subject numbers, inclusion of both sexes and controls such that reliable conclusions are reached. Pre-clinical animal models relevant to ASD must be targeted towards the human phenotype with multiple models developed to encompass the wide spectrum of behavioural and molecular phenotypic characteristics.

With regard to mIA, more research is required to further understand the mechanisms underlying this phenomenon as well as producing standardised protocols across mIA studies. As a result, it will be possible to determine which rodent models are most appropriate for studying different aspects of complex NDDs. In addition, there is a clear requirement for validation of these models in rats. These studies must employ both male and female rats and an extensive battery of behavioural tests so that accurate assessment of the induced behavioural phenotype and associated pathological disturbances is possible.
Figure 7: Outline of the neuroimmune-gut hypothesis for ASD. mIA in the mothers results in inflammatory feedback that can affect the developing foetus. The subsequent disruption to neurodevelopment may lead to chronic sickness behaviours that manifest as the behavioural and gut phenotype seen in ASD. (Figure adapted from MRes dissertation, R. Treleavan).
1.6. Thesis objectives

The overall objective of this project was to develop and validate a translational neurodevelopmental model relevant to ASD using mIA via the administration of poly (I:C). This will be achieved through molecular brain analyses alongside behavioural analysis in both sexes of offspring. A further aim was to determine the presence of any gastrointestinal disturbance in the offspring of mothers subjected to mIA and whether this resulted in functional changes to the gut. Specific objectives are listed below and detailed aims and objectives can also be found at the beginning of each chapter.

- Analyse the acute effect of mIA at GD12.5 in pregnant Wistar rats in an attempt to develop standardised measures of effective mIA induction.
- Determine effects of mIA on foetal development using morphological parameters and _ex vivo_ tissue analysis of brain samples targeting specific genes of interest.
- Determine effects of maternal immune challenge on early postnatal development using morphological parameters and _ex vivo_ tissue analysis on brain samples targeting specific genes of interest.
- Establish effects of mIA at GD12.5 on the gut microbiota of offspring from the mIA model
- Develop a robust behavioural test battery to enable adequate testing of ASD-like behaviours in offspring – social behaviour, repetitive/stereotypic behaviour and communication.
- Use this test battery to determine the longitudinal effect of GD12.5 poly (I:C) administration on behaviour of adolescent and adult offspring of both sexes.
Chapter 2: Establishing mIA methodology
2.1. Introduction

Previous studies using mIA to model NDDs show that differences in dose, strain and route of administration exist (de Souza et al., 2015; Giovanoli et al., 2015; Vernon et al., 2015). These differences may contribute to behavioural and neurochemical variations observed in the offspring. Whilst differences in methodology exist, the rationale for choosing these is rarely justified. Therefore, prior to developing a mIA model of relevance to ASD, it was necessary to determine the optimum dose and strain of rat to use in these studies.

2.2. Materials and methods

2.2.1. Animals

A total of 110 non-pregnant adult rats were used to validate the effects of mIA in a genetically outbred strain across differences doses of poly (I:C) ($n=38$ Wistar, $n=36$ Lister Hooded and $n=36$ Sprague-Dawley) (Charles River Laboratories, UK). This $n$ number was used to enable statistically significant changes to be calculated (based on a power analysis previously performed in the lab). For the induction of mIA during pregnancy only Wistar rats were used ($240-260$ g). Rats were mated in house using male Wistar rats ($540-620$ g). The $n$ numbers of male and female rats used in each study are listed in the materials and methods section of each chapter. Animals were housed in groups or pair-housed (during pregnancy, singly housed from GD19) in individually ventilated cages with two levels (GR1800 Double-Decker Cage, Tecniplast, UK, Figure 8) and under standard conditions at 19-23 °C and 45-65% relative humidity. Rats were maintained on a 12 hour (hr) light:dark cycle (lights on 07:00, lights off 19:00), with free access to standard rat chow (Special Diet Services, UK) and water. All animal procedures were performed under appropriate United Kingdom Home Office licenses (project licence: PPL: 70/8550, personal licence granted to J. Oladipo: PIL: I5A2E2729) in accordance with the UK Animal’s Scientific Procedures Act (1986) and was approved by the University of Manchester’s Animal Welfare and Ethical Review Body (AWERB). The NC3Rs ARRIVE Guidelines were used to design and report the findings of the studies presented in this thesis.


2.2.2. Dose and strain validation

Acute systemic inflammation was induced in female Wistar, Lister Hooded and Sprague Dawley rats (182-281 g; 10-weeks old) with saline or polyinosinic-polycytidylic acid (poly (I:C)) at 5, 10 or 15 mg/kg; P9582 (Sigma, UK) (n= 8-11/ treatment group) using the intraperitoneal (i.p.) route. The 500 mg Sigma poly (I:C) was supplied with 10% active nucleotide in the total weight of potassium salts. To achieve the correct concentration (5, 10 and 15 mg/kg) of poly (I:C), dosing calculations were performed based on the weight of active poly (I:C) content not including potassium salts.

2.2.3. Assessment of systemic inflammation

To assess the extent of acute systemic inflammation, body weight (BW) (Adventurer Pro Balance, AV2101) and core body temperature (CBT) were recorded prior to injection (baseline), 3, 6 and 24 hr post-injection. CBT was measured using a rectal thermistor probe (TES, China). At these same time points, all animals were monitored for the presence of sickness behaviours (i.e. piloerection, lethargy and hunched posture).

Figure 8: Double-Decker Cage, Tecniplast. A) Typical home cage set up with freely available food and water. Cage enrichment was included as a cardboard tube and sizzle nest bedding. B) Home cage without lid showing the shelf.
At 3 hr post-injection, blood was sampled via the lateral tail vein. Rats were placed in a heat-box for 8 min to increase peripheral blood flow to the tail (Figure 9A). Rats were carefully removed and then restrained using a plastic rat restrainer (Vet Tech Solutions Ltd) (Figure 9B). A 21 gauge (G) butterfly needle was inserted into the left or right lateral tail vein and blood collected into a tube with citrate (3.8% in distilled water). If the first entry was unsuccessful, the needle was removed and a new needle inserted into the vein on the opposite side of the tail. No more than two attempts were made at this procedure to prevent the female being held in the restrainer for a long period of time. This procedure took approximately 2 min per animal. After centrifugation for 10 min (13,000 x g, RT), plasma was collected and stored at -20 °C for further analysis.

Figure 9: Equipment and experimental design for assessment of acute systemic inflammation. A) The hotbox used to increase circulation prior to tail vein blood sampling. B) Rat restrainer used during the tail vein procedure.
2.2.4. Enzyme-linked immunosorbent assay (ELISA)

The concentration of IL-6, TNFα and IL-1β in blood plasma was determined using validated rat-specific DuoSet ELISA kits (IL-6 DY506, TNFα DY510, IL-1β DY501 R&D Systems Abingdon, UK). These three cytokines are typically used to monitor the inflammatory state in pregnant mothers following infection as well as in ASD patients and thus were chosen for investigation in these studies. The ELISA kits used employed the avidin-biotin system for detecting proteins in an indirect manner (see Figure 10).

Figure 10: Diagram of the mechanism employed in the ELISA to detect levels cytokines in plasma. The capture antibody (cAb) is used to coat and detect the specific antigen of interest. Then a biotinylated detection primary antibody (dAb) is used to bind the antigen. The addition of Streptavidin forms a complex with the biotin and, when the substrate solution is added, a colour change occurs that can be used for absorbance measurements.

The capture antibody (cAb) for each kit was reconstituted according to manufacturer’s instructions. For IL-6 and TNFα, 720 µg/ml of mouse anti-rat IL-6 or TNFα was reconstituted with 1.0 ml 1x PBS and diluted to a working concentration of 4.0 µg/ml (30 ul cAb in 5370 ul 1x PBS). For IL-1β 144 µg/ml of goat anti-rat IL-1β was reconstituted with 1.0 ml 1x PBS and diluted to a working concentration of 0.8 µg/ml (30 ul cAb in 5370 ul 1x PBS). The diluted cAb was used to coat the Nunc immuno plate (Thermo Scientific) with 50 ul per well. The plate was covered with Parafilm (Bio-Tek) and left to incubate overnight. Blocking was performed the following morning using reagent diluent (RD) (1% BSA in 1x PB), 200 ul per well and left to incubate for 1 hr.
Standards were added to the plate in duplicate (see Table 4, Table 5 and Table 6 for range of standards for IL-6, TNFα and IL-1β ELISAs respectively) and samples were added to single wells, both at 50 ul per well. A negative control was included where distilled water (dH2O) was added in place of the sample. The plate was covered and incubated for 2 hr. The detection antibodies (dAb) were prepared as follows. For IL-6, 72 ug/ml of biotinylated goat anti-rat IL-6 was diluted to a working concentration of 400 ng/ml in RD (30 ul dAb in 5370 ul RD); for TNFα, 40.5 ug/ml of biotinylated goat anti-rat TNFα was diluted to a working concentration of 225 ng/ml in RD (30 ul dAb in 5370 ul RD); for IL-1β 63 ug/ml of biotinylated goat anti-rat IL-1β was diluted to a working concentration of 350 ng/ml in RD with 2 % heat inactivated normal goat serum (NGS) (30 ul dAb in 108 ul NGS and 5262 ul RD) which was prepared 1 hr prior to incubation on the plate. The dAb was added at 50 ul per well and incubated for 2 hr. Following this Streptavidin-HRP was added at 50 ul per well. The plate was covered and incubated for 20 minutes (min) with minimal light exposure. The substrate solution (1:1 reagent A and reagent B, R&D DY999) was added, at 50 μl per well and incubated for a further 20 min. The final stop solution was then added (H2SO4), 50 ul per well before the absorbance was measured.

All incubations were performed at room temperature (RT) in the dark on a plate shaker. With the exception of the addition of the stop solution (H2SO4) between each step the plate was washed 3 times with a wash solution (0.05% Tween20 in 1x PBS). Absorbance was measured at 450 nm using a plate reader (MRX, Dynatech, UK) at RT, and sample concentrations were calculated using a four parameter logistic (4PL) curve fit in GraphPad Prism 7 (GraphPad Software, USA) see Figure 11. For statistical analysis, any samples with an undetectable level of the cytokine were assigned a value of 0 pg/ml.

2.2.5. Statistical analysis

Unless otherwise specified, data are presented as mean ±SEM calculated using GraphPad Prism 7 (GraphPad Software USA). The homoscedasticity (Levene’s test) and normality (Shapiro-Wilk test) of each data set were tested. Unpaired t-test or Mann-Whitney test were used for single comparisons for parametric and non-parametric data respectively. One-way or two-way ANOVA followed by Bonferroni correction for
multiple comparisons was used for parametric data or Kruskal–Wallis test followed by Dunn test for multiple comparisons for non-parametric data.

**Table 4: Range of standards used for the IL-6 ELISA**

<table>
<thead>
<tr>
<th>Standard</th>
<th>Volume Standard (µl)</th>
<th>Reagent Diluent (µl)</th>
<th>Concentration (pg/ml)</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>5.4</td>
<td>494.6</td>
<td>8000</td>
</tr>
<tr>
<td>B</td>
<td>250 A</td>
<td>250</td>
<td>4000</td>
</tr>
<tr>
<td>C</td>
<td>250 B</td>
<td>250</td>
<td>2000</td>
</tr>
<tr>
<td>D</td>
<td>250 C</td>
<td>250</td>
<td>1000</td>
</tr>
<tr>
<td>E</td>
<td>250 D</td>
<td>250</td>
<td>500</td>
</tr>
<tr>
<td>F</td>
<td>250 E</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>G</td>
<td>250 F</td>
<td>250</td>
<td>125</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>250</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 5: Range of standards used for the TNFα ELISA**

<table>
<thead>
<tr>
<th>Standard</th>
<th>Volume Standard (µl)</th>
<th>Reagent Diluent (µl)</th>
<th>Concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>234</td>
<td>4000</td>
</tr>
<tr>
<td>B</td>
<td>120 A</td>
<td>120</td>
<td>2000</td>
</tr>
<tr>
<td>C</td>
<td>120 B</td>
<td>120</td>
<td>1000</td>
</tr>
<tr>
<td>D</td>
<td>120 C</td>
<td>120</td>
<td>500</td>
</tr>
<tr>
<td>E</td>
<td>120 D</td>
<td>120</td>
<td>250</td>
</tr>
<tr>
<td>F</td>
<td>120 E</td>
<td>120</td>
<td>125</td>
</tr>
<tr>
<td>G</td>
<td>120 F</td>
<td>120</td>
<td>62.5</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>120</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 6: Range of standards used for the IL-1β ELISA**

<table>
<thead>
<tr>
<th>Standard</th>
<th>Volume Standard (µl)</th>
<th>Reagent Diluent (µl)</th>
<th>Concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6.4</td>
<td>343.6</td>
<td>4000</td>
</tr>
<tr>
<td>B</td>
<td>175 A</td>
<td>175</td>
<td>2000</td>
</tr>
<tr>
<td>C</td>
<td>175 B</td>
<td>175</td>
<td>1000</td>
</tr>
<tr>
<td>D</td>
<td>175 C</td>
<td>175</td>
<td>500</td>
</tr>
<tr>
<td>E</td>
<td>175 D</td>
<td>175</td>
<td>250</td>
</tr>
<tr>
<td>F</td>
<td>175 E</td>
<td>175</td>
<td>125</td>
</tr>
<tr>
<td>G</td>
<td>175 F</td>
<td>175</td>
<td>62.5</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>350</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 11: Example standard curves. Standard curves were generated using GraphPad Prism 7 for ELISA data. A) IL-6 B) TNFα C) and IL-1β.
2.3. Results

The aim of this pilot work was to determine whether strain and dose have an effect on the response to acute systemic inflammation using poly (I:C) in non-pregnant rats. This would enable the choice of the most suitable strain and dose to optimise the model of mIA.

For this, saline or poly I:C at 5, 10 or 15 mg/kg was injected via the i.p. route to adult female Wistar, Lister Hooded and Sprague Dawley rats. Elevations in the pro-inflammatory cytokine IL-6 (Figure 12A-C) and core body temperature (Figure 12D-F) were observed to a different extent across rat strains. 10 mg/kg i.p., poly I:C induced a significant elevation in plasma IL-6 in all three strains tested ($p<0.05$-$p<0.001$; Figure 12A-C). Elevated IL-6 was most consistently observed with the lowest spread of data in Wistar rats (standard deviation (SD) 121.8 Wistar; 208.3 Lister Hooded; 307.0 Sprague Dawley; Figure 12A-C).
Figure 12: Acute effects of systemic inflammation in adult female rats. A-C) Concentration of IL-6 in blood plasma. D-F) Core body temperature (CBT). Graphs represent group mean ±SEM, n= 8-11 females/treatment group.
A lower, but still significantly elevated, concentration of TNFα was observed 3 hr post-injection of poly (I:C) (Figure 13). This significant elevation was seen in Wistar and Sprague Dawley (p<0.05) (Figure 13A and C), but not Lister Hooded female rats. Only the 10 mg/kg i.p. dose of poly (I:C) was sufficient to cause this effect. No elevation of IL-1β was observed in these rats across any strain or dose (data not shown). Similarly, female animals across all strains did not show any difference in BW following exposure to poly (I:C) (data not shown).

All of the female rats were monitored for sickness behaviour following exposure to the doses of poly (I:C). These included reduced movement in the home cage (lethargy), piloerection or hunched posture. None of the female rats injected with doses 5-15 mg/kg poly (I:C) showed any obvious sickness behaviour.

2.4. Conclusion

IL-6 is a key mediator of mIA (Smith et al., 2007; Choi et al., 2016b) and due to the consistency of IL-6 response alongside TNFα upregulation and increased CBT recorded in Wistar rats, this strain was used for all of my subsequent experiments presented in this thesis. Chapters 3-5 outline the work I conducted to validate a model of mIA for ASD using Wistar rats and exposure to 10 mg/kg i.p. poly (I:C) at GD12.5. The standard methods developed from this pilot work and used in each chapter for the induction of pregnancy and mIA are listed below with a timeline shown in Figure 14.
Figure 13: Acute effects of systemic inflammation in adult female rats on elevation of TNF\(\alpha\). Graphs represent group mean ±SEM, \(n=8\)-11 females/treatment group (* vs. saline * \(p<0.05\)).
2.5. General methods for mIA induction

2.5.1. Time-mating

All rats were handled daily for one week prior to mating in order to habituate the animals to the experimenter. Both male and female animals were weighed and then placed in a prepared mating cage. The mating cages were standard double-decker home cages with the shelf removed. The floor of the cage was covered with a sheet of black card and a raised metal grid with the food hopper placed directly onto the metal grid. Female and male rats were housed together until the appearance of a vaginal plug on the black floor-lining or for a maximum of 5 days to prevent any adverse effects of housing on the metal grid. GD1 was confirmed by the appearance of a vaginal plug. On this day, female and male animals were separated and re-housed. Male animals used for mating were returned to their original cage mates and not prepared for mating again for at least 24 hr. The dams were housed in a fresh cage. Multiple females were prepared for mating at the same time resulting in at least two pregnant dams being housed together at GD1. In an attempt to reduce stress in early pregnancy, dams were left undisturbed until GD8. From this point onwards all females were weighed daily between 08:00-09:00. At GD21 dams were housed separately to ensure that they gave birth in isolation. All of the dams in this study gave birth on GD23 and were not disturbed on this day. This was the first pregnancy for all dams included in this study.

2.5.2. mIA at GD12.5 in pregnant rats

On GD12.5 pregnant rats were injected intraperitoneally (i.p.) with 10 mg/kg i.p. poly (I:C) (Potassium salt, Sigma P9582) or 0.9 % saline using a 26G needle. The needle was aspirated following insertion and prior to injection to ensure correct placement of the needle into the intraperitoneal space. GD12.5 approximately corresponds with human gestational week 7 (mid-first trimester) in terms of cortical neurogenesis (Workman et al., 2013) (http://translatingtime.net/translate). This gestational time point was selected based on previous studies showing that prenatal poly (I:C) exposure during this gestational period causes behavioural and neuroanatomical abnormalities relevant to NDDs in mice.

Dosing was performed between 12:00-12.30 pm in the animal unit procedure bays. Animals were brought into the procedure room 30 min prior to injection to acclimatise
to the new environment. The i.p. route of administration was chosen because it allowed minimal restraint to be applied to the pregnant dams (in an attempt to limit stress exposure) and has been previously validated using poly (I:C) (Forrest et al., 2012; Giulivi et al., 2013; Ratnayake et al., 2014). The poly (I:C) and saline solutions were prepared and aliquoted into individual Eppendorf tubes (400 ul /tube) and placed in the freezer in labelled bags. Ahead of GD12.5, another experimenter in the team labelled the two treatments A and B and kept a record of the corresponding substance. Pregnant animals were then randomly allocated to either treatment group (A or B) and drug administration was performed in a blinded manner on GD12.5.

To assess the extent of mIA in dams, BW and CBT were recorded prior to injection (baseline), 3, 6 and 24 hr post-injection as for non-pregnant rats above. CBT was again measured using a rectal thermistor probe (TES, China). At these same time points, all animals were monitored for the presence of sickness behaviours (i.e. piloerection, lethargy and hunched posture). At 3 hr post-injection, blood was sampled via the lateral tail vein and the plasma used to perform an ELISA to determine levels of IL-6, TNF-α and IL-1β, all procedures were performed as described above.

**Figure 14:** Experimental design for induction of maternal immune activation at GD12.5 (i.p. = intraperitoneal, CBT = core body temperature).
2.5.3. **Data collection and statistical analysis**

Unless otherwise specified, data are presented as mean ±SEM calculated using GraphPad Prism 7 (GraphPad Software USA). In order to draw meaningful conclusions from the validation of this model, standard deviation (SD) where stated was also used to assess the variability within treatment groups or litters. This is an aspect that is typically lacking in published biomedical work (Barde and Barde, 2012). The homoscedasticity (Levene’s test) and normality (Shapiro-Wilk test) of each data set were tested. Unpaired Student’s t-test or Mann-Whitney test were used for mean comparisons of parametric and non-parametric data respectively.

2.5.3.1. **Maternal immune activation**

For comparisons of GD12.5 acute response data (e.g. weight, CBT), repeated measures ANOVA was used with post-hoc Bonferroni correction for multiple comparisons. For comparison of litter numbers and gestational weights a two-way ANOVA was applied (factors treatment, sex, and GD respectively) with post-hoc Bonferroni correction for multiple comparisons. An unpaired Student’s t-test or Mann-Whitney test were used for mean comparisons of parametric and non-parametric data respectively. For all statistical testing, differences were considered significant when $p<0.05$.

Specific experimental analyses are presented in each chapter.
Chapter 3: Analysis of neurodevelopmental changes in the gestational period following mIA
3.1. Introduction

The use of mIA in the preclinical modelling of NDDs has increased in recent years. Indeed, a large number of studies have explored the behavioural and, to some extent, the molecular and cellular changes resulting from immune activation in rodents (Reisinger et al., 2015). The majority of this work has focused on the use of adolescent and adult animals. Fewer studies have investigated the cellular and molecular mechanisms during the embryonic period preceding the appearance of behavioural phenotypes in postnatal life. The investigation of the embryonic period is critical in models of NDDs to identify the temporal changes in development. Indeed, the initial effects of mIA during gestation are likely to influence subsequent developmental processes. Importantly, the study of a complete developmental trajectory within these models may help determine the timing for therapeutic interventions in disease modification.

3.1.1. Maternal immune activation (mIA)

In more recent years, following an initial focus on schizophrenia, the phenotypes resulting from mIA have been considered to be of relevance to ASD in addition to other NDDs (Patterson, 2011a). mIA has also been applied in the field of neurodegeneration with exposure to poly (I:C) during an immune sensitive period, and a second immune challenge given during ageing, producing a phenotype of relevance to Alzheimer’s disease in mice (Krstic et al., 2012). In order to assess the success of mIA induction it is important to monitor the fever response in pregnant dams. In addition to the data presented in the validation of mIA methodology (Chapter 2), examples of the methodology included to monitor mIA induction in pregnant dams are listed in Table 7.

As shown in Table 7, a lack of standardisation exists in previous studies using different models for monitoring the induction of mIA. Indeed, a number of these studies do not employ any methods to assess the extent of immune activation achieved in the pregnant dam although for certain concentrations and dose routes, validation studies have been performed on the effects of poly (I:C) or LPS (Meyer et al., 2005). However, owing to individual differences in immune responses and the variability in dose and strain between these models, it is proposed that methods to assess mIA induction should be employed in each individual study. This is particularly important with regard to determining the reproducibility of results and robust nature of the model.
**Table 7:** An assessment of mIA in pregnant dams across a variety of models (species, strain, gestational day of induction).

<table>
<thead>
<tr>
<th>Author</th>
<th>Species, Strain</th>
<th>mIA*</th>
<th>Methods to determine mIA induction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zuckerman et al. 2003</td>
<td>Rat, Wistar</td>
<td>GD15 poly (I:C) 4 mg/kg (i.v.)</td>
<td>Maternal BW: 1 day post-injection</td>
</tr>
<tr>
<td>Smith, 2007</td>
<td>Mouse, C57BL/6</td>
<td>GD12.5 poly (I:C) 20 mg/kg (i.p.), IL-6 or IFNγ</td>
<td>Cytokine analysis for IL-6, IFNγ, and IL-1β: 3 hr post-injection</td>
</tr>
<tr>
<td>Meyer et al. 2008</td>
<td>Mouse, C57BL/6</td>
<td>GD9 poly (I:C) 5 mg/kg (i.v.)</td>
<td></td>
</tr>
<tr>
<td>Shi et al., 2009</td>
<td>Mouse, BALB/C</td>
<td>GD12.5 poly (I:C) 20 mg/kg (i.p.)</td>
<td></td>
</tr>
<tr>
<td>Ito, 2010</td>
<td>Mouse, C57BL/6</td>
<td>GD12.5 poly (I:C) 5 mg/kg (i.v.) or 20 mg/kg (i.p.)</td>
<td></td>
</tr>
<tr>
<td>Wolff &amp; Bilkey 2010</td>
<td>Rat Sprague-Dawley</td>
<td>GD15 poly (I:C) 4 mg/kg (i.v.)</td>
<td>Maternal BW: 4 days post-injection</td>
</tr>
<tr>
<td>Connor, 2012</td>
<td>Mouse, C57BL/6</td>
<td>GD12.5 or 17.5 poly (I:C) 5 mg/kg (i.v.)</td>
<td>Cytokine analysis for IL-6, TNFα, IL-1β and IFNγ 3 hr post-injection</td>
</tr>
<tr>
<td>Malkova et al., 2012</td>
<td>Mouse, C56BL/6</td>
<td>GD10.5, 12.5 and 14.5 poly (I:C) 5 mg/kg (i.p.)</td>
<td></td>
</tr>
<tr>
<td>Hsiao et al. 2013; 2012</td>
<td>Mouse, C57BL/6</td>
<td>GD12.5 poly (I:C) 20 mg/kg (i.p.)</td>
<td></td>
</tr>
<tr>
<td>Schartner, 2013</td>
<td>Mouse, C57BL/6</td>
<td>GD12.5 poly (I:C) 20 mg/kg (i.p.)</td>
<td></td>
</tr>
<tr>
<td>Arsenault et al., 2014</td>
<td>Mouse, C57BL/6</td>
<td>GD15-17 poly (I:C) 5 mg/kg (i.v.) LPS 120 µg/kg/day (i.v.)</td>
<td>Maternal food intake following last injection Rectal temperature before each injection</td>
</tr>
<tr>
<td>Foley, 2014</td>
<td>Rat, Long Evans</td>
<td>GD12-16 (5 injections) Sodium propionate (PPA) 500mg/kg, LPS 50µg/kg (S.C.)</td>
<td></td>
</tr>
<tr>
<td>Onore, 2014</td>
<td>Mouse, C57BL/6</td>
<td>GD12.5 poly (I:C) 20 mg/kg (i.p.)</td>
<td></td>
</tr>
<tr>
<td>Smolders et al. 2015</td>
<td>Mouse, C57BL/6</td>
<td>GD11.5 and 15.5 poly (I:C) 20 mg/kg (i.p.)</td>
<td>Cytokine analysis for IL-6, 5 hr post-injection</td>
</tr>
<tr>
<td>Vernon et al. 2015</td>
<td>Rat, Sprague Dawley</td>
<td>GD15 poly (I:C) 4 mg/kg (i.v.)</td>
<td></td>
</tr>
<tr>
<td>Giovanoli, 2016</td>
<td>Mouse, C57BL/6</td>
<td>GD12.5 poly (I:C) 5 mg/kg (i.v.)</td>
<td></td>
</tr>
<tr>
<td>Luchicchi, 2016</td>
<td>Rat, Sprague Dawley</td>
<td>GD15 poly (I:C) 4 mg/kg (i.v.)</td>
<td>Maternal BW: 3 days post-injection</td>
</tr>
<tr>
<td>Kirsten and Bernardi, 2017</td>
<td>Rat, Wistar</td>
<td>GD9.5 LPS 100 µg/kg (i.p.)</td>
<td>Sickness behaviour monitored; movement, weight gain, piloerection</td>
</tr>
</tbody>
</table>

*Gestational day of exposure, compound used, route of administration (i.p. intraperitoneal, i.v. intravenous, S.C. subcutaneous; BW= body weight, — no information given)
3.1.2. Early neurodevelopmental changes in mIA models

Previous mIA studies investigating the neonatal period (typically defined as PD0-3), have recorded changes in excitability of hippocampal neurons (Patrich et al., 2016), upregulation of the α, β and γ crystallin gene family (Garbett et al., 2012) as well as multiple molecular changes relevant to schizophrenia. These include reduced N-methyl-D-aspartate receptor (NMDAR) expression in the hippocampus and dopamine hyperfunction (Meyer et al., 2008a; Vuillemot et al., 2010). Further gene expression changes in neonatal offspring at PD3 have been performed in the LPS model of mIA (Baharnoori et al., 2012). In this study, Baharnoori et al. 2012 showed that cortical expression of 5HT1A and 5HT1B mRNA was decreased in mIA offspring at PD3. These findings support a dysregulation of the serotoninergic system alongside behaviours relevant to NDDs such as ASD and schizophrenia.

A limited number of studies have focused on cellular and molecular changes in the embryonic period (Vuillermot et al., 2010; Soumiya et al., 2011; Connor et al., 2012). However, commonly changes in the expression of cytokines in the foetal brain are reported (Gilmore et al., 2005; Meyer, 2006; Arrode-Brusés and Brusés, 2012; Connor et al., 2012; Oskvig et al., 2012; Ratnayake et al., 2014; Arad et al., 2017). Specifically lacking is the analysis of early gene expression changes relevant to ASD. A recent publication by Choi et al. 2016 focused on foetal brain changes in mouse offspring following mIA at GD12.5. The results showed increased expression of the IL-17a receptor in the foetal cerebral cortex. Added to this, cortical cytoarchitecture was disrupted without affecting cortical thickness in GD14.5 foetuses and these animals also showed ASD-like behavioural abnormalities (Choi et al., 2016b). Further studies are clearly required to determine effects of mIA in embryonic offspring including changes at the molecular level that predispose a behavioural phenotype in later stages of development.

3.1.3. Brain regions and genes of interest

Both cortical and cerebellar regions are implicated in ASD pathophysiology, as previously discussed. The frontal cortex (FC) and the cerebellum (CB), therefore, present interesting regions for investigation of changes to brain morphology in models of mIA. In order to validate the effects of GD12.5 mIA on gene expression during the embryonic period, a subset of genes related to neurodevelopment and signalling were
chosen (see Table 8 and section 3.1.3.1 for rationale generated from the literature). These specific genes were also chosen as they had been previously validated in the RT-qPCR protocol used in this study.

Table 8: Genes chosen for model validation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocte-specific enhancer factor 2c (MEF2C)</td>
<td>Synaptic pruning</td>
<td>Elmer et al. 2013</td>
</tr>
<tr>
<td>Major facilitator superfamily domain containing 2A (MFSD2A)</td>
<td>BBB integrity</td>
<td>Ben-Zvi et al. 2014</td>
</tr>
<tr>
<td>Semaphorin 3a (SEMA3A)</td>
<td>Axonal guidance</td>
<td>Nakamura et al. 2000; Eastwood et al. 2003</td>
</tr>
<tr>
<td>SH3 and multiple ankyrin repeat domains 3 (SHANK3)</td>
<td>Synaptic scaffold</td>
<td>Durand et al. 2007; Durand et al. 2012</td>
</tr>
<tr>
<td>Synaptosomal-associated protein 25 (SNAP-25)</td>
<td>Central role in synaptic transmission and plasticity</td>
<td>Braida et al. 2015</td>
</tr>
<tr>
<td>Glutamate decarboxylase 1 (GAD1)</td>
<td>Rate-limiting enzyme for GABA synthesis</td>
<td>Labouesse et al. 2015</td>
</tr>
<tr>
<td>Serotonin receptor 2a (5HT2A)</td>
<td>Involved in serotonergic transmission</td>
<td>Kinast et al. 2013</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein (GFAP)</td>
<td>Expressed in astrocytes</td>
<td>Rajkowska et al. 2002</td>
</tr>
<tr>
<td>Olfactomedin-like protein (OLFML3)</td>
<td>Specifically expressed in microglia</td>
<td>Fatemi et al. 2008; Han et al. 2017</td>
</tr>
</tbody>
</table>
3.1.3.1. Rationale for gene selection

Due to the neural connectivity changes seen in the ASD brain, a number of genes were chosen to investigate synaptic formation, stability and pruning. Indeed, although the patient numbers were small, changes in spine density have been shown in ASD cohorts (Hutsler and Zhang, 2010; Tang et al., 2014). Multiple SNAP-25 polymorphisms have been associated with traits of ASD (Guerini et al., 2011) as well as other neurological disorders including ADHD (Zhang et al., 2011) and schizophrenia (Thompson et al., 2003). Braida et al. 2015 provided further evidence of this in ASD children in their analysis of five different SNAP-25 polymorphisms alongside behavioural phenotyping of SNAP-25 KO mice. Their analysis revealed that reduced levels of the protein SNAP-25 in adolescent mice resulted in behavioural changes akin to an ASD phenotype and a specific SNAP-25 polymorphism was related to the cognitive impairment in ASD children (Braida et al., 2015).

Evidence from genetic association studies alongside clinical data implicating specific developmental pathways were used to select additional genes of interest; SH3 and multiple ankyrin repeat domains 3 (SHANK3) (Peça et al., 2011; Durand et al., 2012) and glutamate decarboxylase 1 (GAD1) (Labouesse et al., 2015; de la Torre-Ubieta et al., 2016). Moreover, the vulnerability of the blood-brain barrier (BBB) to early developmental disruption, as already discussed, was an area of interest. Gene expression changes in the BBB are particularly important for mechanistic understanding of mIA because, if compromised during development, the BBB may allow the infiltration of inflammatory markers with subsequent detrimental effects to the developing brain. The critical role of major facilitator superfamily domain containing 2A (MFSD2A ) in the development of BBB integrity has been shown by the ablation of this gene in mice that resulted in BBB leakage from the embryonic period until adulthood (Ben-Zvi et al., 2014). This evidence stimulated the investigation of MFSD2A in the GD12.5 model of mIA in light of furthering mechanistic understanding.
3.2. Chapter 3 Summary and Objectives

This initial study aimed to investigate the effects of 10 mg/kg i.p. poly (I:C) at GD12.5 on pregnant dams and subsequently, their offspring at GD21. It was very important to monitor the acute effects of poly (I:C) on dams at GD12.5 in an attempt to develop standardised methods for determining the induction of mIA in pregnancy for comparison with other mIA models. In order to provide a longitudinal and in depth analysis of offspring development, the experiments conducted in this chapter were used to determine changes to morphology, growth and early brain development of male and female offspring prior to birth.

3.2.1. Objectives

The aim of this chapter is to add to the knowledge produced by previous mIA work on early embryonic changes in this mIA model using 10 mg/kg i.p. poly (I:C) at GD12.5. In order to achieve this, the following objectives were set.

- Administer mIA using 10 mg/kg i.p. poly (I:C) at GD12.5 and investigate acute sickness behaviour in dams including cytokine analysis at 3 hr post-injection.

- Perform a foetal harvest at GD21 and collect all pups for morphometric analysis to determine any changes to general growth (e.g. body weight, brain weight).

- Perform gene expression analysis on both the FC and CB of GD21 pups measuring specific genes of interest.
3.3. **Materials and methods**

3.3.1. **Experimental design**

Experiments were performed using male and female Wistar rats (Charles River Laboratories, UK). All rats were housed under the conditions described in Chapter 2 section 2.2.1, p.71. For mating $n=5$ male and $n=12$ female rats were used. All of these 12 pregnant dams underwent mIA or the respective control condition ($n=6$ saline, $n=6$ poly (I:C), see section 2.5.2, p.82). In accordance with the principles of the 3Rs (Replacement Reduction and Refinement www.nc3rs.org.uk/the-3rs), at the GD21 foetal harvest, data was collected for all pups (male and female) from each litter. Due to time constraints on processing tissue for the molecular analysis, two pups of each sex were selected at random from each dam (see foetal harvest for further details). **Figure 15** shows an outline of the experimental design used in this study.

**Figure 15: Timeline of GD21 experimental design.** GD= gestational day, CBT= core body temperature, RT-qPCR = reverse transcriptase, quantitative real-time polymerase chain reaction.
3.3.2. GD21 foetal harvest

At GD21 (two days prior to parturition), pregnant female rats were anaesthetised (4%) and maintained (1.5–2%) with isoflurane delivered in 70:30 N₂O/O₂. Pups and respective placentas were removed from the uterine horn and weighed. The direction of dissection from the uterine horn (left to right/right to left) was alternated between dams and counter-balanced within and between treatment groups. The first pup was always removed from the top of the horn and this was labelled as pup number 1 (Figure 16). Pup brains were removed, weighed and regionally dissected to obtain samples of both FC and CB. Brains were placed in RNA later (R0901; Sigma, UK) for processing for mRNA analysis. All samples were then snap frozen in 2-Methylbutane (Fisher Scientific) and stored at -80 °C until processed. The tail tips from each pup were collected and snap frozen in 2-Methylbutane for sex-typing using PCR.

Figure 16: Foetal harvest at GD21 showing uterine horns with foetuses prior to dissection. Each foetus (A) is held in an individual amniotic sac with an individual placenta (B).
3.3.3. Pup sex-typing

Sex-typing was performed on all GD21 pups using the following methods, previously described by Choi et al. 2016. First, DNA was extracted from each tail tissue sample using 44 ul DNase/RNase free water, 5 ul 10x KAPA Express Extract Buffer and 1 ul 1 U/µl KAPA Express Extract Enzyme (Kapa Biosystems). Each sample was vortexed until the tissue was fully submerged in the buffer. Lysis of the tissue was then achieved using a heating block for 15 min at 75 ºC, followed by 10 min at 95 ºC for enzyme inactivation. All samples were briefly centrifuged (1 min at 13,000 x g, at RT) to pellet cellular debris.

A PCR master mix was then prepared to a total volume of 19 ul/sample using the reagents listed in Table 9. 1 ul of DNA was added from each sample to reach a final reaction value of 20 ul. The following primers were used to determine the sex of the pups. The Sry primer was used (described previously by An et al. 1997) to detect a region on the Y chromosome and, therefore, the amplicon (317 bp) was only found in male offspring. Beta-actin (β-actin) was used as a control gene with the amplicon (220 bp) shown in both females and males. The primer sequences are listed below.

Rn_Sry Forward primer (5’ → 3’) TACAGCCAGAGGACATATTA (20),

Rn_Sry Reverse primer (5’ → 3’) GCACTTTAACCCTTCGATGA (20),

Rn-actin Forward primer (5’ → 3’) AGCCATGTACGTAGCCATCC (20),

Rn-actin Reverse primer (5’ → 3’) TGTGGTGGAAGCTGTAGC (20).
Table 9: KAPA Fast Genotyping PCR reaction reagents and volumes

<table>
<thead>
<tr>
<th>Component</th>
<th>Per reaction</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR grade water</td>
<td>5 µl</td>
<td>NA</td>
</tr>
<tr>
<td>2X KAPA2G Fast (HotStart) Genotyping Mix with dye</td>
<td>10 µl</td>
<td>NA</td>
</tr>
<tr>
<td>10 µM Sry Forward primer</td>
<td>1 µl</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>10 µM Sry Reverse primer</td>
<td>1 µl</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>10 µM β-actin Forward primer</td>
<td>1 µl</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>10 µM β-actin Forward primer</td>
<td>1 µl</td>
<td>0.5 µM</td>
</tr>
</tbody>
</table>

3.3.3.1. PCR protocol

For the PCR, samples were first held at 94 °C for 5 min, followed by 94 °C for 30 sec, 60 °C for 30 sec and finally 72 °C for 30 sec, for 30 cycles. The final incubation was for 5 min at 72 °C. Then the products were run on a 2% agarose gel in 1X Tris-acetate EDTA (TAE) buffer with XC loading buffer and a 100bp ladder for determination of size (Figure 17).

![Image of DNA gel]

**Figure 17:** Example DNA gel for sex-typing pups at GD21. The presence on the SRY band was used to confirm the sex of a male pup and the absence of this band for a female pup. β-actin was used as a control gene found in each sex.
3.3.4. Foetal weight frequency distribution curves

Foetal weight distribution curves were generated from a frequency distribution using the Gaussian non-linear regression function (GraphPad Prism 7). Due to the properties of a normally distributed curve the standard deviation can be used to cut off a constant proportion of the distribution of scores. This allows a standard z-score to be used to define a cut-off proportion limit of the distribution. The data from the saline group were used to calculate the 5th centile using the following calculation.

\[
5^{\text{th\ centile}} = (-Z\ score \times \text{Standard\ Deviation}) + \text{Mean}
\]

The z score was set at -1.645 in order to determine what proportion of the fetal weight distribution fell within less than 5% of the population, or 5th centile (i.e. -1.645 will correspond to -45% of the population away from the mean) as previously described (Dilworth et al., 2011; Grice and Barrett, 2014). The resulting graphs show how the data fall in relation to 5% of the population. Data found below the 5th centile for foetal weight are taken to indicate foetal growth restriction in the clinical setting (a shift in the distribution to left indicates an increased proportion of the population falling below 5th centile) (Dilworth et al., 2011; Kusinski et al., 2012).

3.3.5. RNA extraction

3.3.5.1. Tissue preparation

Prior to RNA extraction, sodium dodecyl sulfate (SDS) (10% in dH2O) was used to denature any RNase on surfaces, glassware or other laboratory equipment used for the procedure to prevent RNase contamination.

Total RNA was extracted from isolated FC and CB using TRIzol (Life Sciences, UK) following the manufacturer’s instructions. Tissues were thawed in a fume hood and placed in an Eppendorf containing TRIzol (750 ul TRIzol: ~50 mg of brain tissue). The tissues were disrupted using a rotor-stator homogeniser (The Scientific instrument Centre, Germany) for 15 – 30 sec.
3.3.5.2. **Phase separation**

The homogenised solution was left for 5 min at room temperature (RT) to allow dissociation of the nucleoprotein complex. 150 ul of chloroform was added to the homogenate and vortexed for 20 sec. Samples were then centrifuged at 13,000 rpm for 15 min at 4 °C. The mixture was separated into three sections following centrifugation (Figure 18).

![Diagram of phase separation](image)

**Figure 18: Purification of RNA using phenol-chloroform extraction.** Following addition of chloroform and centrifugation the mixture separates into three distinct layers

3.3.5.3. **RNA isolation and washing**

The aqueous layer containing the RNA was carefully removed and placed in a new Eppendorf. 300 ul of isopropanol was then added to the sample. The samples were vortexed for 20 sec and then left to incubate at RT for 10 min. Centrifugation was then performed at 13,000 rpm for 30 min at 4 °C. The supernatant was removed and the pellet washed with 70% ethanol. Each Eppendorf was briefly inverted to clear any contaminants from the lid. The samples were then centrifuged at 8000 rpm for 5 min at 4 °C. Again the supernatant was removed and the pellets left to air dry for 5-10 min. The RNA pellet was then suspended in 20 ul of RNase free H₂O.
3.3.5.4. **RNA concentration and integrity**

The RNA concentration and quality were measured by NanoDrop 2000 (Thermo Scientific) where the amount of UV light absorbed at 260 nm by nucleic acids is used to calculate their concentration. The NanoDrop was calibrated using RNase free water (also used to dilute the RNA). A 1 ul aliquot of RNA was loaded onto the NanoDrop and the absorbance was measured. The quality of the RNA was assessed through measuring the 260/280 and 260/230 ratios; expected values for high quality RNA are approximately 2.0 and 2.0-2.2, respectively.

Total RNA is comprised of rRNA (major component), tRNA and mRNA which can be visualised on a gel. Prior to conversion to cDNA, all RNA samples were run on an agarose gel (1.5%) to check the RNA integrity by the presence of two discrete rRNA bands comprising 28S rRNA and 18S rRNA (Figure 19). The agarose gel was prepared using 1.5 g of agarose powder in 100 ml 1X TAE buffer. 10 µl Gel Red Stain was added to each 100 ml gel. The gel was left at RT to set for 30 min. RNA was diluted to 100 ng/µl using the NanoDrop readings obtained previously. When the gel was set, it was immersed in 1X TAE buffer and the samples (8 ul) were loaded into wells with 5X loading buffer (2 ul). The gel was run at 120 V for 30-45 min or until bands were well separated for visualization. The gels were visualised using an ultraviolet (UV) transilluminator. Degraded samples (see Figure 19) were excluded from the analysis.

![Agarose gel](image)

**Figure 19**: Agarose gel for measuring RNA quality. **A)** Agarose gel with eight samples showing no degradation. **B)** Agarose gel showing four degraded samples within the gel. * = blank lane
3.3.6. **Reverse transcriptase polymerase chain reaction (RT-PCR)**

3.3.6.1. **RT-PCR protocol**

Target RNA was reversed transcribed using a Veriti Thermal Cycler (Thermo Fisher Scientific). 1 ul of oligo-dT$_{12-18}$ (500 ug/ml) and 1 ul of 10 mM deoxyribonucleotide triphosphate (dNTPS) (Invitrogen Life Sciences, UK) was added to each 1 ug of RNA and held for 5 min at 65 ºC. The samples were then incubated at 37 ºC for 52 min followed by a shorter incubation at 70 ºC for 15 min, in the presence of 4 ul 5X first strand buffer, 2 ul 0.1 M Dithiothreitol (DTT), 1 ul RNaseOUT (40 U/ul) and 1 ul Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT) (200 U). Samples were removed from the machine and kept at – 20 ºC until further processing.

3.3.7. **Quantitative real time PCR (qPCR)**

For the quantitative real time PCR (qPCR) Power SYBR® Green PCR master mix was used (Applied Biosystems, Thermo Fisher). This master mix is optimized for SYBR® Green reagent reactions and contained SYBR® Green I Dye, AmpliTaq Gold® DNA polymerase, dNTPs, ROX passive reference dye and optimized buffer components. For each primer of interest, cDNA and DNase free water were added to the SYBR® master mix (see Table 10 for volumes). Then, the master mix was added to the 384 well plate in triplicate. A negative control was used for each primer set where all the components were added without the cDNA. RNase free water was used to make up to the 10 ul volume in the well. The 384 well plate was sealed with a cover slip and centrifuged at 4000 rpm for 1 min at 4 ºC.

**Table 10**: Reagents used for the qPCR used to make a master mix before adding to the 384 well plate in triplicate

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration</th>
<th>Volume well (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power SYBR® Green PCR master mix</td>
<td>2X</td>
<td>5</td>
</tr>
<tr>
<td>Primer</td>
<td>10X</td>
<td>1</td>
</tr>
<tr>
<td>cDNA</td>
<td>0.1 μg/μl</td>
<td>1</td>
</tr>
<tr>
<td>RNase free water</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>10 μl</strong></td>
<td></td>
</tr>
</tbody>
</table>
A fast, real-time qPCR system (QuantStudio 12K Flex Real-Time PCR System) was used. qPCR cycling conditions for all genes consisted of an initial denaturation step of 95 °C for 10 min, followed by 40 cycles consisting of 95 °C for 15 sec and 60 °C for 1 min. The samples were loaded in triplicate for each gene of interest and the generated cycle threshold (CT) values were used to calculate average CT values for each sample. The CT values represent the number of amplification cycles necessary to achieve the threshold fluorescence signal (above background) for each sample. The CT value is inversely proportional to the amount of target nucleic acid in the sample (e.g. the lower the CT value, the larger the amount of target nucleic acid present in the sample). The average of the triplicate values was used for analysis with the REST 2009 programme to prevent pseudoreplication of results.

Immediately after the end of the qPCR cycling, a built-in melt curve analysis was run as an indicator of whether the primers that were used produced single, specific products. This was performed for all of the primers used in qPCR experiments. During this analysis, fluorescence was measured continuously as the temperature was gradually increased from 65 °C to 95 °C. With increasing temperature, fluorescence decreases as double stranded DNA denatures and the single strands dissociate from the SYBR® Green dye. The change in slope of this curve was plotted against temperature (T) to produce the melt curve. The peak in the curve represents the respective melting point (Tm) of the primer. The presence of two peaks in the analysis can indicate the presence of primer-dimer products. Example melt curves are presented in Figure 20 showing the two HK genes used in these experiments.
Figure 20: The melt curve analysis of PCR products using primers. A) GAPDH and B) SDHA. The appearance of one single peak indicates the specificity of PCR product that had been measured.
3.3.7.1. Primers

All primers were bought pre-made and optimised for use with SYBR® Green from Qiagen (Quantitect Primers Assay, UK). The rat mRNA primers used were: glial fibrillary acidic protein (GFAP), serotonin receptor 2A (HTR2A), myocyte-specific enhancer factor 2C (MEF2C), major facilitator superfamily domain containing protein 2A (MFSD2A), olfactomedin-like protein (OLFML3), Semaphorin 3A (SEMA3A), SH3 and multiple ankyrin repeat domains 3 (SHANK3), glutamate decarboxylase (GAD1), discs large homolog 4 (DLG4 or PSD-95), and synaptosomal-associated protein 25 (SNAP-25) were analysed. These primers were supplied with the exon location for each primer (see Appendix A, section 0, p.248 for individual primer information). The primers were reconstituted in 1.1 ml TAE buffer at pH 8. A number of preclinical studies have investigated the changes in expression of inflammatory markers in the foetal brain, specifically cytokines, following induction of mIA (Gilmore et al., 2005; Arrode-Brusés and Brusés, 2012; Ratnayake et al., 2014). Unpublished data from our lab in this model revealed no changes in the expression of IL-6 and monocyte chemoattractant protein-1 (MCP-1) in the brain of GD21 foetuses and, for this reason, these genes were not explored in the current study.

The expression of two housekeeping (HK) genes, succinate dehydrogenase complex subunit A (SDHA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were also analysed. No significant differences between treatment groups were observed in the expression of these HK genes in either brain region analysed. The expression of each gene was normalised to the geometric mean (GM) of SDHA and GAPDH and, then, a relative expression compared to control was calculated using REST analysis 2009 software (Qiagen) which has been previously validated (Pfaffl, 2002; Yuan et al., 2006). Data are presented as relative expression in gene level in poly (I:C) treated group relative to one (gene expression in the saline control group).

3.3.7.2. Primer efficiency

The serial dilution method was used to assess the efficiency of each primer used. cDNA of naïve animal brain tissue was serially diluted by a factor of 10 over a 5-log range. The threshold cycle levels recorded from the qPCR protocol described above were plotted against the log cDNA dilution in GraphPad prism. Linear regression was used to calculate the regression value $r^2$ and the slope was determined for each primer. The
primer efficiency was calculated using the formula: $\text{Primer efficiency} = 10^{-\frac{1}{\text{slope}}}$. All primers were tested for efficiency in the using the same QuantStudio programme and this data was imported into the analysis programme for generating accurate expression levels (Pfaffl, 2002).

### 3.3.8. Specific experiment analyses

#### 3.3.8.1. Litter comparisons

Data were collected from all of the individual pups from each litter however, nested analysis (as described in section 4.3.9.1, p.136) could not be performed due to failure to meet the assumptions of parametric analysis (normal distribution and equal variance). The mean litter value for each parameter measured at GD21 was taken and, thus, the $n$ number represents the dam rather than the litter number. Normality tests were performed on the mean litter data and unpaired t-test or Mann-Whitney test were used for litter comparisons of parametric and non-parametric data respectively.

#### 3.3.8.2. RT-qPCR

Due to feasibility of carrying out the gene expression analysis on two brain region and multiple litters, only two pups from each sex and treatment group were selected from each dam for RNA extraction and subsequent RT-qPCR. These pups were selected at random using offspring from both sides of the uterine horn with a random number generator (the number of each pup was determined by the dissection protocol as described in section 3.3.2, p.93).

For the analysis of qPCR, the REST programme 2009 was used to calculate the relative expression values and any significant changes compared to the GM of the two HK genes. REST 2009 employs randomisation algorithms to provide an accurate calculation of $p$ values. As recommended for achieving high quality data, 2000 randomisation/iterations were used for the generation of results (see REST 2009 Software User Guide 2009, [http://www.gene-quantification.com/REST_2009_Software_User_Guide.pdf](http://www.gene-quantification.com/REST_2009_Software_User_Guide.pdf)). To confirm that expression of both HK genes were unaffected by treatment, an unpaired t-test was applied within sex groups. Data were assessed for normality and for all statistical testing, differences were considered significant when $p<0.05$. 
3.4. Results

3.4.1. mIA at GD12.5 resulted in no significant sickness behaviour in dams

At GD12.5 core body temperature (CBT) and BW were monitored at 3, 6 and 24 hr post-injection. CBT was not changed at any time point as result of the 10 mg/kg i.p. poly (I:C) injection (Figure 21A). Repeated measures ANOVA showed a significant effect of time on CBT $F_{(3, 30)} = 3.88$, $p= 0.0186$. When compared to baseline, poly (I:C)-treated dams showed significantly reduced CBT at 24 hr post injection ($p= 0.0093$) as shown by Bonferroni’s multiple comparisons. A similar, significant effect of time was found when the data were normalised to baseline as in the repeated measures ANOVA and post-hoc analysis (Figure 21B).

No change was seen in BW between treatment groups post-injection. However, the repeated measures ANOVA showed a significant effect of time $F_{(3, 30)} = 8.75$, $p= 0.0003$ (Figure 21C-D). At 6 hr post-injection saline dams showed a significant reduction in BW compared to baseline ($p= 0.0344$). This is typical for rats during the light cycle due to their reduced food intake. In poly (I:C) dams a significant weight gain was showed 24 hr post injection ($p= 0.0234$). At 24 hr post-injection saline dams recorded a BW in line with baseline measurements. These effects were shown in both the raw mean data and in the baseline-corrected temperature data (Figure 21C-D).

At 3 hr post-injection, no detectable level of IL-6 was found in any dam in either saline- or poly (I:C)-treated groups (data not shown). Levels of plasma TNFα and IL-1β were also analysed. For all dams at GD21 no detectable level of IL-1β was found. Dam FD2 showed a detectable level of TNFα 3 hr post poly (I:C) injection at 346.3 pg/ml.
Figure 21: Effect of mIA at GD12.5 on pregnant dams. A-B) No differences was recorded in core body temperature (CBT) at any time point post injection between treatment groups. Poly (I:C) dams showed a significantly reduced CBT at 24 hr compared to baseline. These data were consistent when normalised to baseline (B). C-D) No significant difference in body weight (BW) was found between treatment groups at any time point (C). Within the saline group a significant reduction in BW was recorded at 6 hr post injection vs. baseline. At 24 hr post injection poly (I:C) dams BW significantly increased compared to baseline weight (D). (* = vs. saline group, # = vs. baseline s. = saline group, p. =poly (I:C), */# p< 0.05, **/## p< 0.01, ***/### p<0.001.) Graphs represent mean ±SEM, n=6 dams/ treatment.
3.4.2. mIA had no effect on gestational weight gain or litter numbers

During gestation, dams gained 109 g on average from GD8 until GD21. Two-way ANOVA revealed no significant interaction of treatment by day (Figure 22). A significant main effect of day was produced $F_{(14, 90)} = 140.0, p= <0.0001$ but no main effect of treatment $F_{(1, 90)} = 1.32, p= 0.2541$. In summary, 10 mg/kg i.p. poly (I:C) at GD12.5 did not affect the gestational weight gain of dams.

![Gestational weights](image)

**Figure 22: mIA had no effect on gestational weight.** No significant difference in body weight was found at any gestational time-point between saline and poly (I:C) dams. Graphs represent mean ±SEM, $n= 6$ dams/treatment.

3.4.2.1. Litter numbers

At GD21 foetal harvest litter numbers from all dams were recorded (Table 11). The sex of the offspring was determined using the sex-typing PCR protocol described above. The distribution of male and female offspring between treatment groups is presented in Figure 23. The average litter size was 15 ±1 for saline and 15 ±2 for poly (I:C) dams and no significant difference was found between total litter numbers ($p= 0.7719$) (Figure 23A). The lowest litter size recorded was poly (I:C) dam FD5 with a litter total of 6 (3 males and 3 females). As already highlighted, this dam showed no detectable level of IL-6 when measured in blood plasma at 3 hr post-injection. A one-way ANOVA revealed no difference in the sex distribution of offspring between treatment
groups $F_{(3, 20)} = 0.10$, $p= 0.44$ (Figure 23B). However, on average there were more male animals in both saline and poly (I:C) litters than female offspring.

**Table 11:** Litter numbers and sex distribution

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dam ID</th>
<th>Total Litter number</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FD1</td>
<td>18</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>FH2</td>
<td>15</td>
<td>12</td>
<td>3</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>FB2</td>
<td>16</td>
<td>10</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>FA2</td>
<td>15</td>
<td>5</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>FC1</td>
<td>14</td>
<td>10</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Poly (I:C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FE5</td>
<td>16</td>
<td>10</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>FD5</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>FD4</td>
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<td></td>
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<tr>
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</tr>
<tr>
<td>FE2</td>
<td>18</td>
<td>11</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

Figure 23: mIA did not affect litter size or sex distribution within litters. Graphs represent group mean ±SEM and individual litter means, $n=6$ dams/ treatment group.
3.4.3. Effects of mIA at GD12.5 on GD21 offspring

3.4.3.1. mIA at GD12.5 did not affect the body weight of offspring

At the GD21 foetal harvest, the body weight (BW) of each pup was recorded. This data was collected due to the relationship between low intrauterine weight and birth weight with NDDs such as intellectual disability and ASD (Leonard et al., 2007; Lampi et al., 2013). However, no significant difference was found in the BW of pups from saline- and poly (I:C)-treated dams. Average BW was recorded as 3.11 ±1.16 g and 3.03 ±1.12 g male and female offspring respectively. The within treatment group variability was higher in poly (I:C) offspring for both sexes. In male offspring the SD was 0.50 g (saline) compared to 1.2 g for poly (I:C) offspring. A similar trend was seen in female offspring with SD of 0.43 g and 0.91 g in saline and poly (I:C) respectively. One poly (I:C) litter had a particularly low BW with male offspring weighing 0.85 ±0.11 g and female offspring 0.87 ±0.10 g (Figure 24A). The range of BW in the poly (I:C) group excluding the FD2 litter were 3.04–4.15 g and 2.98–4.03 g male and female offspring respectively showing the extent of this BW reduction in comparison to other poly (I:C) litters. Interestingly, this dam also recorded the only detectable level of TNFα in plasma 3 hr post-injection.

To explore the BW data further, foetal weight frequency distribution curves were produced. This is a standard method for assessing BW in models of foetal growth restriction (FGR) (Dilworth et al., 2011). The frequency distribution curves show that, at GD21 in poly (I:C) offspring, 24% of male and 14% of female foetuses were below the 5th centile. In both sexes this was made up of pups from the FD2 litter (Figure 24B).
Figure 24: mIA did not affect body weight at GD21. A) Mean litter body weight data showed no difference between treatment groups in either sex. Graphs represent group mean ± SEM and individual litter means, \( n = 6 \) dams/treatment group. B) Frequency distribution curve for male poly (I:C) offspring where 24% of the population were below the 5\(^{th}\) centile (2.6 g). For female poly (I:C) offspring 14% of the population were below the 5\(^{th}\) centile (2.8 g).
3.4.3.2. *mIA GD12.5 had a sex-specific effect on placental weight at GD21*

On average, the placenta weight (PW) was reduced in both male (0.4 g) and female (0.7 g) poly (I:C) groups compared to saline. However, this was only significant in female pups as shown by unpaired Student t-test ($p= 0.031$) (Figure 25A). Placental weight distribution curves show that for male offspring 20% of the population fell below the 5th centile (0.43 g). In the female poly (I:C) population, 7% fell below the 5th centile (0.39 g) (Figure 25B).

**Figure 25:** mIA produced a sexually dimorphic effect on placental weight. 
A) Placental weight was significantly reduced in female poly (I:C) pups only ($p= 0.031$). Graphs represent group mean ±SEM and individual litter means, $n= 6$ dams/ treatment group. B) For placental weight distribution 20% of the male offspring population fell below the 5th centile (0.43 g). In the female poly (I:C) population 7% fell below the 5th centile (0.39 g).
Placenta weight was also analysed as a proportion of BW. No significant difference was found between treatment groups in either sex (Figure 26). For both male and female offspring in the FD2 litter, the ratio of PW to BW was high due to the very small BW recorded for these fetuses.

**Figure 26: Placenta weight as a proportion of body weight.** No significant difference was found between treatment groups. Graphs represent group mean ±SEM and individual litter means, n=6 dams/treatment group.

### 3.4.3.3. mIA at GD12.5 had no effect on brain weight in GD21 offspring

No significant difference was found in brain weight (BrW) or the ratio of brain to body weight between treatment groups. In line with BW data, offspring from the FD2 litter recorded lower BrW on average than the rest of the poly (I:C) litters. In male offspring, the average BrW for the poly (I:C) group was 0.17 g whereas the average BrW mean of the FD2 litter was 0.13 g. Similarly, in poly (I:C) female offspring, the FD2 litter recorded a mean of 0.12 g with the poly (I:C) group mean at 0.17 g (Figure 27).
3.4.3.4. mIA at GD12.5 had no effect on general growth parameters measured at GD21

General growth measurements were taken for all pups with regard to body length, head circumference and abdominal circumference as a further indication of any changes to general development (Dilworth et al., 2011). No significant difference was found in any parameter measured in either sex between treatment groups (Figure 28).
Figure 28: mIA did not affect any morphological parameter tested at GD21. A-C) No significant difference was found between treatment groups for any parameter measured in either sex. Graphs represent group mean ±SEM and individual litter means, n= 6 dams/treatment group.
3.4.4. RT-qPCR

3.4.4.1. mIA at GD12.5 resulted in sex-specific gene expression changes in the frontal cortex of offspring at GD21

To confirm that no difference was found in the reference HK genes SDHA and GAPDH, mean CT values were compared between treatment groups. As shown in Figure 29, no effect of treatment was shown in either HK gene for either sex within the FC.

Figure 29: mIA had no effect on housekeeping gene CT values in the frontal cortex. A-B) No difference was found in the CT values of SDHA or GAPDH between treatment groups in male or female offspring. Graphs represent mean ±SEM, n= 12/sex/treatment.
In male offspring a significant down regulation of the SNAP-25 gene was shown in the FC (Table 12). However, no other changes in gene expression were found. In female offspring, no changes were found in gene expression in the FC at GD21 for the genes of interest (Table 13).

**Table 12:** Gene expression changes in the frontal cortex of GD21 male pups (- no change, ↑ upregulation, ↓ down regulation)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Expression</th>
<th>SEM</th>
<th>P value</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHA</td>
<td>HK</td>
<td>1.169</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>HK</td>
<td>0.856</td>
<td>0.19 - 4.22</td>
<td>0.778</td>
<td>-</td>
</tr>
<tr>
<td>SHANK3</td>
<td>Target</td>
<td>0.887</td>
<td>0.18 - 3.08</td>
<td>0.523</td>
<td>-</td>
</tr>
<tr>
<td>MEF2C</td>
<td>Target</td>
<td>0.76</td>
<td>0.17 - 1.04</td>
<td>0.007</td>
<td>↓</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Target</td>
<td>0.424</td>
<td>0.12 - 5.24</td>
<td>0.654</td>
<td>-</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Target</td>
<td>1.073</td>
<td>0.22 - 4.01</td>
<td>0.909</td>
<td>-</td>
</tr>
<tr>
<td>SEMA3A</td>
<td>Target</td>
<td>1.322</td>
<td>0.37 - 4.88</td>
<td>0.476</td>
<td>-</td>
</tr>
<tr>
<td>MFSD2a</td>
<td>Target</td>
<td>1.557</td>
<td>0.37 – 6.00</td>
<td>0.378</td>
<td>-</td>
</tr>
<tr>
<td>GFAP</td>
<td>Target</td>
<td>1.559</td>
<td>0.54 - 4.53</td>
<td>0.150</td>
<td>-</td>
</tr>
<tr>
<td>OLFML3</td>
<td>Target</td>
<td>1.42</td>
<td>0.29 - 5.90</td>
<td>0.474</td>
<td>-</td>
</tr>
<tr>
<td>GAD1</td>
<td>Target</td>
<td>0.739</td>
<td>0.190 - 3.04</td>
<td>0.468</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 13:** Gene expression changes in the frontal cortex of GD21 female pups

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Expression</th>
<th>SEM</th>
<th>P value</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHA</td>
<td>HK</td>
<td>1.054</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>HK</td>
<td>0.948</td>
<td>0.12 - 2.26</td>
<td>0.235</td>
<td>-</td>
</tr>
<tr>
<td>SHANK3</td>
<td>Target</td>
<td>0.56</td>
<td>0.11 - 2.34</td>
<td>0.350</td>
<td>-</td>
</tr>
<tr>
<td>MEF2C</td>
<td>Target</td>
<td>0.596</td>
<td>0.23 - 3.93</td>
<td>0.978</td>
<td>-</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Target</td>
<td>0.655</td>
<td>0.13 - 2.60</td>
<td>0.380</td>
<td>-</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Target</td>
<td>0.607</td>
<td>0.17 - 2.20</td>
<td>0.384</td>
<td>-</td>
</tr>
<tr>
<td>SEMA3A</td>
<td>Target</td>
<td>0.739</td>
<td>0.21 - 2.98</td>
<td>0.478</td>
<td>-</td>
</tr>
<tr>
<td>MFSD2a</td>
<td>Target</td>
<td>0.713</td>
<td>0.27 - 2.02</td>
<td>0.260</td>
<td>-</td>
</tr>
<tr>
<td>GFAP</td>
<td>Target</td>
<td>0.895</td>
<td>0.25 - 2.98</td>
<td>0.762</td>
<td>-</td>
</tr>
<tr>
<td>OLFML3</td>
<td>Target</td>
<td>0.876</td>
<td>0.24 - 2.63</td>
<td>0.719</td>
<td>-</td>
</tr>
<tr>
<td>GAD1</td>
<td>Target</td>
<td>0.556</td>
<td>0.16 - 1.89</td>
<td>0.162</td>
<td>-</td>
</tr>
</tbody>
</table>
3.4.5. *mIA at GD12.5 resulted in sex-specific gene expression changes in the cerebellum of offspring at GD21*

Similar to the FC, poly (I:C) treatment showed no effect on the expression of the HK genes SDHA and GAPDH in the CB of either male or female offspring (Figure 30).

In male offspring, the expression of SNAP-25 (component of the trans-SNARE complex) and GFAP and OLFML3 were significantly downregulated in the CB (Table 14). Similarly, female offspring showed a significant reduction in the cerebral expression of SNAP-25 as well as MEF2C (a gene implicated in synaptic pruning) (Table 15).

Figure 30: *mIA had no effect on housekeeping gene CT values in the cerebellum at GD21. A-B*) No difference was found in the CT values of SDHA or GAPDH between treatment groups in male or female offspring. Graphs represent mean ±SEM, n= 12/sex/treatment.
Table 14: Gene expression changes in the cerebellum of GD21 of male pups (- no change, ↑ upregulation, ↓ down regulation)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Expression</th>
<th>SEM</th>
<th>P value</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHA</td>
<td>HK</td>
<td>1.027</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>HK</td>
<td>0.973</td>
<td>SEM</td>
<td>0.516</td>
<td>-</td>
</tr>
<tr>
<td>SHANK3</td>
<td>Target</td>
<td>1.084</td>
<td>0.74 - 1.62</td>
<td>0.113</td>
<td>-</td>
</tr>
<tr>
<td>MEF2C</td>
<td>Target</td>
<td>0.448</td>
<td>0.08 - 1.75</td>
<td>0.038</td>
<td>↓</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Target</td>
<td>0.348</td>
<td>0.07 - 1.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSD-95</td>
<td>Target</td>
<td>0.995</td>
<td>0.74 - 1.32</td>
<td>0.950</td>
<td>-</td>
</tr>
<tr>
<td>SEMA3A</td>
<td>Target</td>
<td>0.979</td>
<td>0.43 - 2.47</td>
<td>0.945</td>
<td>-</td>
</tr>
<tr>
<td>MFSD2A</td>
<td>Target</td>
<td>0.99</td>
<td>0.77 - 1.29</td>
<td>0.906</td>
<td>-</td>
</tr>
<tr>
<td>GFAP</td>
<td>Target</td>
<td>0.456</td>
<td>0.13 - 1.40</td>
<td>0.043</td>
<td>↓</td>
</tr>
<tr>
<td>OLFML3</td>
<td>Target</td>
<td>0.553</td>
<td>0.22 - 1.27</td>
<td>0.046</td>
<td>↓</td>
</tr>
<tr>
<td>5HT2A</td>
<td>Target</td>
<td>1.174</td>
<td>0.37 - 5.04</td>
<td>0.775</td>
<td>-</td>
</tr>
<tr>
<td>GAD1</td>
<td>Target</td>
<td>1.075</td>
<td>0.80 - 1.58</td>
<td>0.664</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 15: Gene expression changes in the cerebellum of GD21 of female pups (- no change, ↑ upregulation, ↓ down regulation)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Expression</th>
<th>SEM</th>
<th>P value</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHA</td>
<td>HK</td>
<td>1.059</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>HK</td>
<td>0.944</td>
<td>SEM</td>
<td>0.320</td>
<td>-</td>
</tr>
<tr>
<td>SHANK3</td>
<td>Target</td>
<td>1.156</td>
<td>0.82 - 1.78</td>
<td>0.002</td>
<td>↓</td>
</tr>
<tr>
<td>MEF2C</td>
<td>Target</td>
<td>0.228</td>
<td>0.03 - 0.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Target</td>
<td>0.240</td>
<td>0.03 - 1.12</td>
<td>0.005</td>
<td>↓</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Target</td>
<td>1.114</td>
<td>0.70 - 1.70</td>
<td>0.434</td>
<td>-</td>
</tr>
<tr>
<td>SEMA3A</td>
<td>Target</td>
<td>1.111</td>
<td>0.47 - 2.97</td>
<td>0.740</td>
<td>-</td>
</tr>
<tr>
<td>MFSD2A</td>
<td>Target</td>
<td>0.946</td>
<td>0.70 - 1.31</td>
<td>0.550</td>
<td>-</td>
</tr>
<tr>
<td>GFAP</td>
<td>Target</td>
<td>0.987</td>
<td>0.24 - 4.62</td>
<td>0.971</td>
<td>-</td>
</tr>
<tr>
<td>OLFML3</td>
<td>Target</td>
<td>0.504</td>
<td>0.15 - 1.53</td>
<td>0.076</td>
<td>-</td>
</tr>
<tr>
<td>5HT2A</td>
<td>Target</td>
<td>1.299</td>
<td>0.22 - 12.1</td>
<td>0.665</td>
<td>-</td>
</tr>
<tr>
<td>GAD1</td>
<td>Target</td>
<td>1.302</td>
<td>0.91 - 2.63</td>
<td>0.062</td>
<td>-</td>
</tr>
</tbody>
</table>
3.5. Discussion

3.5.1. mIA produced no significant sickness behaviour at GD12.5

Exposure to 10 mg/kg i.p. poly (I:C) in this study produced no change to CBT at any time point recorded. This indicates that dams did not produce a fever response with regard to CBT. Similarly, no effect of treatment was shown on BW following exposure to poly (I:C). At 24 hr post-injection no treatment effect was shown. However, a significant increase in BW was seen in poly (I:C) dams compared to baseline. Again, this finding indicates that these dams did not experience acute sickness behaviour resulting in reduced food intake but rather gained significant weight in this observation period. Dams did not show any other signs of sickness behaviour as shown by lethargy or piloerection. Moreover, no long-term effect of poly (I:C) exposure was shown on the progression of pregnancy as shown by gestational weight gain. Previous studies record contrasting findings in relation to weight change following mIA with poly (I:C). In agreement with this study, it has been shown that deficits in the offspring following mIA can be independent of maternal weight loss (Wolff and Bilkey, 2010). However, a number of studies have argued that maternal weight loss is a critical determinant of behavioural deficits in the mIA model (Zuckerman et al., 2003; Cunningham et al., 2007; Bronson et al., 2011; Ratnayake et al., 2014). It is likely that the variability in these findings results from the differences in species, strain, dose, dose route and the specific time period at which these experimental parameters were taken. Indeed, in the few studies mentioned here, both rats and mice have been used with dosing regimens of i.p. and i.v. ranging from 0.75-12 mg/kg. Furthermore, the husbandry and handling (particularly of pregnant rats) may greatly influence the responses recorded. While these factors were maintained and controlled carefully in this study, in order to reduce the influence of further maternal stress, these details are absent from the majority of publications (Table 7) and, therefore, cannot be accurately compared. The absence of these measures from the majority of previous studies poses a significant limitation to the progress of this field.

One interesting and unexpected finding from this study was the plasma IL-6 levels in pregnant dams following exposure to poly (I:C). Using the analytical methods described in this study, a complete absence of IL-6 upregulation at 3 hr post-poly (I:C) injection was shown for all six dams. It is known that this viral mimetic exerts an effect via the
activation of TLR-3. Critically, not only has it been shown that IL-6 is involved in the downstream pathway of TLR-3 activation, but it is a key cytokine for mediating the behavioural and transcriptional changes in offspring (Smith et al., 2007). Based on previous studies and other unpublished data from our lab, (including the pilot work discussed in Chapter 2), our hypothesis was that 3 hr following exposure to 10 mg/kg i.p. poly (I:C) an elevated level of IL-6 would be found in the plasma of these dams. As shown in Table 7, relatively few publications report plasma cytokine levels from dams following the induction of mIA preventing meaningful comparison of the effects of poly (I:C) in this study to other mIA models. In order to assess more fully the immune activation resulting from poly (I:C), with regard to cytokine upregulation, plasma level of TNFα and IL-1β was also measured in dams at 3 hr post-injection. While no change in IL-1β was observed, one dam showed an elevation of TNFα. Previous studies show lower levels of both IL-1β and TNFα in the maternal plasma following poly (I:C) exposure compared to IL-6 (Connor et al., 2012). Taken together, these data suggest that, in this study, exposure to 10 mg/kg i.p. poly (I:C) at GD12.5 did not produce sickness behaviour in pregnant dams as shown by cytokine levels, CBT or BW changes. However, as this was the first study in this thesis conducted in pregnant dams with 10 mg/kg i.p. poly (I:C) exposure, it is possible that the time course used to monitor sickness behaviour was insufficient to reveal any effects of mIA. Alternatively, the dose and route of administration may have influenced the physiological responses observed in the dam. As shown in Table 7, a number of previous studies use both i.p. and i.v. routes of administration suggesting that both are sufficient to induce mIA as measured by offspring outcomes. However, it is known that i.v. administration provides the most efficient means of delivery with increased precision and speed of action without the need for solute absorption (Turner et al., 2011). At this dose, therefore, the use of i.p. administration may be influencing the extent of sickness behaviour observed. The current lack of maternal cytokine data across the majority of mIA publications is a significant limitation to mechanistic understanding of mIA and the addition of standardised measurements of the induction of mIA is a key area for future development of these models and standardisation of the methodology (Careaga et al., 2017). As this is the first study in which 10 mg/kg i.p. poly (I:C) has been used at GD12.5, validation of these findings are required before definitive conclusions can be drawn.
3.5.2. At GD21 a sex-specific reduction in placenta weight was shown in offspring from dams that underwent mIA

Despite the absence of mIA following poly (I:C) exposure, as shown by sickness behaviour or plasma cytokines, a significant reduction in placenta weight was found in female offspring from poly (I:C) dams compared to the saline group. This placental change supports the effectiveness of 10 mg/kg i.p. poly (I:C) for the induction of mIA in Wistar rats. Sex-specific phenotypic changes have been recorded in many areas of animal modelling (Keeley et al., 2015) and are a critical aspect of preclinical work that is often ignored. Particularly in the area of ASD, where in the clinic there is an increased incidence in males, observation of female offspring in mIA models, are currently lacking. Further work is required to investigate changes to the placenta at the molecular level. Additional data from our lab has shown that 10 mg/kg i.p. poly (I:C) on GD15 results in a decrease in Dnmt1 expression in female placentas only (Potter et al., 2017). With a role in de novo methylation of gene promoters (implicated in gene silencing), the downstream effects of this reduction in expression remain to be elucidated. Furthermore, in a separate cohort of animals, a sex-specific reduction in the placental Tpbpa gene following poly (I:C) suggests an impairment in the development of the junctional zone in female offspring (Kowash et al., 2017). Analysis of gene expression in the placentas from this study is required to determine any developmental disruption caused by mIA at GD12.5 in addition to the reduction in weight. Evidence exists for the role of the placenta in mediating mIA, however, this has centred on the role of maternal IL-6 elevation and the effect on subsequent levels of essential developmental hormones such as growth hormone and insulin-like growth factor in the placenta (Hsiao and Patterson, 2011). Additional studies are required within our mIA model to determine the effects of 10 mg/kg i.p. poly (I:C) on the maternal foetal interface.

With regard to general growth measurements, no difference was recorded in BW or any morphological measurement taken at GD21. The offspring from poly (I:C)-treated dams showed no general growth restriction or delay compared to pups from saline-treated dams. BrW was similarly unchanged with maternal exposure to poly (I:C) in this study. Interestingly, one litter from a dam treated with poly (I:C) were notably smaller with regard to BW and BrW. This dam was the only dam in this study to record an elevated level of TNFα as shown by the ELISA. Thus, is it possible that this immune activation
disrupted the progression of development in these pups, although further investigation is required to determine any possible mechanism of heightened immune activation with reduced body and brain weight. The clinical findings with regard to brain volume and weight, as well as head circumference, are contrasting. Evidence does exist for increased brain weight and head circumference in young children with ASD that normalises with increasing age (Aylward et al., 2002). The methods employed to monitor brain changes as a result of poly (I:C) exposure in this study are limited and provide only a rudimentary assessment of general development. In order to further determine the effects of mIA on the development of the rat brain, it will be necessary to perform anatomical analysis and more detailed neuronal analysis using Wes™ or immunohistochemistry later in development.

3.5.3. mIA at GD12.5 resulted in specific gene expression changes that were dependent on sex and brain region

In this study, exposure to mIA did not result in any notable maternal immune response as measured via cytokine upregulation, although gene expression changes in the foetal brain were found. Previous studies have shown, however, that IL-6 upregulation following poly (I:C) is necessary for the resulting phenotypes. In this study, and with respect to changes in mRNA in the foetal brain, upregulation of maternal IL-6 following poly (I:C) may not be necessary. This conclusion is made with caution because it is possible that the IL-6 upregulation following poly (I:C) was simply not detected using the ELISA, or the time course of IL-6 upregulation in these dams was different to the time point used in this experiment. However, it has been shown previously in an intrauterine inflammation model that changes in the offspring are not always dependent on maternal cytokine upregulation. Elovitz et al. 2011 reported that exposure to LPS at GD15 caused no significant upregulation of maternal plasma IL-6 when the placenta cytokine response of IL-1β, IL-6 and TNFα were significantly increased. Added to this, significant immune responses were recorded in the foetal brain alongside gene expression changes related to white matter damage and the glutamate-glutamine cycle (Elovitz et al., 2011). Only with additional studies using 10 mg/kg i.p. poly (I:C) at GD12.5 can further conclusions on the maternal cytokine response be made.

A number of studies have explored cytokine upregulation in brain tissue from neonatal offspring at varying time points following mIA induction. Since our brain analysis was
performed eight days following poly (I:C) exposure in the dams (and the inflammatory response is short-lived), cytokine elevations was not explored in the offspring. Due to the variable results observed in the dam’s plasma with regard to inflammatory markers, it is suggested that measurement of cytokine levels in brain tissue and the placenta would be a relevant development for the work presented here. Although much evidence already exists for elevation of cytokine levels in the neonatal brain, these changes are likely to be dependent on species, strain and gestational exposure. These data may further the understanding of the response to mIA in Wistar rats at this gestational time point.

Consistent with current literature proposing a synaptic basis for NDDs (Sarkar et al., 2017) some of the changes in gene expression observed in our rat model of mIA converge on impaired synaptic development. The SNARE protein SNAP-25 is involved in the assembly of the trans-SNARE complexes which are integral to vesicle fusion and regulated exocytosis. SNAP-25 is also associated with some specific voltage-gated calcium channels, thus holding an integral role in controlled neuron function. The altered expression of the SNAP-25 gene and subsequent protein has been associated with neuropsychiatric disorders such as ADHD, ASD and schizophrenia (Thompson et al., 1998; Corradini et al., 2009; Braida et al., 2015). In this study, 10 mg/kg i.p. poly (I:C) at GD12.5 resulted in a down regulation of SNAP-25 at GD21. In male offspring this down regulation was present in both FC and CB at this time point, whereas in female offspring this gene was shown to be altered in the CB only. Although the functional relevance of this gene expression change requires further investigation, the down regulation of SNAP-25 may affect neuronal activity via the exocytosis of neurotransmitters (Noor and Zahid, 2016) or circuitry by changes to axonal growth or dendritic spine morphogenesis (Osen-Sand et al., 1993; Tomasoni et al., 2013). In addition, a reduction in the expression of astrocytic marker GFAP in the CB of male offspring only was observed. A similar reduction in expression of GFAP has been shown previously in an LPS model of mIA in mice (Arsenault et al., 2014). A reduction in the microglial marker OLMFL3 was also shown in male offspring. These gene expression changes may highlight changes in the inflammatory status of the brains of male offspring. However, analysis of mRNA levels alone prevents further definitive conclusions to be made. Protein concentration is affected by different steps in transcription, translation as well as degradation and future studies should be the targeted
towards protein changes in this model. Confirmation of sexually dimorphic changes in the brains offspring are of particular importance owing to the lack of appreciation for sex differences in the development of robust and translatable animal models (Leger and Neill, 2016).

At GD21, the small size of the rat pup brains present a number of difficulties for analysis including accurate isolation of specific brain areas and the small amounts of tissue that are viable for processing. Conflicting literature exists on microglial activation in NDDs, where some have suggested that a second postnatal challenge following mIA is required to induce microglial activation in the offspring (Smolders et al., 2015). The down regulation of GFAP and OLFML3 in the CB of male offspring provides limited information on the effects of mIA on glia. However, this encourages further investigation of these genes at later developmental time points, particularly in light of the fact that this expression change was sex-dependent.

In conclusion, since only a limited number of studies to date have explored embryonic time points in the mIA models of relevance to ASD, the work presented in this chapter provides further insight into gene expression changes present in this mIA model. In order to determine the functional relevance of the gene expression changes highlighted here, confirmation of specific protein expression is required. It should also be considered that, in this study, a targeted approach was used for determining the genes of interest and this may have resulted in important expression changes being missed in the analysis. While the current study investigated certain genes in each pathway in the neonatal brain, providing limited information regarding overall gene expression patterns, the data presented has highlighted target proteins, which are specific to sex, that should be explored within this model. A number of the significant changes in offspring following mIA were sex-dependent, highlighting the importance of investigating both sexes. In order to accurately determine the effects of 10 mg/kg i.p. poly (I:C) on sickness behaviour in pregnant dams at GD12.5, additional studies are required (for example, adding other time points for IL-6 measurement). Furthermore, investigation of later developmental time points will enable the longitudinal effects of poly (I:C) on offspring to be determined, and these will be the focus of the next chapter.
Chapter 4: Analysis of neurodevelopmental changes in the postnatal period following mIA
4.1. Introduction

To continue the investigation into early developmental changes in this mIA model, analysis of offspring was conducted in the early adolescent, post-natal period. Often, analysis at the molecular level is included following the application of a behavioural test battery resulting in a majority of work being performed in adulthood (Zuckerman et al., 2003; Onore et al., 2014; Buschert et al., 2016; da Silveira et al., 2017). Although this analysis is crucial for mechanistic understanding of behavioural correlates, there is a critical need for investigation of early developmental changes which may predispose to the altered behavioural phenotypes.

A number of previous studies that have investigated changes in the adolescent postnatal brains of mIA offspring have been focused on schizophrenia pathologies. This is likely due to the growing evidence showing that adolescence is a key period for the appearance of schizophrenia symptomatology. A number of studies have investigated these developmental pathologies. In a model using repeated gestational exposure to poly (I:C) at GD14, 16 and 18 in Wistar rats, a reduction in the expression of GluN1 N-methyl-D-aspartate receptor (NMDAR) subunit was found in the cerebral cortex of PD21 mIA exposed offspring (sex of pups not stated) (Forrest et al., 2012). Changes in the expression of the metabotropic glutamate receptor 5 (mGluR5) have also been reported at early developmental stages (PD10) with increased expression shown in offspring from repeated LPS and poly (I:C) exposure on GD15-GD17 in the mouse (Arsenault et al., 2014). Coupled with these changes related to glutamate neurotransmission, further investigation of Wistar rat offspring in the adolescent period has detected synaptic dysfunction and increased cannabinoid receptor expression similarly related to schizophrenia pathophysiology (Oh-Nishi et al., 2010; Verdurand et al., 2014). There is a requirement for further analysis of early development across the various mIA models involving different gestational exposure of LPS or poly (I:C). In this study, gene expression was investigated using the same methodology described for the analysis at GD21. The same genes and brain regions of interest were investigated in order to determine any effects that were sustained into the postnatal period or indeed that only appeared at this developmental stage.
4.1.1. Gut microbiota

The importance of alterations in gut pathophysiology in human ASD has already been discussed (see section 1.1.3, p. 27) with a number of changes reported in composition of the gut microbiota in patients with ASD (see Table 16). In order to determine potential changes to gut function in models of mIA, it is important to assess any changes to the gut microbiota in offspring.

Table 16: Gut microbiota changes in patients with ASD (table adapted from Fung et al., 2017)

<table>
<thead>
<tr>
<th>Gut microbiota change</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Increased:</strong> <em>Lactobacillus, Desulfovibrio, Clostridium</em></td>
<td>Tomova et al., 2015</td>
</tr>
<tr>
<td><strong>Decreased:</strong> <em>Bacteroides / Firmicutes</em>, ratio</td>
<td></td>
</tr>
<tr>
<td><strong>Increased:</strong> <em>Sutterella</em></td>
<td>Wang et al., 2013</td>
</tr>
<tr>
<td><strong>Increased:</strong> <em>Clostridium, Bacteroides, Porphyromonas, Prevotella, Pseudomonas, Aeromonas, Enterobacteriaceae</em></td>
<td>De Angelis et al., 2013</td>
</tr>
<tr>
<td><strong>Decreased:</strong> <em>Enterococcus, Lactobacillus, Streptococcus, Lactorcoccus, Staphylococcus, Bifidobacteria</em></td>
<td></td>
</tr>
<tr>
<td><strong>Increased:</strong> <em>Lactobacillus</em></td>
<td>Kang et al., 2013</td>
</tr>
<tr>
<td><strong>Decreased:</strong> <em>Prevotella, Coprococcus, Veillonellaceae</em></td>
<td></td>
</tr>
<tr>
<td><strong>Increased:</strong> <em>Sutterella, Lachnospiraceae, Ruminococcaceae</em></td>
<td>Williams et al., 2011</td>
</tr>
<tr>
<td><strong>Increased:</strong> <em>Lactobacillus</em></td>
<td>Adams et al., 2011</td>
</tr>
<tr>
<td><strong>Decreased:</strong> <em>Bifidobacterium, Enterococcus</em></td>
<td></td>
</tr>
<tr>
<td><strong>Increased:</strong> <em>Bacteroidetes, Proteobacterium</em></td>
<td>Finegold et al., 2010</td>
</tr>
<tr>
<td><strong>Decreased:</strong> <em>Actinobacterium, Bifidobacterium</em></td>
<td></td>
</tr>
<tr>
<td><strong>Increased:</strong> <em>Clostridium</em> clusters 1 and 2</td>
<td>Parracho et al., 2005</td>
</tr>
</tbody>
</table>

A standard method for analysis of the gut microbiota is metagenetic sequencing using the prokaryotic 16S ribosomal gene (16S rRNA). This gene contains variable regions interspersed between conserved regions, and the variable regions of the 16S rRNA can be used in phylogenetic classifications in humans and rodents (Tap et al., 2009; deTheije et al., 2014b). At the time of writing, only a few studies were found to have explored changes to the gut microbiota in mouse models relevant to ASD including genetic (Coretti et al., 2017) and maternal insult using VPA (deTheije et al., 2014b) and...
poly (I:C) (Hsiao et al., 2013). Hsiao et al., 2013 provided convincing evidence of an ASD-like behavioural phenotype in mice injected with poly(I:C) at GD12.5 alongside an increase in intestinal permeability which was detectable at three weeks of age. Moreover, dysbiosis of the gut microbiota was reported as measured by changes in the diversity of both Clostridia and Bacteroidia bacteria (Hsiao et al., 2013). These findings stimulated our investigation of gut microbiota changes as part of the validation of the GD12.5 mIA model in Wistar rats.

4.2. Chapter 4 Summary and Objectives

The target genes outlined in Chapter 3 were analysed in the FC and CB of PD21 offspring. This was performed in an attempt to assess any sustained changes in gene expression and to determine the appearance of any subsequent gene expression changes in the postnatal period relevant to ASD in both male and female offspring. This more longitudinal approach to the assessment of NDD models is critical but performed less often (Samuelsson et al., 2006; Shi et al., 2009; Coiro et al., 2015). The analysis of the gut microbiota of offspring in the mIA model was employed to establish any effect of in utero poly (I:C) on altered development of the gut microbial environment.

4.2.1. Objectives

- Induce mIA using 10 mg/kg i.p. poly (I:C) at GD12.5 and investigate acute sickness behaviour in dams including cytokine analysis at 3 hr post-injection.

- Determine any effect of mIA on the social communication behaviour of offspring in early postnatal development.

- Harvest brain tissue from pups at PD21 and perform gene expression analysis on both FC and CB using specific genes of interest as defined and investigated in Chapter 3.

- Collect faecal boles from the offspring at PD21 and determine any changes in the microbial composition between treatment groups using metagenetic sequencing.
4.3. Materials and methods

4.3.1. Experimental design

Experiments were performed using male and female Wistar rats (Charles River Laboratories, UK). All rats were housed under the conditions described in Chapter 2 section 2.2.1, p.71. For mating $n=5$ male and $n=16$ female rats were used. Only 14 of these pairs resulted in successful pregnancy and, therefore, only 14 dams underwent mIA or the respective control condition ($n=8$ saline, $n=6$ poly (I:C), see section 2.5.2 p.82). Total offspring numbers from these dams were, $n=31$ males and $31$ females in the saline group, $n=23$ males and 22 females in the poly (I:C) group. Figure 31 shows an outline of the experimental design used in this study.

Figure 31: Timeline of experimental design. GD= gestational day, PD= postnatal day, CBT= core body temperature, RT-qPCR = reverse transcriptase, quantitatitive real-time polymerase chain reaction.
4.3.2. Offspring

4.3.2.1. Litter numbers

On postnatal day (PD) 1 (PD0 = day of parturition), pups were counted and total litter numbers (including ratio of males to females) were recorded. In accordance with the principles of the 3Rs and to correct limitations of previous studies, both male and female offspring were used. However, following counting, in order to control for growth differences due to different litter sizes (Azar et al., 1991), all of the litters were culled to contain only 4 males and 4 females. For this procedure, the dam was first removed from the cage to prevent undue stress. Pups were carefully removed from the nest for counting and sexing. The sex of each pup was determined by assessing external anatomy (Figure 32). After 4 males and 4 females had been counted, these pups were replaced in the nest or cage as they had been found. The mother was then returned to the cage and monitored briefly. In all cases, mothers did not show any abnormal behaviour when returned to their litters in the nest.

There were differences in the ratio of males and females between litters and, in some cases, there were not sufficient pups to obtain $n=4$ of each sex. In two litters (FB1 and FB3, both saline-treated dams), an extra pup of the opposite sex was kept with the dam in attempt to control the growth rate of the offspring across litters and keep litter sizes consistent.

![Male and Female Pups](image)

**Figure 32: Anatomy of rat pups at PD1.** Male offspring shown on the left have more pronounced genitalia and a gap (approx. 0.5cm) between these and the anus. Female offspring shown on the right have less pronounced genitals with a smaller distance between the genitals and anus.
4.3.2.2. Animal identification

During early development, pups were identified using non-toxic permanent marks on one of their hind paws (Table 17). Due to nursing and allogrooming from the mothers, these marks quickly faded and were redrawn every three days. It is known that maternal care (including licking and grooming) can have an effect on the development of offspring (Liu et al., 2000). Therefore, all pups had the foot marks redrawn even when marks were still visible to prevent any covariate effect of some pups being handled or marked more frequently than others. The mother was not removed from the cage during this time to prevent any effect of maternal separation during early development and pups were handled as little as possible.

Table 17: Animal identification during early postnatal life

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Colour ID</th>
<th>Paw</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>Black</td>
<td>Hind Left</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Blue</td>
<td>Hind Right</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>Purple</td>
<td>Hind Right</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>Red</td>
<td>Hind Left</td>
</tr>
</tbody>
</table>

4.3.2.3. Body weight monitoring

BW was recorded from all pups throughout early development, specifically at PD1, 12, 18 and 21. Otherwise pups were left undisturbed with their mothers. As described in (Chapter 3 section 3.3.4, p.96), weight distribution curves were constructed for these postnatal weights.

4.3.3. Ultrasonic vocalisations

At PD9, each pup was tested in an isolation paradigm where ultrasonic vocalisations (USVs) were monitored in the individual pup as previously described (Hofer et al., 2002; Baharnoori et al., 2012; Choi et al., 2016b). 15 min prior to the start of the test the dam was removed from the home cage and placed in a separate cage on the opposite side of the test room. The dam was given the tube, shelf (including food hopper) and lid.
from her home cage in attempt to reduce the stress from being placed in a novel environment away from the litter. The litter were left in the nest and not disturbed at this time. The nest was placed on a heating-pad set to the lowest temperature. The pups were left undisturbed for 15 min after removing the dam from the nest to ensure steady-state conditions in the litter. The test chamber (28 cm L; 18 cm W; 13 cm H, made from Plexiglas) was prepared with wood chippings covering the floor. A Batbox Duet (Batbox.com) tuneable bat detector was suspended approximately 15 cm above the test chamber floor. The detector’s frequency range was set at 50 kHz. The bat detector was connected to a recorder (Roland, R-05) with a sampling rate of 96 kHz.

For the experiment, each pup was taken in turn at random from the nest and placed gently in the centre of the test chamber. The USVs were then recorded for 3 min. At the end of the test, the pup was removed, weighed and the sex and paw marking noted. This was repeated for each pup using a fresh, clean test chamber for each animal.

Files were analysed using BatScan 9 software (NHBS, UK) to produce a spectrogram of each test. These spectrograms (Figure 33) were used to manually score the number of USV calls produced during the 3 min test in the range of 30-60 kHz (highlighted on Figure 33).

**Figure 33: Example spectrogram from BatScan software** (data taken from male pup, litter FD3). Each single call (blue arrow) shown in the spectrogram was counted for analysis. Only calls in the range of 30-60 kHz were included (red arrow showing excluded call).
4.3.4. PD21 experiment

4.3.4.1. Tissue collection

At PD21 all pups were anaesthetised (4%) and maintained (1.5–2%) with isoflurane delivered in 70:30 N₂O/O₂. Each pup was transcardially-perfused with 0.1% Diethyl pyrocarbonate (DEPC; D5758; Sigma, UK) treated sterile saline for 8 min. The saline was incubated with DEPC for 1 hr before autoclaving. Brains were removed, weighed and preserved in RNA later. After incubation overnight, brains were frozen in 2-Methylbutane (Fisher Scientific) before storing at -80 °C. Brains were later regionally dissected and the FC and CB were collected for processing.

A faecal sample was collected from the large intestine of each pup using aseptic method prior to the perfusion. These were stored in individual Eppendorf tubes and immediately frozen in 2-Methylbutane before storing at -80 °C.

4.3.5. RNA extraction, Quantitative real time PCR and Reverse transcriptase polymerase chain reaction (RT-PCR)

RNA extraction and quantitative RT-qPCR were performed on PD21 FC and CB as described in Chapter 3 sections 3.3.5-3.3.7, pp.96-99.

4.3.6. Analysis of faecal microbiota

4.3.6.1. Faecal DNA extraction

Faecal DNA extraction was performed using the QIAamp® Fast DNA stool mini kit (Qiagen, Cat. No. 51604) and the protocol was performed according to manufacturer’s instructions. 180–220 mg of the frozen faecal bole collected from each of the PD21 offspring was placed on ice in an individually labelled Eppendorf tube. 2 ml of InhibitEX buffer was added to each faecal sample. The tubes were vortexed continuously until the sample was thoroughly homogenized. This step was performed to ensure a maximum DNA concentration was collected in the final elutes. All samples were centrifuged for 1 min at 14,000 rpm to pellet faecal particles. A new, labelled 1.5 ml microcentrifuge tube was prepared for each sample and 25 μl of Proteinase K was added. 600 μl of supernatant from the original sample tube was pipetted into the 1.5 ml microcentrifuge tube containing Proteinase K. To this new tube, 600 μl buffer AL was
added and vortexed for 15 sec to form a homogenous solution. The samples were then incubated at 70 °C for 10 min in a water bath.

After 10 min, 600 μl of ethanol absolute (96–100%) was added to the lysate and mixed by vortexing. 600 μl of this lysate was loaded into a QIAamp spin column and centrifuged for 1 min at 14,000 rpm. Following centrifugation, the QIAamp spin column was placed in a new 2 ml collection tube and the filtrate discarded. This step was repeated until all of the lysate had been loaded into the column. 500 μl buffer AW1 was loaded into each QIAamp spin column and again centrifuged for 1 min. The QIAamp spin column was placed in a new 2 ml collection tube and the filtrate discarded. This step was repeated with 500 μl buffer AW2 loaded into the QIAamp spin column. Centrifugation for this step was performed for 3 min and the resulting filtrate discarded. The QIAamp spin column was transferred into a new 2 ml microcentrifuge tube, and 400 μl buffer ATE was flushed directly onto the QIAamp membrane. Each column was incubated for 1 min at room temperature. A final centrifugation step was performed for 1 min to elute DNA. Excluding the initial faecal bole homogenisation, all steps were performed at room temperature (15–25 °C) and all centrifugation steps were performed at 14,000 rpm. DNA was also extracted from a sample of E. Coli that was grown on an agar plate overnight in a cabinet incubator (37 °C) and the following morning a few colonies were homogenised in 2 ml of InhibitEX buffer. 200 ul of this homogenised solution was processed using the protocol described above from the first centrifugation step.

4.3.7. 16S microbial sequencing

The extracted DNA samples were sent to the Centre for Genomic Research at the University of Liverpool for metagenetic processing on the Illumina MiSeq® platform.

4.3.7.1. 16s dual index nested PCR and sequencing on the MiSeq® System

The required universal amplicon primers were added to each DNA sample to amplify the V4 region of the 16S rRNA gene. These target-specific sequences were taken from (Caporaso et al., 2011). The designed primer sequences for the 16S rRNA gene amplicon PCR are listed below.
Forward Primer =
5'ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNGTGCCAGCMGCCGCGGTAA3' 

Reverse Primer =
5'GTGACTGGAGTTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWC TAAT3'.

First stage PCR was run using an automated thermal cycler and the protocol followed as described by Illumina. All primers were ordered as lyophilised stocks (Integrated DNA Technologies) and made up to 100 uM in dH₂O before being further diluted to 10 uM working concentration. An excess of the master mix (see Table 18) was made up for the first PCR reaction and 15 ul was added to each well of the 96 plate. Each DNA sample was diluted to a concentration of 1 ng/ul. 5 ul was added to the well to create a 20 ul PCR reaction with an input of 5 ng of DNA. The PCR cycling conditions for amplification are listed below in Table 19.

Table 18: Reagents for first stage PCR

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
<th>Concentration</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Kapa 2x Master Mix</td>
<td>10</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Forward Primer</td>
<td>0.25</td>
<td>10 μM</td>
<td></td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>0.25</td>
<td>10 μM</td>
<td></td>
</tr>
<tr>
<td>dH₂O</td>
<td>4.5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>15</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 19: PCR cycling conditions

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td>98</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>20 sec</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>15 sec</td>
<td>x15</td>
</tr>
<tr>
<td>70</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>
Samples were then purified from PCR by-products (free primers and primer dimer species) with Axygen SPRI Beads before entering the second PCR. This second stage was performed to incorporate Illumina adapter sequences containing indexes (i5 and i7) for sample identification. The original primer design incorporated these recognition sequences to allow a secondary nested PCR process (these sections of the primer are highlighted above in red). 20 cycles of PCR were performed using the same conditions as described in Table 19.

Samples were again purified using Axygen SPRI Beads. These final libraries were pooled in equimolar amounts using the Qubit and Fragment Analyzer data and size selected on the Pippin prep (Sage Science) using a size range of 300-600 base pairs (bp). The quantity and quality of each pool was assessed by Bioanalyzer and, subsequently by qPCR. This was performed using the Illumina Library Quantification Kit from Kapa on a Roche Light Cycler LC480II according to manufacturer's instructions. Successfully generated amplicon libraries were taken forward for sequencing. The pool of libraries was sequenced on one lane of the MiSeq® System at 2x250 bp paired-end sequencing. To help balance the complexity of the amplicon library 15% PhiX was spiked in. A second pool was made and a further sequencing lane was run as described above. This was due to the low sequence coverage of two libraries obtained in the first run that were below desired clustering levels on the flow-cell during sequencing of the original pool. The data was then extracted and analysed using the open-source bioinformatics pipeline Quantitative Insights into Microbial Ecology (QIIME).

4.3.8. Analysis using QIIME

Following the barcoded amplicon sequencing of samples on the MiSeq platform, the GreenGenes database (DeSantis et al., 2006) was used in QIIME to assign species equivalent operational taxonomic units (OTUs) to the samples. These OTUs are reference sequences that can be used to produce a phylogenetic tree of the microbiota in these samples. DESeq2 phyloseq was used to determine the significance of abundance changes of OTUs between treatment groups following data stabilisation transformation (McMurdie and Holmes, 2013).

Beta diversity analysis was performed in phyloseq 1.13.2 and comprised non-metric multidimensional scaling (NMDS) ordination of Bray distances following a log
transformation of the data. Bray distance matrices were further analysed using ADONIS in order to explain the variation by mapping file categories. Alpha diversity metrics were generated from raw datasets using the “estimate_richness” command in phyloseq.

4.3.9. Specific experimental analyses

4.3.9.1. Litter comparisons

To determine the effects of treatment on offspring, all 8 animals per litter (4 male and 4 female where possible) were used. A mixed-effects model, (nested-ANOVA), was performed. Treatment was used as a between-subject fixed factor and litter, as a random variable to account for any confounding effect of the dam (IBM SPSS Statistics Version 22). Throughout development in utero, each pup is contained in an individual amniotic sack and receives nutrition from a single placenta. These pups were considered, therefore, to have an individual exposure to the effects of poly (I:C) at GD12.5. Thus, each pup was considered a separate individual. Nesting of the offspring in litters enabled appropriate handling of the data without artificially inflating the sample size (Lazic and Essioux, 2013). Where data sets did not fulfil the assumptions for nested parametric data analysis, non-parametric analyses were conducted on litter means using Mann-Whitney U test (GraphPad Prism 7).

4.3.9.2. RT-qPCR

Due to feasibility of testing, only 2 pups from each sex and treatment group were selected from each dam for RNA extraction and subsequent RT-qPCR. These pups were selected at random from saline and poly (I:C) litters.

For the analysis of qPCR the REST programme 2009 was used to calculate the relative expression values as described in Chapter 3, section 3.3.8.2 p.103.
4.4. Results

4.4.1. mIA at GD12.5 resulted in sickness behaviour as shown by CBT and IL-6 elevation

Following administration of 10 mg/kg i.p. poly (I:C) on GD12.5 CBT and BW were monitored at 3, 6 and 24 hr. CBT was elevated at 3 hr post-injection. Repeated measures ANOVA showed a significant interaction of treatment by time $F_{(3, 36)} = 4.60, p= 0.008$. At 3 hr post-injection poly (I:C) dams had a significantly higher CBT compared to saline treated dams ($p= 0.0271$). At 24 hr post-injection CBT had returned to baseline level in all dams. Saline dams showed a small but significant reduction in CBT throughout the day. This reduction from 36.5 °C to 36 °C was present at 3 and 6 hr, $p =0.0421$ and $p= 0.0372$ respectively. When the data was normalised to baseline, the same significant interaction of treatment by time was shown. Post-hoc Bonferroni multiple comparison test revealed a significant increase in CBT at 3 hr post-injection.

A significant reduction in BW was shown by the repeated measures ANOVA for the factor time $F_{(3, 36)} = 41.27, p= 0.0001$. However, no difference between treatment groups was shown in post-hoc Bonferroni test. Both saline and poly (I:C) ($p= 0.0001$) dams showed a reduction in BW at 6 hr post-injection compared to baseline which is standard for rats during the light cycle due to reduced food intake. At 24 hr post-injection, BW from both treatment groups showed increased BW. Saline dams showed a significant increase in BW gain at 24 hr compared to baseline ($p =0.0017$). These effects were shown in both the raw data and in the baseline-corrected temperature data.

Plasma IL-6 levels measured at 3 hr post-injection were significantly elevated in the poly (I:C) compared to the saline treatment group ($p= 0.0182$). However, an undetectable level of IL-6 was recorded in 2 poly (I:C) dams. In all of the saline dams the level of IL-6 measured in blood plasma was below the level of detection.
Figure 34: Effect of 10mg/kg poly (I:C) at GD12.5 on pregnant dams. A-B) At 3 hr post-injection poly (I:C) dams showed a significantly higher core body temperature (CBT) compared to saline treated dams (A). Saline dams showed a significant reduction in CBT at 3 and 6 hr from baseline. These effects were maintained when normalised to baseline (B). C-D) A significant reduction in body weight (BW) was shown at 6 hr post-injection in both saline- and poly (I:C)-treated dams compared to baseline with no difference between treatment groups. (D) At 24 hr post injection, saline- but not poly (I:C)-treated dams showed significantly increased BW compared to baseline. (* = vs. saline group, # = vs. baseline s. = saline group, p. =poly (I:C), */# p< 0.05, **/## p< 0.01, ###/#### p<0.001.) Graphs represent mean ±SEM, n= 8 saline dams and n= 6 poly (I:C) dams.
4.4.2. mIA had no effect on gestational weight gain or litter number

Dams gained 125 g on average during gestation, from GD8 to GD21. Two-way ANOVA revealed no significant interaction of treatment by day. A significant main effect of day was produced $F_{(14, 90)} = 140.0, p < 0.0001$ but no main effect of treatment $F_{(1, 90)} = 1.32, p = 0.2541$. 10 mg/kg i.p. poly (I:C) at GD12.5 did not affect the gestational weight gain of dams (Figure 35).

![Gestational weights](image)

Figure 35: mIA had no effect on gestational weight of pregnant dams. No significant difference in gestational weight was seen between saline and poly (I:C) dams. Graphs represent mean ±SEM, $n = 8$ saline dams and $n = 6$ poly (I:C) dams.

At PD1 (PD0= day of birth) litter numbers from all dams were recorded (Table 20). The offspring were also sexed at this time to determine the distribution of male and female offspring between treatment groups. The average litter size was 14 ±1 for saline and 16 ±1 (mean ±SEM) for poly (I:C) dams and no significant difference was found between total litter numbers ($p = 0.383$) (Figure 36A). A one-way ANOVA revealed no difference in the sex distribution of offspring between treatment groups $F_{(3, 24)} = 1.48, p = 0.250$ (Figure 36B).
Table 20: Litter numbers and sex distribution including corresponding IL-6 level

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dam ID</th>
<th>Litter number</th>
<th>Male</th>
<th>Females</th>
<th>IL6 Level (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>FE2</td>
<td>16</td>
<td>6</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>FD3</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>FH3</td>
<td>16</td>
<td>9</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>FG4</td>
<td>18</td>
<td>8</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>FB1</td>
<td>9</td>
<td>6</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>FB3</td>
<td>10</td>
<td>3</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>FA3</td>
<td>16</td>
<td>7</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>FC4</td>
<td>16</td>
<td>9</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Poly (I:C)</td>
<td>FG1</td>
<td>17</td>
<td>9</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>FG2</td>
<td>14</td>
<td>8</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>FB4</td>
<td>14</td>
<td>7</td>
<td>7</td>
<td>129.1</td>
</tr>
<tr>
<td></td>
<td>FA4</td>
<td>17</td>
<td>11</td>
<td>6</td>
<td>335.8</td>
</tr>
<tr>
<td></td>
<td>FC3</td>
<td>14</td>
<td>8</td>
<td>6</td>
<td>588.7</td>
</tr>
<tr>
<td></td>
<td>FG3</td>
<td>19</td>
<td>10</td>
<td>9</td>
<td>755.5</td>
</tr>
</tbody>
</table>

Figure 36: No effect of mIA was shown on litter size or sex distribution within litters. A-B) No significant difference was found between litter sizes (A) or numbers of males and female pups within a litter (B) from saline and poly (I:C) treated dams. Graphs represent group mean ±SEM and individual litter means, n = 8 saline dams and n = 6 poly (I:C) dams.
4.4.3. Offspring from dams exposed to mIA showed a significant reduction in body weight throughout early postnatal life

All offspring were weighed regularly throughout early postnatal life PD 1, 12, 18 and 21. At every time point, a significant reduction in BW was found in poly (I:C) offspring compared to saline (Figure 37). Male and female offspring were similar in weight within treatment groups during early postnatal time points (see Table 21 for all early postnatal weights).

Table 21: Early developmental body weights

<table>
<thead>
<tr>
<th>Postnatal Day</th>
<th>Saline</th>
<th>Poly (I:C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>PD1</td>
<td>7.2 ±0.6</td>
<td>6.6 ±0.6</td>
</tr>
<tr>
<td>PD12</td>
<td>34.1 ±3.0</td>
<td>32.8 ±2.4</td>
</tr>
<tr>
<td>PD18</td>
<td>52.7 ±3.1</td>
<td>50.1 ±3.2</td>
</tr>
<tr>
<td>PD21</td>
<td>66.6 ±3.8</td>
<td>63.0 ±3.7</td>
</tr>
</tbody>
</table>
Figure 37: mIA at GD12.5 resulted in a reduction in early postnatal body weight (BW) of offspring. A-D) A significant decrease in BW was seen at all postnatal days monitored (PD1, 12, 18, 21) between saline and poly (I:C) pups (* vs. saline *** p<0.001). Graphs represent group mean ±SEM and individual pup values, saline n=31 males and 31 females, poly (I:C) n= 23 males and 22 females.
4.4.4. Offspring from dams exposed to mIA recorded a change in brain weight relative to body weight

In male poly (I:C) offspring a significant reduction was found in brain weight (BrW) compared to saline $F_{(1, 40)} = 16.2$, $p < 0.001$. The difference between mean BrW of saline and poly (I:C) offspring was $41 \pm 1.68$ mg for male offspring. For female offspring a non-significant mean difference of $16 \pm 1.96$ mg was shown $F_{(1, 39)} = 1.5$, $p = 0.23$ (Figure 38A). Brain weight is also presented as a proportion of body weight (Figure 38B). Both male and female offspring from poly (I:C) dams showed increased BrW as a proportion of BW, $F_{(1, 40)} = 11.3$, $p = 0.002$, $F_{(1, 39)} = 14.8$, $p = <0.001$ male and female respectively.
Figure 38: mIA GD12.5 resulted in asexually dimorphic reduction in brain weight (BrW) at PD21. 

A) A significant decrease in BrW was seen in male but not female offspring from poly (I:C)-treated compared to saline-treated groups. 

B) BrW as a proportion of BW showed a significant increase in both male and female offspring from poly (I:C)-treated compared to saline-treated dams (* vs. saline ** p<0.01, *** p<0.001). Graphs represent group mean ±SEM and individual pup values, saline n= 31 males and 31 females, poly (I:C) n= 23 males and 22 females.
4.4.5. RT-qPCR

4.4.5.1. mIA at GD12.5 resulted in sex-specific gene expression changes in the frontal cortex of offspring at PD21

No difference was found in the reference HK genes SDHA and GAPDH, and mean CT values were compared between treatment groups (Figure 39). In male offspring a significant upregulation of the SHANK3 (synaptic scaffold) gene was shown. In female offspring, a significant downregulation of the gene MFSD2A (BBB integrity) was found. No other changes in gene expression were found in the FC of PD21 poly (I:C) offspring as shown in Table 22 and Table 23.

Figure 39: mIA had no effect on housekeeping gene CT values from frontal cortex. A-B) No difference was found in the CT values between treatment groups in male or female offspring. Graphs represent mean ±SEM, n= 12/sex for saline offspring and 16/sex for poly (I:C) offspring.
Table 22: Gene expression changes in the frontal cortex of PD21 male pups (- no change, ↑ upregulation, ↓ downregulation)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Expression</th>
<th>SEM</th>
<th>P value</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHA</td>
<td>HK</td>
<td>0.982</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>HK</td>
<td>1.018</td>
<td>0.56-3.45</td>
<td>0.011</td>
<td>↑</td>
</tr>
<tr>
<td>SHANK3</td>
<td>Target</td>
<td>1.78</td>
<td>0.45-1.78</td>
<td>0.215</td>
<td>-</td>
</tr>
<tr>
<td>MEF2C</td>
<td>Target</td>
<td>1.379</td>
<td>0.53-1.68</td>
<td>0.618</td>
<td>-</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Target</td>
<td>0.914</td>
<td>0.50-1.83</td>
<td>0.887</td>
<td>-</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Target</td>
<td>0.977</td>
<td>0.41-2.48</td>
<td>0.673</td>
<td>-</td>
</tr>
<tr>
<td>SEMA3A</td>
<td>Target</td>
<td>0.931</td>
<td>0.44-1.67</td>
<td>0.980</td>
<td>-</td>
</tr>
<tr>
<td>MFSD2A</td>
<td>Target</td>
<td>1.008</td>
<td>0.43-1.61</td>
<td>0.446</td>
<td>-</td>
</tr>
<tr>
<td>GFAP</td>
<td>Target</td>
<td>0.864</td>
<td>0.54-2.14</td>
<td>0.241</td>
<td>-</td>
</tr>
<tr>
<td>OLFML3</td>
<td>Target</td>
<td>0.805</td>
<td>0.35-1.56</td>
<td>0.831</td>
<td>-</td>
</tr>
<tr>
<td>5HT2A</td>
<td>Target</td>
<td>1.038</td>
<td>0.63-2.40</td>
<td>0.167</td>
<td>-</td>
</tr>
<tr>
<td>GAD1</td>
<td>Target</td>
<td>0.758</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 23: Gene expression changes in the frontal cortex of PD21 female pups (- no change, ↑ upregulation, ↓ downregulation)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Expression</th>
<th>SEM</th>
<th>P value</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHA</td>
<td>HK</td>
<td>0.865</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>HK</td>
<td>1.156</td>
<td>0.36-1.67</td>
<td>0.945</td>
<td>-</td>
</tr>
<tr>
<td>SHANK3</td>
<td>Target</td>
<td>0.983</td>
<td>0.46-2.11</td>
<td>0.230</td>
<td>-</td>
</tr>
<tr>
<td>MEF2C</td>
<td>Target</td>
<td>0.774</td>
<td>0.26-1.52</td>
<td>0.719</td>
<td>-</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Target</td>
<td>0.927</td>
<td>0.41-1.34</td>
<td>0.105</td>
<td>-</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Target</td>
<td>0.708</td>
<td>0.23-1.36</td>
<td>0.061</td>
<td>-</td>
</tr>
<tr>
<td>SEMA3A</td>
<td>Target</td>
<td>0.528</td>
<td>0.57-2.42</td>
<td>0.030</td>
<td>↓</td>
</tr>
<tr>
<td>MFSD2A</td>
<td>Target</td>
<td>1.113</td>
<td>0.51-2.15</td>
<td>0.605</td>
<td>-</td>
</tr>
<tr>
<td>GFAP</td>
<td>Target</td>
<td>1.012</td>
<td>0.59-1.37</td>
<td>0.946</td>
<td>-</td>
</tr>
<tr>
<td>OLFML3</td>
<td>Target</td>
<td>0.86</td>
<td>0.43-1.81</td>
<td>0.284</td>
<td>-</td>
</tr>
<tr>
<td>5HT2A</td>
<td>Target</td>
<td>0.905</td>
<td>0.49-2.61</td>
<td>0.612</td>
<td>-</td>
</tr>
<tr>
<td>GAD1</td>
<td>Target</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.4.5.2. mIA exposure at GD12.5 did not affect gene expression changes in the cerebellum of offspring at PD21

As shown in Figure 40, poly (I:C) treatment did not affect the expression of either HK in the CB of male or female offspring. Furthermore, no significant changes were found in any of the target genes tested in the CB at this postnatal time point in either sex (Table 24 and Table 25).

Figure 40: mIA had no effect on housekeeping gene CT values from the cerebellum. A-B) No difference was found in the CT values of SDHA or GAPDH between treatment groups in male or female offspring. Graphs represent mean ±SEM, n= 12/sex for saline offspring and 16/sex for poly (I:C) offspring.
Table 24: Gene expression changes in the cerebellum of PD21 male pups

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Expression</th>
<th>SEM</th>
<th>P value</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHA</td>
<td>HK</td>
<td>1.144</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>GAPDH</td>
<td>HK</td>
<td>0.874</td>
<td>SEM</td>
<td>0.317</td>
<td>-</td>
</tr>
<tr>
<td>SHANK3</td>
<td>Target</td>
<td>0.869</td>
<td>0.53 - 1.41</td>
<td>0.317</td>
<td>-</td>
</tr>
<tr>
<td>MEF2C</td>
<td>Target</td>
<td>0.842</td>
<td>0.46 - 0.49</td>
<td>0.395</td>
<td>-</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Target</td>
<td>0.966</td>
<td>0.56 - 1.67</td>
<td>0.807</td>
<td>-</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Target</td>
<td>0.943</td>
<td>0.56 - 1.52</td>
<td>0.690</td>
<td>-</td>
</tr>
<tr>
<td>SEMA3A</td>
<td>Target</td>
<td>0.961</td>
<td>0.65 - 1.40</td>
<td>0.723</td>
<td>-</td>
</tr>
<tr>
<td>MFSD2A</td>
<td>Target</td>
<td>1.1</td>
<td>0.61 - 1.90</td>
<td>0.553</td>
<td>-</td>
</tr>
<tr>
<td>GFAP</td>
<td>Target</td>
<td>0.956</td>
<td>0.62 - 1.43</td>
<td>0.706</td>
<td>-</td>
</tr>
<tr>
<td>OLFML3</td>
<td>Target</td>
<td>0.912</td>
<td>0.61 - 1.32</td>
<td>0.413</td>
<td>-</td>
</tr>
<tr>
<td>5HT2A</td>
<td>Target</td>
<td>1.045</td>
<td>0.57 - 1.83</td>
<td>0.781</td>
<td>-</td>
</tr>
<tr>
<td>GAD1</td>
<td>Target</td>
<td>0.88</td>
<td>0.49 - 1.51</td>
<td>0.454</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 25: Gene expression changes in the cerebellum of PD21 female pups

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Expression</th>
<th>SEM</th>
<th>P value</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHA</td>
<td>HK</td>
<td>1.092</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>GAPDH</td>
<td>HK</td>
<td>0.916</td>
<td>SEM</td>
<td>0.306</td>
<td>-</td>
</tr>
<tr>
<td>SHANK3</td>
<td>Target</td>
<td>0.836</td>
<td>0.49 - 1.52</td>
<td>0.306</td>
<td>-</td>
</tr>
<tr>
<td>MEF2C</td>
<td>Target</td>
<td>0.775</td>
<td>0.35 - 1.58</td>
<td>0.304</td>
<td>-</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Target</td>
<td>0.914</td>
<td>0.47 - 1.83</td>
<td>0.617</td>
<td>-</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Target</td>
<td>0.948</td>
<td>0.55 - 1.78</td>
<td>0.753</td>
<td>-</td>
</tr>
<tr>
<td>SEMA3A</td>
<td>Target</td>
<td>0.947</td>
<td>0.57 - 1.74</td>
<td>0.747</td>
<td>-</td>
</tr>
<tr>
<td>MFSD2A</td>
<td>Target</td>
<td>0.861</td>
<td>0.49 - 1.63</td>
<td>0.390</td>
<td>-</td>
</tr>
<tr>
<td>GFAP</td>
<td>Target</td>
<td>0.913</td>
<td>0.59 - 1.51</td>
<td>0.497</td>
<td>-</td>
</tr>
<tr>
<td>OLFML3</td>
<td>Target</td>
<td>0.932</td>
<td>0.54 - 1.66</td>
<td>0.656</td>
<td>-</td>
</tr>
<tr>
<td>5HT2A</td>
<td>Target</td>
<td>0.992</td>
<td>0.54 - 1.81</td>
<td>0.962</td>
<td>-</td>
</tr>
<tr>
<td>GAD1</td>
<td>Target</td>
<td>0.821</td>
<td>0.49 - 1.37</td>
<td>0.201</td>
<td>-</td>
</tr>
</tbody>
</table>
4.4.6. Behaviour

4.4.6.1. mIA at GD12.5 did not affect social communication in offspring as assessed by production of ultrasonic vocalisations (USVs) on PD9

During the 3 min isolation test, no difference was found in the USVs produced by saline and poly (I:C) offspring of either sex ($p = 0.241$ and 0.145 respectively) (Figure 41). The range of USV production within treatment groups was large. In the male saline group, the mean USV production was 278 ±122 and female pups 275 ±135 (mean ±SD). Offspring in the poly (I:C) treatment group showed a similarly high variability with USV production at 338 ±133 and 346 ±128 respectively (mean ±SD).

Figure 41: mIA did not affect the production of ultrasonic vocalisations (USVs) during the 3 min isolation test. Graphs represent mean ±SEM, $n= 8$ saline dams and $n= 6$ poly (I:C) dams.
4.4.7. 16S microbial sequencing

4.4.7.1. mIA resulted in a subtle alteration of the gut microbiota of PD21 offspring

The MiSeq and subsequent QIIME analysis showed, as expected, that the main phyla making up the composition of the gut microbiota were *Firmicutes* and *Bacteroidetes* (Figure 42) (deTheije et al., 2014b; Jandhyala et al., 2015). The NMDS ordination plots for beta diversity (community diversity divergence between samples), are shown in Figure 43 and Figure 44. An ordination plot shows the similarity between the samples based on the species abundance data represented by the distances between the points in the ordination space. There was no clear effect of treatment on the composition of gut microbiota as shown by the distribution in the ordination plot in Figure 43. However, a significant effect of treatment was highlighted in the ADONIS analysis ($p=0.001$). As shown in Figure 44, the data were clearly clustered by litter and this effect was shown to be significant ($p=0.001$) by the ADONIS. This similarity between microbiota from rodents sharing the same home cage and environment has been shown previously (Friswell et al., 2010).
Figure 42: Stacked bar plot summarising bacterial phyla isolated from the gut of saline and poly (I:C) offspring. Graphs represent individual pup values, saline $n=16$ males and 16 females, poly (I:C) $n=12$ males and 12 females.
Figure 43: NMDS ordination plot of Bray-Curtis dissimilarity between saline and poly (I:C) treatment groups. ADONIS analysis showed a significant effect of treatment and litter ($p = 0.001$). The clustering by treatment is not obvious in the ordination plot. However the blue line drawn highlights a potential pattern in this data set which may have driven this significant effect. Graphs represent individual pup values, saline $n= 16$ males and $16$ females, poly (I:C) $n= 12$ males and $12$ females.
Figure 44: NMDS ordination of Bray-Curtis dissimilarity between saline and poly (I:C) samples faceted by litter. Samples are differentiated by shape to represent male and female offspring and colour to represent treatment. Graphs represent individual pup values, saline \( n = 16 \) males and 16 females, poly (I:C) \( n = 12 \) males and 12 females.
The alpha diversity metrics, showing the microbial diversity within samples by total observed OTUs for saline and poly (I:C) samples are shown in Figure 45. On average, the mean observed OTUs in the poly (I:C) offspring showed a decreased diversity compared to saline offspring (Figure 45A), although this was not significant. Figure 45B shows the mean observed OTUs for each litter, faceted by treatment. This shows the variability between litters while still highlighting that, on average, there was increased diversity of bacterial species in the saline offspring.

Table 26 shows the abundance of gut microbiota following the DeSeq2 analysis and a differential stabilisation transformation using base mean data. The base mean is the mean of the normalised counts of all samples, normalising for sequencing depth. Low bases mean values for an OTU show that a very small amount of the sample is represented. The bacteria listed in this table are significant at the $p<0.05$ level, after false discovery rate (FDR) adjustment for multiple comparisons, between saline and poly (I:C) offspring, calculated using individual OTUs. The p value adjustment was performed using the `p.adjust()` function in the DeSeq2 package which calculates a FDR using the Benjamini-Hochberg method (Benjamini et al., 2001).

As shown in Table 26, the base means for the differentially abundant OTUs were low in general. The exception to this was the 5 OTUs (highlighted in green) that were greater than 1000 sequences (see base mean values, column 1 Table 26). These 5 bacteria have base means that account for a larger proportion of OTUs in the samples and therefore may infer greater functional relevance. Interestingly, with the exception of a single OTU, the fold change in Clostridiales was always increased in poly (I:C) compared to saline offspring. However, with very low base mean values, the biological relevance of these significant changes is hard to assess and the majority of these findings should be interpreted with some caution.
Figure 45: Alpha diversity metrics (total observed OTUs) for saline and poly (I:C) samples. A) Mean observed OTUs sectioned by treatment. B) Mean observed OTUs for each litter faceted by treatment. Graphs represent saline \( n = 16 \) males and 16 females, poly (I:C) \( n = 12 \) males and 12 females.
Table 26: The abundance of gut bacteria in samples following the DeSeq2 analysis and a differential stabilisation transformation. Data shown are significant ($p<0.05$, adjusted for multiple comparisons) changes in individual OTUs following comparison between saline and poly (I:C) with data pooled from male and female offspring.

<table>
<thead>
<tr>
<th>Base Mean</th>
<th>OTU Number</th>
<th>Log Fold Change</th>
<th>P value</th>
<th>Adjusted p value</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.513</td>
<td>OTU_1032</td>
<td>3.594</td>
<td>0.002</td>
<td>0.018</td>
<td>[Clostridiales]</td>
</tr>
<tr>
<td>2.195</td>
<td>OTU_374</td>
<td>3.199</td>
<td>0.008</td>
<td>0.050</td>
<td>[Clostridiales]</td>
</tr>
<tr>
<td>3.004</td>
<td>OTU_866</td>
<td>3.754</td>
<td>0.002</td>
<td>0.018</td>
<td>[Coriobacteriaceae]</td>
</tr>
<tr>
<td>3.164</td>
<td>OTU_516</td>
<td>3.825</td>
<td>0.005</td>
<td>0.039</td>
<td>Oscillospira sp.</td>
</tr>
<tr>
<td>4.513</td>
<td>OTU_351</td>
<td>3.220</td>
<td>0.002</td>
<td>0.018</td>
<td>[Ruminococcaceae]</td>
</tr>
<tr>
<td>4.718</td>
<td>OTU_370</td>
<td>2.719</td>
<td>0.002</td>
<td>0.019</td>
<td>[Clostridiales]</td>
</tr>
<tr>
<td>5.589</td>
<td>OTU_559</td>
<td>2.820</td>
<td>0.004</td>
<td>0.028</td>
<td>[Lachnospiraceae]</td>
</tr>
<tr>
<td>5.598</td>
<td>OTU_340</td>
<td>5.124</td>
<td>0.000</td>
<td>0.004</td>
<td>[Clostridiales]</td>
</tr>
<tr>
<td>6.676</td>
<td>OTU_396</td>
<td>5.167</td>
<td>0.001</td>
<td>0.015</td>
<td>[Clostridiales]</td>
</tr>
<tr>
<td>7.680</td>
<td>OTU_868</td>
<td>1.701</td>
<td>0.005</td>
<td>0.039</td>
<td>[Ruminococcaceae]</td>
</tr>
<tr>
<td>9.744</td>
<td>OTU_266</td>
<td>5.422</td>
<td>0.001</td>
<td>0.014</td>
<td>Coprococcus sp.</td>
</tr>
<tr>
<td>9.838</td>
<td>OTU_314</td>
<td>5.637</td>
<td>0.000</td>
<td>0.001</td>
<td>Oscillospira sp.</td>
</tr>
<tr>
<td>9.960</td>
<td>OTU_864</td>
<td>-6.158</td>
<td>0.000</td>
<td>0.000</td>
<td>[Erysipelotrichaceae]</td>
</tr>
<tr>
<td>10.232</td>
<td>OTU_298</td>
<td>3.772</td>
<td>0.001</td>
<td>0.009</td>
<td>Clostridiales</td>
</tr>
<tr>
<td>13.740</td>
<td>OTU_877</td>
<td>2.662</td>
<td>0.005</td>
<td>0.038</td>
<td>Clostridium saccharogumia.</td>
</tr>
<tr>
<td>15.854</td>
<td>OTU_852</td>
<td>4.184</td>
<td>0.000</td>
<td>0.000</td>
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</tr>
<tr>
<td>15.975</td>
<td>OTU_327</td>
<td>4.097</td>
<td>0.000</td>
<td>0.001</td>
<td>[Clostridiales]</td>
</tr>
<tr>
<td>18.082</td>
<td>OTU_211</td>
<td>4.494</td>
<td>0.003</td>
<td>0.022</td>
<td>[Lachnospiraceae]</td>
</tr>
<tr>
<td>18.683</td>
<td>OTU_233</td>
<td>5.882</td>
<td>0.001</td>
<td>0.013</td>
<td>[Lachnospiraceae]</td>
</tr>
<tr>
<td>21.908</td>
<td>OTU_126</td>
<td>6.146</td>
<td>0.000</td>
<td>0.000</td>
<td>[RF32]</td>
</tr>
<tr>
<td>22.794</td>
<td>OTU_112</td>
<td>5.704</td>
<td>0.002</td>
<td>0.018</td>
<td>[RF32]</td>
</tr>
<tr>
<td>24.248</td>
<td>OTU_131</td>
<td>6.640</td>
<td>0.000</td>
<td>0.001</td>
<td>Marvinbryantia formatexigens.</td>
</tr>
<tr>
<td>24.618</td>
<td>OTU_848</td>
<td>6.930</td>
<td>0.000</td>
<td>0.000</td>
<td>[RF39]</td>
</tr>
<tr>
<td>24.993</td>
<td>OTU_240</td>
<td>3.523</td>
<td>0.001</td>
<td>0.013</td>
<td>[Ruminococcaceae]</td>
</tr>
<tr>
<td>26.079</td>
<td>OTU_147</td>
<td>-4.933</td>
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<td>Value</td>
<td>P_Value</td>
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<td>formicigenerans</td>
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<td>0.033</td>
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</tr>
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<td>Rank</td>
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<td>Taxonomic Abundance</td>
<td>Phylum</td>
<td>Genus</td>
</tr>
<tr>
<td>------</td>
<td>------</td>
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<tr>
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<td>Streptococcus</td>
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<tr>
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<td>0.001</td>
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<td></td>
</tr>
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<td>0</td>
<td>Anaeroplasma</td>
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4.5. Discussion

The aim of the work presented in this chapter was to investigate whether exposure to mIA during early pregnancy significantly impacted on development as shown by general growth, gene expression in the brain and communication in the early postnatal period, and whether these effects varied by sex. In addition, investigation into the relationship between development of the brain and GI tract was attempted by monitoring changes to the composition of faecal microbiota. Owing to the post-natal development of the brain (Knuesel et al., 2014), this was a critical time point for investigation in this NDD model and enabled comparison against the results that were found at GD21.

4.5.1. mIA produced a sickness response at GD12.5 as shown by CBT and IL-6 elevation

Exposure to 10 mg/kg i.p. poly (I:C) in this study resulted in an acute upregulation in CBT as well as a significant elevation in plasma IL-6 3 hr post-injection. In this cohort of animals, 4 out of 6 dams showed a detectable level of IL-6 using the described analytical methods with no elevation of TNFα or IL-1β observed in any dam. The acute increase in CBT following 10 mg/kg i.p. poly (I:C) at GD12.5 returned to baseline 24 hr post-injection. As already shown in Table 7, CBT changes following induction of mIA are generally not recorded in mIA studies. However, previous work in male Sprague-Dawley rats has shown that poly (I:C) can induce a significant, but short-lived increase in body temperature (Fortier et al., 2004). Since this effect was shown in a different sex of rat and in the absence of pregnancy, the findings in this study cannot be directly compared. However, CBT changes may predict successful induction of a fever-like response in the dam which may affect the developing offspring. Therefore, it is proposed that measures of mIA induction such as CBT should be included as standard in models of mIA.

These results are in contrast to those of Chapter 3, where no significant physiological responses related to sickness behaviour were shown following poly (I:C) exposure. These contrasting results are particularly interesting because the same batch of poly (I:C) was used in both GD21 and PD21 studies. While it is the case that differences between batches of poly (I:C) may underlie the variability in study outcomes across
different labs, it is unusual to produced different effects in two studies using the same batch of poly (I:C). The variability in the molecular weight (MW) of poly (I:C) supplied by Sigma (average MW of 200-500 kilodaltons (KDa)) is likely to influence the resulting level of immune activation. However, this variability between dams injected with the same batch of poly (I:C) highlights important questions about dam specific responses to poly (I:C) that require further investigation. Cautious interpretation of these results supports a hypothesis of dam-dependent responses to immune activation. This may be due to variability in the baseline immune status of pregnant dams. It is possible that mIA above a certain threshold (i.e. IL-6 upregulation) may lead to a maternal immune activation strong enough to cause the phenotype. This threshold is yet to be determined in this model and may require the analysis of additional cytokines and/or sickness parameters. Determination of such a threshold is important for the future validation of mIA models in the future, particularly in light of achieving standardisation between different labs.

4.5.2. Analysis of offspring at PD21

In order to determine the effect of 10 mg/kg i.p. on general growth and development postpartum, pups were monitored from PD1 until PD21. Offspring from poly (I:C)-treated dams showed a significant reduction in BW that was maintained throughout this period, suggesting a disruption to general growth. In the previous study, no difference was shown in the BW of offspring at GD21. Due to the difference in response to poly (I:C) recorded in the dams between these two studies it is difficult to determine the origin of this reduction. It is possible that this reduction in BW only appears postpartum, (perhaps due to disrupted maternal care from poly (I:C)-treated dams in the first 24 hr of life), which meant that at GD21 no change in BW was observed. Alternatively, the absence of a BW change in the GD21 experiment may have been due to the lack of immune activation in the dams, and as a result the general growth of the pups was not affected. The effects of 10 mg/kg i.p. poly (I:C) on the maternal care of dams have yet to be elucidated in this model and are currently under investigation in our lab. Considering the recent evidence showing that mIA using poly (I:C) can produce transgenerational effects on mother-pup interactions (Ronovsky et al., 2016), this is a key area for investigation in this model.
At PD21, when tissue was harvested from the pups, total BrW in male offspring from poly (I:C)-treated dams was reduced compared to male saline offspring. Due to the significant reduction in total BW, BrW was normalised to BW for each individual pup, and BrW was presented as a proportion of BW (Figure 38B). This data shows how the relativity of organ growth is altered with respect to whole body growth. If proportionality was maintained (i.e. smaller organs due to the smaller foetus), then this relative ratio would be expected to be the same as control (i.e. constant proportion of BrW:BW). The data suggest that BrW, relative to BW, is increased in poly (I:C) group for both sexes. This could be interpreted as an increased growth in brains of poly (I:C) offspring or that general brain development, (as measured by weight), has been protected relative to body. Due to the BW being a composite of tissue, bone, and fat mass, all of these may be altered in the poly (I:C) pups.

4.5.2.1. mIA at GD12.5 resulted in specific gene expression changes that were dependent on sex and brain region

In this study, 10 mg/kg i.p. poly (I:C) exposure at GD12.5 was sufficient to cause significant gene expression changes in the FC of offspring. Interestingly, the gene expression changes found at GD21 (downregulation of SNAP-25), were not present at this postnatal time point. In the PD21 cerebellum no changes to gene expression were shown in either sex, showing that the downregulation of MEF2C, GFAP and OLFML3 was not sustained into this developmental period. Previous work in the influenza, IL-6 and poly (I:C) models of mIA, have reported acute embryonic changes in crystallin gene expression (3-6 hr post mIA) that do not persist in postnatal life as shown by analysis of 15 week old and 6 month old offspring (Garbett et al., 2012). Although the changes seen at GD21 were returned to control levels at PD21, this does not rule out their influence on developmental processes. It is possible that the gene expression analysis performed at GD21 and PD21 only captures a snapshot of the molecular changes occurring during development. Furthermore, early gene expression changes may be viable early biomarkers for NDDs and would be missed if gene expression was only investigated later in life. In order to better determine the temporal changes in gene expression, multiple time points should be investigated in post-natal life. Ideally this would involve the study of offspring from the same litter over a longitudinal period.
SHANK3 deletion has been shown to cause disruption to synaptic protein composition at the postsynaptic density and reduce synaptic plasticity, leading to ASD-like behaviours (Peça et al., 2011; Wang et al., 2011b). Moreover, SHANK3 mutations are observed in families with ASD (Durand et al., 2007). In the FC of male poly (I:C) offspring, the gene for major scaffolding protein SHANK3 was over expressed at PD21, which is the time when synapses are being formed (Flavell, 2006). In light of validating this model in relation to ASD pathophysiology, the hypothesis was that a similar disruption to the SHANK3 gene might be shown in these offspring. However, the opposite effect of SHANK3 gene expression was shown. Over expression of SHANK3 has been linked with the appearance of manic-like behaviour in mice (Han et al., 2013). SHANK3 mutants in the study by Han et al., 2013 showed increased locomotor activity in the OFT which was augmented by treatment with amphetamine. Further behavioural work in this model will likely determine the presence of a behavioural phenotype relevant to psychiatric disorders.

Reduced expression of MFSD2A (associated with BBB formation) at PD21 could suggest delayed brain development following pre-natal poly I:C exposure, although investigation of these genes and their functional relevance at further time points is required to confirm this. The reduced expression of MFSD2A was also only shown in female offspring and cautious interpretation of this finding supports a sex-dependent mechanism for the effects of 10 mg/kg i.p. poly (I:C) at GD12.5 on the developing foetus. Recent work has suggested that the BBB becomes functional at GD15.5 in mice (Ben-Zvi et al., 2014). Thus, exposure to poly (I:C) at GD12.5 may have delayed normal development of the BBB in these male offspring. However, because this effect was not observed at GD21, this study requires replication to determine the reliability of this finding. A potential delay in BBB formation may allow the infiltration of blood-borne factors and activation of an immune response which could be damaging to the developing brain and makes this a key area for future investigation.

Another important area for further investigation is sex differences in the gene expression shown in the brains of both PD21 and GD21 offspring. In both studies the gene expression changes were very different between male and female offspring highlighting the need for investigation into sex differences in mIA models. Specifically in the area of ASD and owing to the increased incidence of this condition in males in the clinic, it is unfortunately standard practice that only male animals are used. Sex
specific outcomes are essential for increased mechanistic understanding of developmental disorders and are increasingly becoming recognised in recent research work (Makinson et al., 2017). It was not feasible to investigate microglial activation in this study. However, as the resolution of small animal PET scanners is sufficient for imaging rat brains it will be possible to perform a detailed assessment of microglia structure and function in different brain areas post-mIA in the future (Yao et al., 2012; Boutin et al., 2013).

4.5.2.2. mIA did not affect pup USVs during the isolation test

In this study social communication was assessed by the production of USVs during an isolation test of offspring from their mother at PD9. This test was chosen because disruptions in social communication are a key symptom of ASD. Assessment of USVs in rats can be made at different points of development to assess different aspects of communicative behaviour. These include USVs emitted during isolation from mother and litter mates, USVs emitted in aversive situations (i.e. predator exposure), and USVs emitted by juvenile rats during bouts of play behaviour (Yee et al., 2012; Wöhr and Schwarting, 2013; Raza et al., 2015). It is difficult to monitor behaviour before weaning in young offspring, so USV analysis was one test that could be adequately performed in these animals. The data showed that, at this postnatal age, no difference in the number of social calls made during the isolation test was present between treatment groups. Notably, the within-litter variation was very high for offspring in this test. With more advanced apparatus, it would be possible to monitor differences in the frequency or duration of the calls made during the isolation test which may provide a more comprehensive analysis of the effects of poly (I:C). This time point was chosen so that the pups received minimal disturbance in the days following birth as this may have caused stress for the offspring. In addition, data from an mIA pilot study performed in our lab showed that the USV test could be successfully conducted on PD3, 5 9 and 11 but only on PD9 was USV production reduced on average in the poly(I:C) offspring. Therefore, it was interesting to determine if this effect was shown in the GD12.5 mIA model. Previous studies have shown a disruption in these social calls at PD3-11 (Umeda et al., 2010; Baharnoori et al., 2012) so this is an area for further investigation.

It has been shown that the characteristics of offspring calls can be affected by maternal care which may have influenced resulted presented here (Wöhr et al., 2008). In the
current study, changes to maternal-pup interactions during early postnatal life were not monitored. Therefore, although it is known that early life maternal interactions are significant predictors of subsequent behavioural development (van Oers et al., 1998; Zimmerberg et al., 2003), any effect of mIA on maternal behaviour in this model remains to be elucidated.

4.5.2.3. mIA resulted in a subtle alteration of the gut microbiota of PD21 offspring

In relation to ASD, a few studies have shown disruptions to the gut microbiota in mouse models using VPA at GD12.5 (deTheije et al., 2014a). However, since the original work by Hsiao et al., 2013, exploration of gut microbiota changes following mIA with poly (I:C) have been limited (Lim et al., 2017).

In this study, litter mates were housed together until analysis on PD21, when faecal samples were taken from the offspring. This is likely to explain the clustering of microbiota from both male and female offspring in the same litter due to rats being coprophagric and having a shared environment with each other and the mother. This highlights that, for future development of these findings, it may be necessary to house offspring singly in order to better determine individual microbiota changes. Due to the confounding effects of social isolation on development, it was not possible to do this in the current study.

Offspring from poly (I:C)-treated dams showed, a tendency towards decreased diversity in their faecal microbiota. Although this was not significant in this study, decreased microbial diversity in the gut of PD21 male offspring has been shown in another poly (I:C) GD12.5 model in mice (Lim et al., 2017). As well as in ASD patients (Kang et al., 2013). Although the biological significance of the statistical changes in specific OTUs cannot be confirmed from this study, increased expression of the order Clostridiales is an interesting finding considering the frequency with which the bacterial genus Clostridium is upregulated in the gut microbiota of some ASD patient cohorts (see Table 16). Additional analyses of the gut microbiota of mIA offspring are required at the genus level as these were not identified in this study. Alternative techniques that enable the generation of longer amplicon lengths or sequencing with the V3 region of the 16S rRNA gene could be employed to achieve this. While the results are interpreted with caution, in part due to the small cohort of poly (I:C) offspring that were analysed, these results suggest that exposure to 10 mg/kg i.p. poly (I:C) in utero may be sufficient
to cause a disruption to the development of the faecal microbiota. Additional evidence is required to determine the functional relevance of these microbial alterations particularly considering the apparent involvement of the gut-brain axis in ASD pathophysiology, as well as other neurodevelopmental and psychiatric disorders (Bravo et al., 2012; Dinan and Cryan, 2016).

In conclusion, the results presented in this chapter contribute to our understanding of the gene expression changes following mIA, and provide an insight into how these change throughout development. As mentioned in the previous chapter, determination of the functional relevance of these changes requires the analysis of protein expression. However, additional candidate proteins have been identified for investigation in PD21 brains. The variability in the acute response to poly (I:C) at GD12.5 confirms that it is critical to monitor sickness behaviour and physiological response in each individual dam across all mIA studies. This is particularly important for determining the correlation between the nature and extent of immune activation in the dam and the phenotype produced in offspring. It remains to be determined whether these early developmental changes in the brains of offspring following mIA result in a measurable behavioural phenotype in later life. This will be explored in the next chapter using an ethologically relevant battery of behavioural tests.
Chapter 5: Analysis of behavioural changes in adolescent and adult offspring following mIA
5.1. **Introduction**

Numerous epidemiological studies have provided evidence for the role of inflammatory processes in the aetiology of neuropsychiatric disorders (Knuesel et al., 2014). mIA, particularly using the viral mimetic poly (I:C), has emerged as a key model for studying behavioural phenotypes of NDDs and has been employed in rodents in addition to non-human primates (Reisinger et al., 2015). The emerging evidence suggests that mIA can affect the development of a foetus, owing to the vulnerability of the central nervous system (CNS) and that paradigm may be relevant to a wide variety of disorders particularly considering the array of behavioural changes that have been demonstrated.

5.1.1. **Models of mIA**

For the development of mIA models relevant to NDD phenotypes, both the timing of gestational exposure to mIA and the dosing regimen applied (single vs. repeated exposure) have been investigated. Indeed, an early study showed that the timing of the maternal infection had a specific effect on the cytokine upregulation observed in fetal brains, alongside differences seen in postnatal brain development and subsequent behavioural phenotype (Meyer, 2006). The majority of models using rodents induce mIA between days 9-17 of gestation. Gestational days 15-17 (sometimes as late as 19) are typically associated with a schizophrenia-like pathology and behavioural phenotype whereas GD9-12.5, are associated with ASD (with GD12.5 as the most widely used see Table 27). The neural events occurring at this time point are considered to approximate the late first and early second trimester in humans (Workman et al., 2013; Knuesel et al., 2014). As shown in Table 27 mice are more commonly used in ASD modelling than rats. Indeed, at the time of writing there were no known studies published using a rat mIA model at GD12.5 even though rats provide similar benefits to mice with their low cost and short gestational periods. Interestingly, there are a large number of studies that have used GD12.5 exposure to the AED VPA for modelling ASD-like behavioural and anatomical phenotypes in rats (Ingram et al., 2000; Schneider et al., 2005, 2008; Favre et al., 2013; Roullet et al., 2013). Briefly, the VPA model produces an ASD-like behavioural phenotype including deficits in social behaviour and communication and the appearance of stereotypic behaviours. Molecular and cellular correlates to human ASD have also been highlighted including, disruption to neuronal morphology and circuitry as well as diminished expression of Neuroligin-3 (NLGN3) (Ingram et al.,
2000; Snow et al., 2008; Gandal et al., 2010; Mehta et al., 2011). This evidence highlights the potential for GD12.5 exposure in modelling ASD-like behaviours in rats. Owing to the desirable benefits of using rats in behavioural modelling over mice, (for example, their complex social behaviour repertoire and reduced aggressive behaviour) it is proposed that the validation of a rat model of mIA using the GD12.5 exposure to the viral mimetic poly (I:C) would expand this field.

Considering the overlap in the behavioural phenotypes induced, it might be considered limiting to restrict the analysis of a single mIA model to a specific human disorder. However, while the current work is firmly in support of mIA as a potential priming mechanism for a wide variety of disorders, this study has been conducted with a focus on ASD behaviour. This approach provides a platform to systematically evaluate the effect of mIA on brain development relevant to ASD as well as corresponding changes to behaviour that are characteristic of this disorder. The overall aim is that the translational impact of preclinical modelling will be achieved.
Table 27: Maternal immune activation models

<table>
<thead>
<tr>
<th>Author</th>
<th>Species, Strain</th>
<th>mIA *</th>
<th>Sex*</th>
<th>Behavioural read-out</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smith, 2007</td>
<td>Mouse, C57BL/6</td>
<td><strong>GD12.5</strong> poly (I:C) 20 mg/kg (i.p.), IL-6 or IFNγ</td>
<td>—</td>
<td>Latent inhibition (LH) ↓, prepulse inhibition (PPI) ↓, open field test (OFT) (anxiety) ↑, social interaction test (SIT) ↓</td>
</tr>
<tr>
<td>Shi et al., 2009</td>
<td>Mouse, BALB/C</td>
<td><strong>GD12.5</strong> Poly (I:C) 20 mg/kg (i.p.)</td>
<td>M &amp; F</td>
<td>LH ↓, OFT (anxiety) ↑, novel object recognition (NOR) ↓, PPI ↓</td>
</tr>
<tr>
<td>Ito, 2010</td>
<td>Mouse, C57BL/6</td>
<td><strong>GD12.5</strong> poly (I:C) 5 mg/kg (i.v.) or 20 mg/kg (i.p.)</td>
<td>—</td>
<td>Water maze ↓, NOR ↓</td>
</tr>
<tr>
<td>Connor, 2012</td>
<td>Mouse, C57BL/6</td>
<td><strong>GD12.5 or 17.5</strong> poly (I:C) 5 mg/kg (i.v.)</td>
<td>M &amp; F</td>
<td>T-maze ↓</td>
</tr>
<tr>
<td>Malkova et al., 2012</td>
<td>Mouse, C56BL/6</td>
<td><strong>GD10.5, 12.5 and 14.5</strong> poly (I:C) 5 mg/kg (i.p.)</td>
<td>M</td>
<td>PPI ↓, OFT ↑, ultrasonic vocalisations (USVs) ↓, SIT and scent marking ↓</td>
</tr>
<tr>
<td>Hsiao, 2012</td>
<td>Mouse, C57BL/6</td>
<td><strong>GD12.5</strong> poly (I:C) 20 mg/kg (i.p.)</td>
<td>—</td>
<td>PPI ↓, OFT (anxiety) ↑, repetitive marble burying ↑, SIT ↓</td>
</tr>
<tr>
<td>Schwartz, 2013</td>
<td>Mouse, C57BL/6</td>
<td><strong>GD12.5</strong> poly (I:C) 20 mg/kg (i.p.)</td>
<td>M &amp; F</td>
<td>SIT ↓, repetitive marble burying ↑, USVs ↓, grooming ↑</td>
</tr>
<tr>
<td>Foley, 2014</td>
<td>Rat, Long Evans</td>
<td><strong>GD12-16</strong> (5 injections) Sodium propionate (PPA) 500mg/kg, LPS 50µg/kg (SC)</td>
<td>M &amp; F</td>
<td>OFT (anxiety) ↑, adolescent and adult SIT ↓, Novel object vs. novel rat choice ↓</td>
</tr>
<tr>
<td>Onore, 2014</td>
<td>Mouse, C57BL/6</td>
<td><strong>GD12.5</strong> poly (I:C) 20 mg/kg (i.p.)</td>
<td>M &amp; F</td>
<td>SIT ↓, marble burying ↑</td>
</tr>
<tr>
<td>Giovanoli, 2016</td>
<td>Mouse, C57BL/6</td>
<td><strong>GD12.5</strong> poly (I:C) 5 mg/kg (i.v.)</td>
<td>M</td>
<td>PPI ↓</td>
</tr>
<tr>
<td>Luchicchi, 2016</td>
<td>Rat, Sprague Dawley</td>
<td><strong>GD15</strong> Poly (I:C) 4 mg/kg (i.v.)</td>
<td>M</td>
<td>PPI ↓, NOR ↓, SIT ↓</td>
</tr>
<tr>
<td>Kirsten and Bernardi, 2017</td>
<td>Rat, Wistar</td>
<td><strong>GD9.5</strong> LPS 100 µg/kg (i.p.)</td>
<td>M</td>
<td>OFT (stereotypy) ↑, self-grooming ↑</td>
</tr>
</tbody>
</table>

*Gestational day of exposure, compound used, route of administration (i.p. intraperitoneal, i.v. intravenous); Sex M= male, F= female, — no information given; behavioural read-out ↑ increased, ↓ decreased.
5.1.2. Behaviours relevant to ASD

ASD is characterised by early onset of deficits in social behaviour and communication as well as repetitive behaviours and restricted interests (DSM-5, American Psychiatric Association). As previously mentioned, high levels of anxiety have been reported in addition to these characteristic behaviours. Even in the earliest descriptions of infantile autism a substantial observation of anxiety was made (Kanner, 1943). More recent evidence from meta-analyses supports a substantial comorbidity, with 40% of children and adolescents with ASD reporting clinically elevated levels of anxiety and at least one anxiety disorder (van Steensel et al., 2011). To achieve sufficient face validity, these core behaviours are the focus for developing relevant tests of behavioural changes in animal models. Importantly for disorders such as ASD, it is possible to measure behaviours in rodents relevant to the human such as social (van Kerkhof et al., 2013) and anxiety-like (Bailey and Crawley, 2009) behaviours. Behavioural phenotyping in animal models is of considerable importance for understanding the relationship between brain and behaviour. Of course, anatomical or molecular changes to the brain are critical to enhance our understanding of the neurobiology of neurodevelopmental disorders. However, if we are limited to molecular changes in the absence of a behavioural phenotype, the biological relevance and translational application of such models remain incomplete. Therefore, a combination of molecular and behavioural approaches is best.

As shown in Table 27, a large number of behavioural tests are employed in these studies and a number of these target behaviours that arguably translate to certain aspects of the human condition. A limited number of studies produce robust behavioural phenotypes that are longitudinal (i.e. that develop in adolescence and are also observed adulthood) to monitor the long-term effects of mIA. In order to consolidate the findings across the many mIA studies, it is necessary for there to be a more standardised approach to behavioural test batteries as well as a focus on tests that measure aspects of behaviour that are relevant to the disorder phenotype. Considering that no single animal model of mIA (or an alternative) will ever achieve a complete phenotype of a human disorder, this is particularly important to enable comparisons between different studies to achieve a clear picture of the validity of these models. This is of particular relevance to NDDs such as ASD with a spectrum of disorder symptoms and severity. Although ASD is almost certainly a product of multiple environmental factors combined with complex genetic interactions, it is likely that different species, strains and gestational
exposures may be useful for producing models relevant to distinct clinical phenotypes within the same disorder.

5.2. Chapter 5 summary and objectives

Behavioural modelling is critical to understanding the translational validity of an animal model. Following the investigation of molecular brain changes in offspring from poly (I:C)-treated dams, it was important to establish the behavioural phenotype induced by this gestational stressor in a longitudinal manner. The tests were chosen to target core behaviours relevant to this condition such as social behaviour, repetitive behaviour and anxiety-like behaviour.

5.2.1. Objectives

- Administer mIA using 10 mg/kg i.p. poly (I:C) at GD12.5 and follow acute sickness behaviour in dams including cytokine analysis in a new cohort of animals

- Measure behavioural changes in offspring during adolescence and adulthood in a battery targeted towards core symptoms of ASD to investigate the translational validity of our proposed rat mIA model
5.3. Materials and methods

5.3.1. Experimental design

Experiments were performed using male and female Wistar rats (Charles River Laboratories, UK). All rats were housed in the conditions as described in Chapter 2, section 2.2.1, p.71. For mating $n=5$ male and $n=10$ female Wistar rats were used. Only 8 of the 10 dams were successfully mated and underwent mIA ($n=4$ saline, $n=4$ poly (I:C)) as described section 2.5.2, p.82. All offspring were used in the behavioural battery performed at adolescence and adulthood. See Figure 46 for diagram of experimental design used in this study.

**Figure 46:** Timeline of experimental design. GD= gestational day, PD= postnatal day, CBT= core body temperature, EPM= elevated plus maze
5.3.2. Offspring

5.3.2.1. Litter numbers

As described in Chapter 4 (see section 4.3.2.1) on PD1, the pups were counted and total litter numbers (including ratio of males to females) were recorded. Following this all litters were culled and identified using methods previously described (see section 4.3.2.2). In this study, one poly (I:C) treated dam had a litter of only 5 pups (3 males and 2 females). Due to resulting differences in maternal care, these pups were excluded from analysis. Pups were weaned on PD21 and group housed with same-sex littermates. At weaning and into adulthood pups were tail-marked to identify them within their same-sex litter groups. Tail marks were redrawn every week (using the same non-toxic permanent marker at the base of the tail) to ensure each animal was tracked throughout the entire study.

In accordance with the principles of the 3Rs, all offspring (male and female) were used for behavioural testing (saline n=15-16 from 4 dams; poly (I:C) n=12 from 3 dams). For certain behavioural tasks exclusion criteria were applied (see specific behavioural test descriptions). Where an animal was excluded from the analysis the relevant n numbers used in the statistical analysis are listed in the figure legend. The individual pups were considered to be the experimental unit and appropriate statistical analysis was performed to account for litter effects (see section 4.3.9.1, p.136).

5.3.2.2. Growth monitoring

All offspring were weighed regularly; during early development this was performed at PD6, 12, 18, 21, at adolescence daily from PD30-38, and at adulthood on PD68, 70 and 72.

5.3.3. Behavioural testing

A battery of behavioural tests was applied during adolescence and adulthood for phenotyping. Tests were chosen to measure behaviours relevant to the core symptoms of ASD (e.g. social and stereotypic/repetitive behaviours) as well as anxiety-like behaviour. Ultrasonic vocalisations (USVs) were not monitored in this study as no effect of 10 mg/kg i.p. poly (I:C) was shown on this aspect of social communication in the previous study. At adolescence behavioural tests were performed between PD30-37.
At adulthood testing was performed at PD68-72. See Figure 47 for the behavioural test battery timeline. Behavioural testing was performed in the light-cycle between 07:00 and 13:00. For each behavioural test the experimenter remained blinded to the treatment of the rats. The order of testing was balanced between treatment groups A and B and testing was performed in a randomised manner within each behavioural test. Where multiple tests were performed on one day, the order was balanced by cage, for treatment and sex groups across the morning. All behavioural scoring was performed manually from videotapes by the same experimenter who was blinded to the treatment and sex of the rats.

![Behavioural test battery timeline](image)

**Figure 47: Behavioural test battery.** Each individual behavioural test is shown with the corresponding postnatal day for both adolescence and adulthood testing. (EPM = elevated plus maze, SIT = social interaction). The same offspring were tested at each time point.

### 5.3.3.1. Open field test (OFT)

All offspring were tested for anxiety-like, general exploratory and repetitive behaviour using the OFT. The test box was a PVC box (52 cm L; 52 cm W; 31 cm H) with a 3 x 3 grid on the base (Figure 48). All offspring were naïve to the test box. The rat was placed in the front right corner of the test box facing the wall and exploratory behaviour was recorded via video camera for 10 min. On completion of the test all boxes were cleaned to remove faeces and wiped with 70% ethanol solution in an attempt to remove the olfactory trails in subsequent testing. Time spent in the corners, perimeter, and centre of the boxes was analysed alongside time spent grooming. General locomotor
activity was measured using line crossings of the arena. A line crossing was defined as every time the base of the rat’s tail crossed a line of the 3 x 3 grid on the base of the test box (Figure 48). The test rat was considered to be inside the centre zone when all four paws and the base of the tail were in the centre square of the grid. Percentage time spent in the centre was calculated as the measure of anxiety-like behaviour using the formula:

\[
\text{% Time centre} = \frac{\text{Time centre}}{\text{Total Time in arena}} \times 100
\]

Time spent grooming in the OFT arena was recorded as a measure of repetitive behaviour relevant to an ASD-like phenotype (Silverman et al., 2010). A bout of grooming typically began with the rat licking the front paws and using them to wash the face and ears. The rat would then move to nibbling and licking the fur on its shoulders, flank and hind quarters. Any of the above behaviours were recorded as grooming.

Figure 48: Open field test arena. Middle quadrant (highlighted red) used for % time centre calculation.
5.3.3.2. Elevated plus maze

All offspring were tested for anxiety-like behaviour in the elevated plus maze (EPM), a commonly used behavioural test in rodents (Pellow et al., 1985; Walf and Frye, 2007). The EPM was a four armed maze raised 50 cm above the ground (arm dimensions: 50 cm L; 10 cm W) (Figure 49). Two of the arms are “closed” with high walls (40 cm). The other two arms are “open” with no walls. All offspring were naïve to the maze on testing. Offspring were placed in the centre of the maze facing an open arm. The arm chosen (L/R) was balanced across treatment and sex to prevent any influence of directional bias. The test duration was 5 min and behaviour was recorded using a video camera. Between rats, the maze was wiped with blue towel in attempt to remove olfactory trails using 70% ethanol. Time spent in the open and closed arms as well as the entries between these arms were recorded. Percentage time and entries in the open arms was calculated using the formula (where total time \( or \) entries represents time in open plus time in closed arms):

\[
\text{\% Time or entries in open arm} = \frac{\text{Time or entries in open arm}}{\text{Total Time or entries}} \times 100
\]

Latency to the open arms was also recorded in seconds (sec), as the time spent in the maze before the test animal made the first entry to an open arm. If an animal failed to enter the open arms for the whole duration of the test then it was excluded from the analysis.
5.3.3.3. Habituation

On PD32-33, all offspring and conspecific rats were habituated to the arenas used for the OFT and social interaction test (SIT). Conspecific rats were ordered from Charles River to match the age of the adolescent offspring bred in-house. Animals were sex and weight matched ahead of testing. The test arena was a PVC box (52 cm L; 52 cm W; 31 cm H) with a 3 x 3 grid on the base (Figure 48). Rats at this age showed reduced exploratory behaviour and a high amount of defecation during the first habituation session suggesting stress from the novel environment. As a result, two days of habituation was chosen. All offspring and conspecifics were habituated to the boxes for 30min in cage groups on both habituation days.

5.3.3.4. Dyadic Social interaction test (SIT)

The SIT was performed as previously described (Neill et al., 2016) in adolescent offspring at PD34 and adult offspring at PD72. A novel, inanimate object, (aluminium can) was placed in the middle of the arena and acted as a control for preference to general novelty (Figure 50). All offspring were weight-matched (within 20 %) with an untreated conspecific of the same sex. All conspecifics were only used once in each testing period. These conspecific rats were ordered by age from Charles River Laboratories, UK. Conspecific rats were left undisturbed for 1 week prior to testing and then handled by the experimenter.
At the start of the SIT, the test rat (offspring from saline- or poly (I:C)-treated dams) and the conspecific were placed in the arena at opposite corners. Social behaviour was recorded for 10 min via video recorder. The videos were scored in a blinded manner, following testing for specific social behaviours performed by the test rat only. The experimenter was trained to score the social behaviour before completing the scoring on the test animals.

Time spent engaging in play behaviours (pinning/pouncing, boxing), “sniffing”, “following” and “object exploration” were recorded. “Sniffing” was defined as time spent sniffing any part of the body of the conspecific. “Following” was recorded when the test rat was closely following the conspecific within 1-2 cm of its hind-quarters. Object exploration was considered to be any time that the test rat had its nose within 1-2 cm of the inanimate object and was actively exploring. Rearing up prior to climbing or climbing on top of the object was not considered as object exploration.

The adolescent SIT was performed on PD34 as play behaviour is pronounced at this time and almost completely absent by PD40 (Trezza and Vanderschuren, 2008). “Pouncing” was defined as the rat attempting to rub or nip the back of the neck of the other. In a successful play response, the target rat rotates on to its dorsal surface while the other rat is standing over it, which is defined as “pinning” (Vanderschuren et al., 1997; Trezza et al., 2010). “Boxing” behaviour was also recorded which is defined as the two rats standing on their hind legs, face-to-face, pushing or pawing at one another around the head and neck. Sniffing and following performed by the test rat was considered a measure of more general social interaction.

In adulthood, time spent fighting was included in the analysis. These encounters were sometimes preceded by tail rattling where the rats would move their tails sinuously on the floor of the test arena. A bout of fighting behaviour began with biting of the nape or boxing. Once escalated the two rats would roll around while interlocked for short bursts.
5.3.3.5. Habituated burrowing

Offspring were tested using a modified version of a habituated burrowing test previously described in adult rats (Wodarski et al., 2016). Testing was performed over three days with two sessions of habituation and a single test session. On the first day of habituation, pairs of rats (cage mates) were placed into the testing box for 15 min without any digging equipment. Offspring were then removed and the digging tube placed in the back right hand corner of the test box. The same pairs of offspring were replaced into the testing boxes and left for 60 min. The digging tube was made from a 30 cm long gutter tube which was raised on one end with two screws (Figure 51A-B). 2500 g of fine gravel was placed in the tube. This protocol was repeated on day two of habituation. If no burrowing was seen in the first habituation session then offspring were placed in different litter pairs so that they were exposed to the burrowing behaviours. On the third (test) day the protocol was repeated but this time offspring were placed singly into the test cages for burrowing. Following the 60 min digging session, the gravel remaining in the tube was weighed and recorded. If no gravel was expelled from the tube during the test session then the animals were excluded from the analysis. All equipment (including gravel) was cleaned between testing in attempt to remove olfactory cues.

Figure 50: Social interaction test arena. An aluminium can was placed in the middle of the arena to act as a control for a general preference for novelty.
5.3.4. Specific experimental analyses

5.3.4.1. Offspring comparisons

To determine the effects of treatment on offspring, all 8 animals per litter (4 male and 4 female where possible) were used. A mixed-effects model, (nested-ANOVA), was performed as described in Chapter 4, section 4.3.9.1. p.136.

Figure 51: Habituated burrowing apparatus. A-B) The burrowing tube used showing the fixed and raised angle. C) Fine fish gravel that was used to fill the tube for the training and burrowing sessions. At the beginning of each test 2500 g of gravel was placed in the tube.
5.4. Results

5.5. mA at GD12.5 induced an acute increase in CBT and serum IL-6 in pregnant dams

To validate the effect of 10 mg/kg i.p. poly (I:C) at GD12.5 core body temperature (CBT) and BW were monitored at 3, 6 and 24 hr post-injection. CBT was elevated at 3 and 6 hr (Figure 52A-B). Repeated measures ANOVA showed a significant interaction of treatment by time $F_{(3, 18)} = 3.97$, $p = 0.025$. At 3 and 6 hr post-injection poly (I:C) dams had a significantly higher CBT compared to saline treated dams ($p = 0.0058$ and $p = 0.0305$ respectively) (Figure 52A). At 24 hr post-injection, CBT was returned to baseline level. Saline dams showed a reduction in CBT throughout the day, but this was not significant. When the data was normalised to baseline, a significant interaction of treatment by time was shown $F_{(3, 18)} = 3.97$, $p = 0.025$. However, post-hoc Bonferroni multiple comparison test showed only a significant increase in CBT at 3 hr post-injection ($p = 0.0165$) (Figure 52B).

A significant reduction in BW was shown by repeated measures ANOVA for factor time $F_{(3, 18)} = 20.27$, $p = 0.0001$. However, no difference between treatment groups was shown in post-hoc testing. Both saline ($p = 0.0381$) and poly (I:C) ($p = 0.0002$) dams showed a reduction in BW at 6 hr post-injection which is typical for rats during the light cycle due to reduced food intake. At 24 hr post-injection BW from both treatment groups returned to baseline level. Saline dams showed a slightly increased BW gain at 24 hr but this was not significant. These effects were shown in both the raw data and in the baseline-corrected temperature data (Figure 52B-C).

Plasma IL-6 levels recorded at 3 hr post-injection were not significantly different between treatment groups ($p = 0.0689$). However, a detectable level (125 pg/ml) of IL-6 was found in 3 out of the 4 dams injected with 10 mg/kg i.p. poly (I:C). In all of the saline dams the level of IL-6 found in the blood plasma sample was below the level of detection.
Figure 52: Effects of mIA at GD12.5 on pregnant dams. A-B) At 3 and 6 hr post-injection poly (I:C) dams recorded a significantly higher core body temperature (CBT) compared to saline treated dams (A). When normalised to baseline, Bonferroni post hoc showed a significantly higher CBT at 3 hr post-injection only (B). C-D) No significant difference in body weight (BW) was found between treatment groups at any time point. C) Weight loss at the 6 hr time point was greater on average but this did not reach significance in poly (I:C) dams compared to saline (D). (*= vs. saline group, #= vs. baseline s.= saline group, p.= poly (I:C), */#= p< 0.05, **/#= p< 0.01, ***/###= p<0.001.) Graphs represent mean ±SEM, n=4 dams/ treatment.
mIA had no effect on gestational weight gain or litter numbers

During gestation dams gained 125 g on average from GD8 until GD22. Two-way ANOVA revealed no significant interaction of treatment by day $F_{(14, 90)} = 1.52, p=0.1215$ (Figure 53). A significant main effect of day was produced $F_{(14, 90)} = 140.0, p<0.0001$ but no main effect of treatment $F_{(1, 90)} = 1.32, p=0.2541$. 10 mg/kg i.p. poly (I:C) at GD12.5 did not affect the gestational weight gain of dams. At GD22, (the day prior to birth), the mean difference between saline and poly (I:C) dams was 19.28 ±6.84 g but this difference was not significant.

![Gestational weights](image)

**Figure 53: mIA had no effect on gestational weight of pregnant dams.** No significant difference in body weight was seen throughout gestation between saline and poly (I:C) dams. Graphs represent mean ±SEM, $n=4$ dams/treatment.

5.5.1.1. Pup numbers

At PD1 (PD0= day of birth) litter numbers from all dams were recorded (Table 28). The offspring were also sexed at this time to determine the distribution of male and female offspring between treatment groups (Figure 54B). The average litter size was 14 ±1 for saline and 12 ±3 for poly (I:C) dams and no significant difference was found between total litter numbers ($p=0.5437$) (Figure 54A). A one-way ANOVA revealed no difference in the sex distribution of offspring between treatment groups.
\( F_{(3, 12)} = 1.38, p= 0.30 \) (Figure 54B). The lowest litter size recorded was poly (I:C) dam FB4 with a litter total of 5 (3 males and 2 females). This dam showed the highest level of IL-6 (584 pg/ml) when measured in blood plasma at 3hr post-injection.

On average, dams treated with poly (I:C) appeared to have more male (8 ±2 vs. 6 ±1 poly (I:C) vs. saline) and less female (5 ±1 vs. 8 ±1 poly (I:C) vs. saline) offspring in their litter than saline treated dams. However, a two-way ANOVA revealed no significant effect of treatment on the distribution of males or females \( F_{(1, 12)} = 0.44, p= 0.5190 \) (Figure 54B).

Table 28: Litter numbers and sex distribution including corresponding IL-6 level

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dam ID</th>
<th>Litter number</th>
<th>Male</th>
<th>Females</th>
<th>IL6 Level (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>FA2</td>
<td>14</td>
<td>6</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>FB2</td>
<td>16</td>
<td>5</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>FC1</td>
<td>15</td>
<td>8</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>FB3</td>
<td>11</td>
<td>6</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>FA3</td>
<td>16</td>
<td>11</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>FB1</td>
<td>13</td>
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<td>214.8</td>
</tr>
<tr>
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<td>FA4</td>
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</tr>
<tr>
<td></td>
<td>FB4</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>584.0</td>
</tr>
</tbody>
</table>

Figure 54: mIA did not affect litter size or sex distribution within litters. 
A-B) No significant difference was found between litter sizes or numbers of males and female pups within a litter from saline and poly (I:C) treated dams. Individual data points are shown with mean ±SEM \( n=4 \) dams/ treatment group.
5.5.2. mIA did not affect offspring body weight throughout development

5.5.2.1. Early postnatal life

All offspring were weighed regularly throughout early postnatal life at PD6, 12, 18 and 21 before weaning (Figure 55A-D). At every time point, no significant difference was found between saline and poly (I:C) offspring. Male and female offspring were similar and within treatment groups in weight during early postnatal time points (see Table 29) for all early postnatal weights and SD).

Table 29: Early postnatal body weights

<table>
<thead>
<tr>
<th>Postnatal Day</th>
<th>Saline</th>
<th>Poly (I:C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male ±SD (g)</td>
<td>Male ±SD (g)</td>
</tr>
<tr>
<td></td>
<td>Female ±SD (g)</td>
<td>Female ±SD (g)</td>
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<td>PD6</td>
<td>14.9 ±1.0</td>
<td>14.8 ±1.0</td>
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<td>13.9 ±1.0</td>
<td>14.3 ±0.9</td>
</tr>
<tr>
<td>PD12</td>
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<td>31.5 ±2.0</td>
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<tr>
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<td>30.4 ±2.4</td>
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<tr>
<td>PD18</td>
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<td>49.6 ±3.5</td>
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<tr>
<td></td>
<td>46.9 ±4.3</td>
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</tr>
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<td>PD21</td>
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<tr>
<td></td>
<td>55.5 ±4.0</td>
<td>57.2 ±2.6</td>
</tr>
</tbody>
</table>

5.5.2.2. Adolescence and adulthood

During adolescence offspring were weighed everyday between PD30-38. At each of the time points tested, no significant difference was found between saline and poly (I:C) offspring. Poly (I:C) offspring showed a normal trajectory of development with regard to weight-gain during adolescence and adulthood. In contrast to early postnatal body weight monitoring, a significant difference was found between male and female offspring at all time points during adolescence \( F_{(3, 455)} = 228.0, \ p < 0.0001 \) (Figure 56A-B). Male Wistar offspring were significantly heavier than females at all time points tested having showed equal BW at the day of weaning.
Figure 55: mIA did not affect body weight of early postnatal offspring. A-D) No significant difference in body weight was seen throughout the early postnatal period (PD6-21) between saline and poly (I:C) pups. Graphs represent mean ±SEM and individual pup values, saline n= 16 males and 15 females, poly (I:C) n= 12 males and 12 females.
Figure 56: mIA had no effect on adolescent or adult body weight of offspring. 

A-B) No significant difference in body weight was seen throughout adolescence (A) and into adulthood (B) between saline and poly(I:C) pups. At adolescence and adulthood female offspring had a significantly reduced body weight compared to male offspring that was seen at all time points tested. (### $p > 0.001$, # = male vs. female comparison). Graphs represent mean ±SEM, saline $n$ = 16 males and 15 females, poly (I:C) $n$ = 12 males and 12 females.
5.5.3. mIA produced behaviour relevant to an anxiety-like phenotype at adolescence but not for any other behavioural task employed

To optimise this model of mIA in Wistar rats an extensive behavioural battery was performed at adolescence and adulthood.

5.5.3.1. Open field test

Anxiety-like behaviour: All offspring from saline and poly (I:C) treated dams were tested in the OFT to ascertain changes in anxiety-like behaviour. A percentage was calculated for time spent in the centre of the arena. A rat showing an increased time spent in the edges (including corners) of the arena versus the centre would suggest a risk-averse, anxiety-like phenotype. Locomotor activity in the OFT was determined by monitoring line crossings in the arena. Both male and female offspring from poly (I:C) treated dams showed increased locomotor activity in the OFT (Figure 57A). Line crossings for male offspring from saline treated dams were recorded at 85 ±7 compared to 119 ±10 in poly (I:C) offspring ($F_{(1, 20)} = 7.46, p= 0.013$). Similarly in female offspring from saline-treated dams, line crossing were recorded at 84 ±8 whereas offspring from poly (I:C)-treated dams were 119 ±10 ($F_{(1, 20)} = 14.7, p= 0.001$). With regard to locomotor activity, male and female animals from each treatment group behaved similarly in this test.

It is known that rodents show a general aversion to open spaces and a preference for enclosed or edged areas (thigmotaxis). This phenomenon was shown in our OFT where saline-treated animals spent on average 17 ±3.78 and 14 ±2.69 sec (male and female respectively) in the centre quadrant of the arena out of the 600s test period. Male offspring from poly (I:C)-treated dams showed a significant decrease in the percentage time spent in the centre of the open field test $F_{(1, 20)} = 7.20, p= 0.014$. There was no significant difference shown in female animals for percentage time spent in the centre ($F_{(1, 20)} = 0.65, p= 0.651$) (Figure 57B). Within-group variability was higher in male offspring from saline-treated dams (2.85 ± 2.06, mean ±SD) compared to those from poly (I:C)-treated dams (0.84 ±0.70, mean ±SD).

Repetitive behaviour, grooming: Time spent grooming during the OFT was recorded as a measure of repetitive-like behaviour in these rodents. This technique is frequently applied in mice (Silverman and Crawley, 2014). No significant difference was shown in
time spent (sec) grooming during the OFT between treatment groups for either sex of offspring \( (F_{(1, 20)} = 3.282, p = 0.084, F_{(1, 20)} = 1.129, p = 0.301 \) male and female offspring respectively). However, offspring from poly (I:C)-treated dams spent more time grooming on average than saline-treated offspring, male (52 ±9 vs. 75 ±10) and female (47 ±5 vs. 62 ±10) (Figure 57C).
Figure 57: mIA produced an anxiety-like phenotype in male offspring as shown by exploratory behaviour in the open field test (OFT). A) Locomotor activity during 10min OFT. Poly (I:C)-treated offspring showed significantly increased movement during this task. B) A significant reduction in % time spent in the centre (% Time Centre = Time in centre/total exploration time x 100) was found in male offspring from poly (I:C) offspring. In female offspring no difference was found between treatment groups. C) Offspring from poly (I:C) treated dams showed an increased time spent grooming during the OFT however this effect did not reach significance. (* vs. saline group, \( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \). Graphs represent mean ±SEM, saline \( n = 16 \) males and 15 females, poly (I:C) \( n = 11 \) males and 12 females.
5.5.3.2. Elevated plus maze (EPM)

The EPM is a commonly used behavioural test for investigating anxiety-like behaviour in mice and rats. A rat showing anxiety-like behaviour would be expected to spend less time in the open arms compared to the walled-closed arms during the 5 min test. At adolescence, one male was excluded from the poly (I:C) group as it did not enter the open arms during the test.

Percentage time spent in the open arms was calculated and no significant difference was found between saline and poly (I:C) offspring in either sex ($F_{(1, 20)} = 1.20, p = 0.285$, $F_{(1, 20)} = 1.62, p = 0.218$, males and females respectively) (Figure 58A). On average, male animals from saline-treated dams spent 27% of the test time in the open arms whereas offspring from poly (I:C)-treated dams spent 22%. In females, offspring from poly (I:C)-treated dams spent more time on average in the open arms of the maze (35%) whereas offspring from saline-treated dams recorded similar percentage time in the to their male litter mates, at 28%.

Although both saline- and poly (I:C) offspring spent similar percentage time in the open arms, a significant reduction in entries into the open arms was found in male poly (I:C) offspring ($F_{(1, 20)} = 6.54, p = 0.018$). This effect was not shown by female offspring ($F_{(1, 20)} = 0.11, p = 0.744$) (Figure 58B).

Added to this, latency to entry into the open arms was longer on average in both male and female offspring from poly (I:C)-treated dams (Figure 58C). This effect did not reach significance in either sex ($F_{(1, 20)} = 4.08, p = 0.057$, $F_{(1, 20)} = 2.50, p = 0.129$ male and female respectively.)
Figure 58: mIA had an effect on entries to the open arms of the EPM in male offspring only. A) % time spent in the open arms was not significantly different between saline and poly (I:C) offspring of either sex. B) % open entries was significantly reduced in male poly (I:C) offspring compared to saline ($p= 0.018$). In female offspring, no significant difference in % open entries was found. C) In male and female offspring the latency to entry into the open arms was longer on average in poly (I:C) compared to saline groups but this effect did not reach significance in either sex. $^*p< 0.05$. Graphs represent mean ±SEM, saline $n= 16$ males and 15 females, poly (I:C) $n= 11$ males and 12 females.
5.5.3.3. **Habituated burrowing**

Burrowing is a natural behaviour performed readily by mice and rats and thus represents an ethologically relevant measure of behaviour in rodents (Deacon, 2006).

During the habituated burrowing test, male offspring from poly (I:C)-treated dams burrowed less on average compared to saline offspring, as shown by the weight of dug gravel at the end of the test ([Figure 59A](#)). Although saline offspring burrowed three times more gravel than poly (I:C) offspring, this effect did not reach significance, $F_{(1, 8)} = 0.067, p = 0.802$. No difference in burrowing behaviours was shown in female offspring, $F_{(1, 14)} = 1.71, p = 0.213$. The weight of dug gravel at the end of the test was similar between treatment groups (saline $681.5 \pm 173.6$ g and poly (I:C) $571.6 \pm 133.5$ g) ([Figure 59B](#)).

In both saline and poly (I:C) treatment groups, some of the male offspring did not burrow at all during the test ($n = 7$ offspring/treatment group). Notably male offspring showed freezing behaviour during the test session. For female offspring, $n = 3$ offspring/treatment group did not burrow at all during the test session.

![Figure 59: mIA had no effect on habituated burrowing. A-B](#) No significant difference was found in the amount of dug gravel at adolescence between saline and poly(I:C) offspring in either sex. Graphs represent mean ±SEM, saline $n = 9$ males and 12 females, poly (I:C) $n = 4$ males and 9 females.
5.5.3.4. *Dyadic social interaction test*

Rats are very sociable rodents with a vast social behaviour repertoire which can be measured during general interaction as well as during play. When tested in our dyadic SIT no effect of mIA was shown in the behaviour either sex for social sniffing (Male; $F_{(1, 20)} = 0.35$, $p = 0.563$, female; $F_{(1, 20)} = 0.20$, $p = 0.662$) or following (Male; $F_{(1, 20)} = 0.02$, $p = 0.902$, female; $F_{(1, 20)} = 0.51$, $p = 0.484$) (Figure 60A-B).

Similarly, no difference was seen in time spent exploring the novel object in the arena (Male; $F_{(1, 20)} = 0.34$, $p = 0.565$, female; $F_{(1, 20)} = 1.67$, $p = 0.211$). This suggests that there was no effect on general preference for novelty in offspring from saline- and poly (I:C)-treated dams (Figure 60C). Offspring showed similar exploratory behaviour during the SIT, as shown by locomotor activity, with no significant difference found between treatment groups in either sex (Male; $F_{(1, 20)} = 1.58$, $p = 0.223$, female; $F_{(1, 20)} = 1.12$, $p = 0.303$) (Figure 60D).

The behaviours for all forms of social play measured showed no significant difference between treatment groups (Figure 61A-C).
Figure 60: mIA showed no effect on general social interaction in the dyadic SIT. No difference was shown in any parameter tested. A) Total time sniffing. B) Total time following. C) Total time exploring the inanimate object. D) General locomotor activity. Graphs represent mean ±SEM, saline n= 16 males and 15 females, poly (I:C) n= 11 males and 12 females.
Figure 61: mIA did not affect social play behaviour in the SIT. A) Frequency of pinning behaviour. B) Frequency of pouncing behaviour. C) Frequency of boxing behaviour. Graphs represent mean ±SEM, saline n= 16 males and 15 females, poly (I:C) n= 11 males and 12 females.
5.5.4. Adulthood behaviour

5.5.4.1. Elevated plus maze (EPM)

At adulthood, 5 males were excluded from the analysis of open arm exploration for all parameters as they did not enter the open arms during the entire test (n= 3 saline and n=2 poly (I:C)). During the EPM task at adulthood, male animals from saline- and poly (I:C)-treated dams spent less time in the open arms than female offspring. However, no significant difference was found between saline and poly (I:C) offspring in either sex (F(1, 14) = 0.10, p= 0.757, F(1, 20) = 0.05, p= 0.820, males and females respectively) (Figure 62A). Compared to the adolescent EPM task exploration time in the open arms was lower for both male and female animals however this was more pronounced in the male offspring.

In line with both saline- and poly (I:C) offspring spending a similar amount of the test time in the open arms, entries to the open arms were not different between groups (F(1, 14) = 0.32, p= 0.579, F(1, 20) = 0.51, p= 0.482, males and females respectively) (Figure 62B).

Similarly to adolescence, average latency to entry into the open arms was longer in both male and female offspring from poly (I:C)-treated dams. However, this was not as pronounced as in the first testing and again did not reach statistical significance in either sex (F(1, 14) = 0.77, p= 0.394, F(1, 20) = 1.61, p= 0.219 male and female respectively) (Figure 62C).
Figure 62: mIA had no effect on behaviour in the EPM. 

A) % Time spent in open arms was not significantly different between saline and poly (I:C) adult offspring in either sex. 

B) % open entries was not significantly different between saline and poly (I:C) adult offspring in either sex. 

C) No significant difference was shown in either sex for latency to entry into the open arm. 

Graphs represent mean ±SEM, saline n= 13 males and 15 females, poly (I:C) n= 9 males and 12 females.
5.5.4.2. Habituated burrowing

At adulthood a number of offspring did not show any burrowing behaviour during the test session. In the saline group, 2 male offspring and 3 female offspring did show any burrowing behaviour and, thus, no gravel was expelled during the test session. In the poly (I:C) treatment group, 5 male offspring and 2 female offspring did not burrow during the test. All of these animals were excluded from the analysis. Similarly to adolescence, some male offspring showed freezing behaviour during the test session.

During the test session, male offspring from poly (I:C)-treated dams burrowed less on average compared to saline offspring, as shown by the weight of dug gravel at the end of the test (Figure 63A). This effect did not reach significance ($F_{(1, 13)} = 2.66, p = 0.127$). Female offspring showed a similar amount of digging during the test session in saline and poly (I:C) treatment groups (Figure 63B).

![Figure 63: mIA did not affect habituated burrowing at adulthood. A-B) No significant difference was found in the amount of dug gravel at adulthood between saline and poly (I:C) offspring in either sex. Graphs represent mean ±SEM, saline $n = 14$ males and 12 females, poly (I:C) $n = 6$ males and 10 females.](image)
5.5.4.3. Dyadic social interaction test

Similarly to adolescence, when tested in our dyadic SIT no effect of mIA was shown in either sex of adult offspring for social sniffing (Male; $F_{(1, 20)} = 0.94$, $p = 0.345$, female; $F_{(1, 20)} = 0.22$, $p = 0.646$) or following (Male; $F_{(1, 20)} = 1.01$, $p = 0.327$, female; $F_{(1, 20)} = 0.02$, $p = 0.886$) behaviour (Figure 64A-B). Similarly, no difference was seen in time spent exploring the novel object in the arena (Male; $F_{(1, 20)} = 3.11$, $p = 0.093$, female; $F_{(1, 20)} = 1.12$, $p = 0.302$). This suggests that there was no effect on general preference for novelty in offspring from saline- and poly (I:C)-treated dams (Figure 64C). Offspring showed similar exploratory behaviour during the SIT, as shown by locomotor activity, with no significant difference found between treatment groups in either sex (Male; $F_{(1, 20)} = 1.58$, $p = 0.223$, female; $F_{(1, 20)} = 1.12$, $p = 0.303$) (Figure 64D).

During the SIT at adulthood, fighting behaviour was seen frequently between test animals and the conspecific, particularly in males. This behaviour was scored, and although on average offspring from poly (I:C)-treated dams showed less fighting behaviour than those from saline-treated mothers, no significant difference was found ($F_{(1, 20)} = 2.62$, $p = 0.121$). Female animals showed fighting behaviour much more infrequently and, similarly, no significant difference was found between treatment groups ($F_{(1, 20)} = 0.83$, $p = 0.374$) (Figure 65A-B).
Figure 64: mIA had no effect on general social interaction in the dyadic SIT. No difference was shown in any parameter tested. A) Total time sniffing. B) Total time following. C) Total time exploring the inanimate object. D) General locomotor activity. Graphs represent mean ±SEM, saline n= 16 males and 15 females, poly (I:C) n= 11 males and 12 females.
Figure 65: mIA had no effect on fighting behaviour during adulthood SIT. A-B) No difference was shown between treatment groups in either sex. Graphs represent mean ±SEM, saline $n=16$ males and 15 females, poly (I:C) $n=11$ males and 12 females.
5.6. Discussion

The poly (I:C) model of mIA has been used extensively to investigate the effects of immune activation on the developmental profile of offspring. It has been shown that different doses and timing of the mIA insult can induce different behavioural phenotypes because of the disruption to different developmental processes (Careaga et al., 2017). The aim of this study was to characterise the behavioural phenotype induced by mIA at GD12.5 using 10 mg/kg i.p. poly (I:C) in Wistar rats in both male and female offspring. While ASD pathophysiology is manifested in early childhood deficits are sustained into adolescence/adulthood therefore behavioural analysis as well as molecular correlates must be investigated at multiple developmental time points. To my knowledge, this gestational timing of exposure of poly (I:C) and behavioural outcomes in offspring, while extensively investigated in mouse models, has not been explored in rats.

5.6.1. mIA produced a sickness response at GD12.5 as shown by CBT and IL-6 elevation

In this third study using exposure of 10 mg/kg i.p. at GD12.5, similar results were shown in the pregnant dams, as described in Chapter 4. An acute increase in CBT was seen in poly (I:C)-treated dams at 3 hr post-injection. In agreement with all three studies using 10 mg/kg i.p. poly (I:C), no change in BW was shown post-injection of poly (I:C). It was also found that, again, poly (I:C) did not affect the weight gain of dams throughout the period of gestation showing that this exposure to the viral mimetic does not have an effect on maternal weight gain. Thus it is concluded in this model of mIA that BW cannot be used to predict any effects of 10 mg/kg i.p. poly (I:C) at GD12.5. Again in line with the previous studies, none of the dams showed any obvious sickness behaviours (piloerection, lethargy or hunched posture) at the time-points measured highlighting that this is not a robust measurement of the induction of mIA in this model.

In this cohort of animals, only 3 dams showed a detectable level of IL-6 using the described analytical methods. Furthermore, the dam that recorded the highest serum concentration of IL-6 had a litter of only 5 offspring (3 males and 2 females). Since the growth weight of the pups and the potential differences in maternal care that may have resulted in a smaller litter, these pups were excluded from the analysis. As a result, the
behavioural findings were from offspring of dams with low or undetectable levels of IL-6. Due to the complexity of the immune response, a limitation of this study was the cytokine analysis of only IL-6. This was due to the low levels of whole blood that were obtained from these dams resulting in only enough plasma for a single ELISA. This study confirms the hypothesis that measuring IL-6 alone is not sufficient to gain a clear picture of the response to poly (I:C) at GD12.5. Indeed, the complex interactions with IL-17 have been a focus for investigation in the pathways underlying mIA particularly with regard to models of ASD (Choi et al., 2016b; Wong and Hoeffer, 2018). Taken together, the plasma cytokine data collected across all three studies, shows that IL-6 is often elevated in plasma following injection of 10 mg/kg i.p. poly (I:C) at GD12.5; however this upregulation is highly variable between dams. Due to the constraints of the current project licence, I was unable to measure a time course of the IL-6 response. A critical area for further validation of this model would include the addition of a femoral or jugular vein cannula to rats prior to mating. This would enable the assessment of a number of different cytokines in plasma at multiple time points. This could include baseline measurements (prior to pregnancy), during pregnancy and at multiple time points following exposure to poly (I:C). Not only would this enable a much fuller picture of the time course of cytokine production, but also a clearer indication of dam-to-dam variation in cytokine levels prior and following exposure to poly (I:C).

Data from previous studies and data from our own lab has shown that plasma levels of IL-6 can range from 1000 to as high as 9000 pg/ml following injection of poly (I:C) at GD12.5 (mice), GD15 and GD17.5 (rats) (Connor et al., 2012; Smolders et al., 2015; Grayson et al., 2017). In this study, the plasma levels of IL-6 were much lower at GD12.5, ranging from 200-600 pg/ml. It is possible that at this gestational time point in the Wistar rat the immune system may be suppressed in order to protect developing foetuses. However, the studies in mice at GD9-12.5 suggest that in the early stages in pregnancy, an upregulation of IL-6 is seen at 3 hr post-injection (Meyer, 2006; Connor et al., 2012). Due to the low number of dams included in this study it is proposed that further work is required to determine the validity of this finding and whether it is strain-dependent. Although isolated to one behavioural test, the presence of an anxiety-like phenotype in the male offspring from these dams, suggests that poly (I:C) did cause a disruption to development that manifested in this particular behavioural paradigm.
Further work is the cytokine profile in Wistar rats following 10 mg/kg i.p. poly (I:C) exposure at GD12.5.

5.6.2. mIA produced an anxiety-like phenotype that was only shown in adolescent offspring

5.6.2.1. Adolescence

In order to validate this mIA model in rats it was necessary to employ a range of behavioural tests to produce a full representation of the phenotype induced. Furthermore, it was critical to include offspring of both sexes as this is typically missing in published work. Overall, offspring from poly (I:C)-treated dams were found to behave typically with regard to social behaviour, although a sexually dimorphic anxiety-like phenotype was recorded. This was seen in the OFT where only male offspring from poly (I:C)-treated dams showed a reduced time spent in the central area of the arena. Interestingly this effect was found despite male offspring showing hyperactivity in the OFT arena. In addition, these male offspring showed reduced entries and an increased latency into the open arms supporting an anxiety-like phenotype. Both male and female offspring exhibited repetitive grooming behaviours that were, on average, more frequent in offspring from poly (I:C) treated dams but this increase was not significant. The results from the habituated burrowing task did not support the presence of repetitive or stereotyped behaviours in these offspring. During the burrowing test, male offspring that did not engage in as frequent burrowing behaviour and exhibited freezing behaviour during the task. This provides some further evidence of anxiety-like behaviour in these animals. With regard to the behaviour shown during the social interaction test, male offspring did not behave differently to offspring from saline dams, highlighting the specificity of this anxiety-like phenotype. Thus, our mIA model shows aspects of an ASD-like phenotype that have been previously shown in mouse models (Hsiao et al., 2012) but, as predicted, does not incorporate the entire spectrum of relevant behaviours to the human condition. At this developmental time point this model of mIA may represent a platform for investigating specifically anxiety-like behaviour and may be relevant to specific cohorts of patients with NDDs such as ASD.
5.6.2.2. Adulthood

At adulthood, offspring did not maintain a significant anxiety-like phenotype and exhibited similarly neurotypical social behaviour as in adolescence. This highlights that for this particular model, it may be that adolescence is the key time to analyse aspects of anxiety-like behaviour. There are a number of potential reasons for the lack of behavioural deficits seen at this time point. Most simply it is proposed that this model of mIA does not result in changes in the behaviour of either male or female offspring at adulthood. However, it may also be that the tests employed in this study are not sensitive enough to highlight any deficits in the offspring. It is also possible that a single 10 mg/kg i.p. dose of poly (I:C) may not be sufficient by itself to produce a phenotype in these animals. A key limitation to the current work is the number of dams that were used. This may not have been a big enough cohort to adequately assess the effects on offspring. It is also possible that, in order to assess the chronicity of behavioural changes in this model, the offspring should be tested even later in life rather than at the young-adulthood time point chosen within this study. Indeed, it has been shown that some behavioural phenotypes do not appear until later in adulthood ((Meyer et al., 2008b).

It is proposed that the offspring may require a “second-hit” to expose the vulnerable or “built to fail” neural system that has developed as a result of gestational mIA. These offspring may be considered as “primed” so that on receiving a stressor or subsequent immune challenge during a critical postnatal period, a more severe and widespread behavioural phenotype would be observed. This has been proposed in the field of schizophrenia with the period of adolescence as a critical period for a second hit. It is known that in human ASD, it is not only acute maternal immune activation in that increases the risk for ASD but that ASD children show a heightened immune state in development (Onore et al., 2012). It is also thought that the increased circulation of these immune markers may further affect the brain and worsen behaviour (Breeze et al., 2013). Mimicking this in offspring exposed to mIA will be a key area for further development of this model.

In conclusion, the behavioural data presented in this chapter expand our knowledge of mIA modelling in rats by applying ethologically relevant tests in line with the characteristic behaviours known to be altered in ASD. Furthermore, these tests were
performed in both male and female rats and using a longitudinal analysis from adolescence to early adulthood that is currently lacking in the majority of studies. The anxiety-like phenotype produced in male offspring, following exposure to 10 mg/kg i.p. poly (I:C) at GD12.5 presents a useful platform for the study of NDDs including ASD.
Chapter 6: General conclusions
6.1. Main findings and clinical relevance

During the course of this project, a rodent model of mIA using exposure to 10 mg/kg i.p. poly (I:C) at GD12.5 in Wistar rats has been developed. The series of experiments reported in this thesis aimed to validate this model for NDDs with molecular and behavioural analyses targeted towards an ASD phenotype. A summary of the main findings from both poly (I:C)-treated dams and their offspring across all studies are shown in Table 30 and Table 31 respectively.

Table 30: Response in pregnant dams across experiments (↔ no change, ↑ increase, ↓ decrease, ND not determined)

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<thead>
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<th>Experiment</th>
<th>GD21 (Chapter 3)</th>
<th>PD21 (Chapter 4)</th>
<th>Adolescence &amp; Adulthood (Chapter 5)</th>
</tr>
</thead>
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<td>Maternal Cytokines</td>
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<td>IL-6: ↑</td>
<td>IL-6: ↑</td>
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<td>TNFα: ↔</td>
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<td></td>
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<td>IL-1β: ↔</td>
<td>IL-1β: ND</td>
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**Table 31:** Response in poly (I:C) offspring across experiments (M= male, F= female; ↔ no change, ↑ increase, ↓ decrease, ND not determined, FC= frontal cortex, CB= cerebellum, USV= ultrasonic vocalisations.)

<table>
<thead>
<tr>
<th>Experiment</th>
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<th>PD21</th>
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<th>Adulthood</th>
</tr>
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<td>F</td>
<td>M</td>
<td>F</td>
<td>M</td>
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<tr>
<td><strong>Gene Expression FC</strong></td>
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<td>↓</td>
<td>↔</td>
<td>SHANK3</td>
</tr>
<tr>
<td><strong>Gene Expression CB</strong></td>
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<td>↓</td>
<td></td>
<td>MEF2c, SNAP-25</td>
</tr>
<tr>
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<td>↔</td>
<td>↓</td>
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<td><strong>Body Weight</strong></td>
<td>↔</td>
<td>↓</td>
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<td>↔</td>
</tr>
<tr>
<td><strong>Brain Weight</strong></td>
<td>↔</td>
<td>↑</td>
<td></td>
<td>ND</td>
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<tr>
<td><strong>Microbiota</strong></td>
<td>ND</td>
<td>Bacterial diversity (on average)</td>
<td>↓</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clostridiales</td>
<td>↑</td>
<td></td>
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<tr>
<td><strong>Behaviour</strong></td>
<td></td>
<td>USV ↔</td>
<td></td>
<td>Anxiety</td>
</tr>
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</table>
This work has attempted to introduce improvements to methodology in comparison with current research in this area. These have included the breeding of animals in house, to improve control over the maternal and offspring environment, as well as reducing stress during transport of pregnant animals likely to exacerbate any developmental disruption in offspring. In addition, the most appropriate strain of rat and dose of poly (I:C) to be used was identified in the initial pilot work, whereas, typically, this type of validation is either ignored or omitted from experimental methods. This work has shown that physiological responses (CBT, cytokine elevation) in the dam following poly (I:C) exposure are variable, even when the timing of exposure and the dose of the viral mimetic is controlled. This has highlighted a significant limitation of many previous mIA studies where no analysis of the response in the dam is made following exposure to a viral mimetic (see Table 7, Chapter 3). My results have demonstrated that it cannot be assumed that an immune response will be triggered following poly (I:C) exposure, (even at a dose shown to be sufficient to produce a significant response in a separate cohort of animals). Instead, it is suggested that CBT and elevation of plasma cytokine levels (at multiple time-points), as a minimum requirement, should be monitored in all dams as standard procedure for mIA induction.

Another key limitation identified by the work conducted for this thesis was a focus on only IL-6 elevation (Babri et al., 2014; Smolders et al., 2015; Meehan et al., 2017) or indeed the absence of any cytokine measurements following mIA (Malkova et al., 2012; Schwartzer et al., 2013; Onore et al., 2014; Farrelly et al., 2015; Zhang and van Praag, 2015; Giovanoli et al., 2016; Patrich et al., 2016). In order to understand the variability in phenotypes across different mIA models, including differential gestational exposure, viral mimetic used, concentration and route of administration, dose, species and strain differences, quantification of the immune activation is essential. In addition, standardised measures for confirming mIA induction are required to understand what role immune markers (i.e. cytokines) have in disrupting development and how subtle changes to the mIA protocol can affect this.

The majority of previous studies do not analyse the full developmental trajectory of offspring in mIA models. However, here, the analysis of multiple developmental time points, (GD21, PD21, adolescence and adulthood) has provided a longitudinal overview of the effects of mIA at GD12.5 in offspring. The standard approach of only studying
male offspring has been challenged also with the inclusion of both sexes of rats, and the 
observation of sex dependent effects.

Significant effects of mIA on gene expression, morphometric parameters, and the 
presence of a behavioural phenotype, have been shown to be sex-specific, highlighting 
the necessity of this approach. Interestingly, gene expression changes related to synaptic 
function shown prior to birth were not sustained in offspring at PD21 and different 
genes were shown to be significantly altered at this time point. This confirms the 
importance of evaluating the full developmental trajectory in mIA studies. The 
sequencing of faecal microbiota from offspring suggests subtle effects on the GI system, 
as a result of this developmental insult. Due to recent improved understanding of the 
relationship between GI comorbidities and the severity of ASD symptoms, the 
biological significance of such microbial changes requires further investigation. Finally, 
the behavioural work revealed an anxiety-like phenotype in adolescent male offspring, 
providing additional evidence for the validity of using this mIA model for studying 
aspects of an ASD phenotype. It is possible that the behavioural battery was not 
sufficiently sensitive to assess any deficit outside of anxiety-like behaviour either due to 
lack of sensitivity in the tests used, or the timing of the behavioural battery. However, 
as anxiety is a significant clinical symptom of ASD as well as other NDDs, this finding 
strengthens the clinical relevance of the model.

To my knowledge, this is the first mIA study investigating the effects of 10 mg/kg i.p. 
poly (I:C) in Wistar rats at GD12.5. I have investigated the immune response in 
pregnant dams following poly (I:C) exposure alongside an in depth, developmental 
analysis of brain, behaviour and gut in both male and female offspring. In doing so, 
previously unknown effects of mIA at this time point have been determined which are 
useful for the continued application of poly (I:C) in preclinical mIA models, as well as 
for allowing translatability to the clinical setting to be better determined. The focus on 
infection and inflammation as a key risk factor for neurodevelopment or psychiatric 
disorders has increased ever further since the start of this project (Depino, 2017; 
Spencer and Meyer, 2017) reinforcing the clinical relevance of mIA.
6.1.1. Variable insult hypothesis

In the introduction, the “variable insult hypothesis” for NDDs was introduced and a figure was presented outlining the potential mechanisms involved. In an attempt to summarise the results across all of the experiments conducted in this thesis, an updated figure is presented below showing how the findings from the GD12.5 mIA model may support this hypothesis (Figure 66). In addition, the areas for future work, (see section 6.3, p.217), that are essential for a more complete validation of this model are highlighted.

As already discussed, the heterogeneity of the ASD patient population provides the most significant challenge to mechanistic understanding and the development of therapeutics. With such varied phenotypic outcomes among ASD patients, in addition to the presence of a number of comorbid conditions, it is likely that there are multiple pathophysiological pathways behind this “whole body” disorder. It is proposed that in contrast to other animal models for ASD, that involve an extreme mechanism behind the developmental disruption (e.g. gene KO), mIA primes or produces susceptibility in the developing offspring and that is manifested in subtle phenotypic changes that are specific to the timing or intensity of the immune activation. As a result, different models of mIA will be useful different aspects of ASD (e.g. the presence of anxiety-like behaviour in the absence of other characteristic traits), and each model may be specifically relevant to a subtype of patient. This is arguably more relevant to the clinical setting where similarly ASD patients present with vastly different behavioural and neuroimmunological phenotypes. Disruption to the developing neurological and immune system may produce susceptibility to subsequent, and specific insults later in development (e.g. an additional immune challenge) and this hypothesis can be tested by the introduction of a “two-hit” model which is discussed in section 6.3 p.217.

A single animal model will never be sufficient to model a complex NDD in its entirety. Indeed, NDD phenotypes overlap heavily and likely share common aetiologies. The true validity of this model is recognised when taken in the context of previous mIA findings, using a different gestational exposure or strain/species of animal. This new model provides an additional platform which can be used in conjunction with other models to achieve a comprehensive mechanistic understanding of the wide-ranging effects of NDDs with the ultimate aim of furthering understanding of NDDs in the clinical setting.
Figure 66: Variable insult hypothesis with the addition of experimental findings from this thesis. 10 mg/kg poly (I:C) exposure to the pregnant dam results in a variable immune response that can affect the developing foetus, perhaps through altered placenta function or cytokine exposure. The subsequent disruption to neurodevelopment may lead to chronic sickness behaviour that manifests as the anxiety-like behaviour and altered gut microbiota in offspring.
6.2. Limitations

As this was the first time developmental work was carried out in our lab, some of the limitations are partly due to practical issues. For example, I faced a number of issues with the mating of rats. This included rats that did not successfully mate, or the presence of a vaginal plug that did not accurately predict pregnancy which could only be confirmed after the administration of drugs at GD12.5. As a result, some females were excluded from a study and the \( n \) number of dams was low, particularly for the behavioural study. The application of nested analysis enabled the appropriate use of all offspring from a litter, in the future a greater number of dams would be used considering both between and within litter variability. For the postnatal studies, only four offspring per sex were kept to control the growth of the pups. The knowledge gained from these studies however, show that it would be appropriate to keep five males and female from each litter which would also increase the \( n \) number for the analysis of offspring.

The variability in response to poly (I:C) between cohorts has underlined the importance of standardised methods for measuring mIA induction. However, it also poses a significant limitation for the development of reliable and reproducible preclinical models for studying NDD pathophysiology, within and across labs. The variability in molecular weight (MW) and therefore the length of the double-stranded inosine and cytidine chains of the poly (I:C), can affect the immune response induced in animals. Indeed, it has been shown previously that poly (I:C) from two different suppliers (Sigma and GE healthcare), produced distinctly different immune responses in cells due to the variability in MW. In addition, different cell types respond to poly (I:C) in a length dependent manner (Mian et al., 2013). A limitation of the poly (I:C) used in this study (supplied by Sigma), is the lack of information provided about the MW of the dsRNA analogue. As already highlighted, the MW of a Sigma poly (I:C) batch ranges, on average, between 200-500 kDa. Despite this, Sigma poly (I:C) is the most widely used across previous mIA studies and has been shown to be effective for inducing mIA in rodents (Meyer et al., 2008b; Shi et al., 2009; Malkova et al., 2012; Yee et al., 2012; Garay et al., 2013; Giuliani et al., 2013; Onore et al., 2014; Labouesse et al., 2015; Choi et al., 2016b). Notably, the discussion of MW variability is omitted from all of these studies but has been observed in one previous study where three batches of poly (I:C)
from Sigma were shown to produce different immune activation as shown by IL-6 elevation in non-pregnant females (Harvey and Boks, 2012). In addition to MW variability, the endotoxicity (LPS contamination) of the poly (I:C) supplied by Sigma is not reported and may range between 25-1250 EU/mg. This information was not known for the batches used in these experiments so it cannot be ruled out that this may have further influenced the findings. The variability between batches of poly (I:C) may explain, in part, the differences between the cytokine responses shown in my results. In order to circumvent the issue of between batch variability, it is suggested that the MW is first tested for all batches of poly (I:C) used in mIA work, for example using the multi-angle light scattering technique. Alternatively, poly (I:C) could be purchased from Invivogen, a company that supply the poly (I:C) at specified low or high MW. Moreover, Invivogen measure the levels of gram negative bacteria in the poly (I:C) batch (using a kinetic chromogenic LAL assay), providing improved quality control of contamination across batches. Accurate quantification of the differences between batches, particularly of MW, would enable correlation with maternal IL-6 upregulation, placental measures, and subsequent changes in the offspring.

It is clear that consistency in methodology is required to further improve the suitability of mIA for a robust animal model. Another limitation of the current work was the lack of investigation of the temporal profile of cytokine responses following poly (I:C) exposure. Due to the constraints of the project licence, it was not possible to take multiple blood samples via a cannula. With the opportunity to take a blood sample from the animals at baseline (before pregnancy), on the day of poly (I:C) administration, and then at multiple time points after the poly (I:C), a more complete picture of the inflammatory profile and subsequent responses to mIA in these dams would be determined. In addition, the ELISA technique employed in these studies meant that only single cytokines could be measured, and each run required a reasonably large amount of rat plasma. The use of an array kit would enable the detection of multiple cytokines and chemokines simultaneously, improving the scope of results presented here.
6.3. Future research directions

There are two potential mechanisms by which mIA may influence neural development and the associated atypical molecular and behavioural outcomes as previously proposed by Schwarz & Bilbo 2011. Exposure to maternal immune activation in neonatal life could permanently alter or disrupt the development of pathways important for neural processing and the expression of certain behaviours over time. Alternatively, early life immune activation could alter the programming of immune function manifested in the adolescent or adult, negatively affecting how an individual responds to a subsequent immune challenge, resulting in atypical behaviours (Schwarz and Bilbo, 2011).

The molecular and behavioural changes produced in the “one-hit” model, presented in this thesis, may be explained by the former mechanism, where exposure to poly (I:C) at a single time point during early pregnancy altered the development of neural pathways. The aim of developing and validating this “one-hit” model of relevance to ASD has, to an extent, been met by the studies presented in this thesis which shows subtle changes to early brain development as well as changes to the gut microbiota and appearance of a behavioural phenotype. In order to more fully validate this “one-hit” model, a few key developments are suggested. As previously discussed, and potentially the most important follow-up experiment, will be to determine protein expression of the target genes highlighted in the GD21 and PD21 work. With regard to mechanistic understanding, analysis of the placentas collected from the GD21 study are likely to provide additional information about how mIA results in a developmental disruption in offspring. The inclusions of additional tests to investigate the effects of mIA on cognition (e.g. novel object recognition, attentional set shifting) would expand the behavioural analysis of this model. Similarly, the study of maternal care following mIA is critical to determine whether poly (I:C) may also have an on maternal behaviour (nest building, nursing etc.) that may influence neonatal development and, subsequently, the phenotype shown in offspring.

As the phenotype observed in these offspring was subtle, it is possible that “one-hit” of poly (I:C) may have primed a “built to fail” system, as outlined by the latter mechanism. Therefore, a more robust change to brain and behaviour may occur with subsequent immune challenge or another stressor. In order to determine whether a more widespread phenotype is inducible in these animals, a combination of stressors or “hits” may be
required. The “two-hit” hypothesis has received attention in schizophrenia modelling due, to the second-wave hits that have been identified in adolescence (such as social stressors or high potency cannabis use) and “booster” hits identified in adulthood following the first psychotic episode (Maynard et al., 2001; Millan et al., 2016). Ultimately, future NDD research will benefit greatly from the inclusion of genetic susceptibility and neonatal/postnatal environmental manipulations (Powell, 2010). My initial work did not incorporate dual or triple hits as it was important to establish the effects of mIA in this model first. However, now that the “one-hit” model has been reasonably well validated, there is the opportunity to extend this established mIA model to incorporate ethologically and ASD relevant stressors.

Clinical evidence supports a mechanism of altered immune system as a result of mIA. The development of the GD12.5 model with a second-hit, for example an additional immune challenge in postnatal life, is a logical step for further research. This may be achieved by exposing offspring to poly (I:C) or LPS in the first few postnatal days and monitoring the immune system response (i.e. plasma cytokine levels). With the appropriate control groups included, it will be possible to determine if the addition of a second immune challenge produces a more widespread phenotype in the offspring. As already discussed, the variable insult hypothesis suggests that it may be the presence of a comorbid gut pathology that causes a heightened inflammatory state, resulting in chronic sickness behaviour manifesting as the phenotypes seen in ASD patients. The microbiota changes found in PD21 offspring and clinical findings make this is a particularly interesting area for further work. Recent evidence also suggests a role for maternal diet which, in addition to mIA, may produce a more robust ASD phenotype in offspring. Indeed, maternal obesity and the presence of a high-fat diet during pregnancy have been suggested as additional risk factors for ASD (Connolly et al., 2016). The biological mechanism of how high-fat diet affects neurodevelopment has not been identified, although a heightened inflammatory state has been reported in both dams (elevated cytokine levels) and offspring (microglial activation in the hippocampus) (Bilbo and Tsang, 2010). In the dual model, in addition to the exposure to poly (I:C) at GD12.5, pregnant dams would be maintained on a high-fat diet throughout pregnancy and until offspring were weaned. The same behavioural battery used in this thesis could then be used to tease out any differences in the offspring phenotype following the additional developmental challenge. Interestingly, a high-fat diet has been shown to
influence maternal care, the programming of anxiety and social behaviour as well as disrupting development of the gut microbiota of offspring (Sullivan et al., 2015; Buffington et al., 2016; Connolly et al., 2016). The development of a dual hit model, including detailed investigation of the maternal foetal interface, the role of maternal care, and a combination of analytical methods, (including behavioural testing, neuroimaging and post-mortem pathology), is warranted for future studies conducted in this area.

In summary, this project has succeeded in producing a model of mIA that may be useful to investigate molecular and behavioural aspects of a phenotype relevant to NDDs such as ASD. It is suggested that this “one-hit” exposure to poly (I:C) produced a model of mIA with a priming mechanism, relevant for investigating NDDs at the preclinical level. Subject to the future work suggested here, there is great potential for this model to be used, in conjunction with existing mIA models, to determine specific time periods and new neuronal and GI tract targets for therapeutic intervention for the prevention and/or treatment of NDDs.
References


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Thompson, P.M., Sower, A.C., and Perrone-Bizozero, N.I. (1998). Altered levels of


Wolff, A.R., and Bilkey, D.K. (2010). The maternal immune activation (MIA) model of schizophrenia produces pre-pulse inhibition (PPI) deficits in both juvenile and adult rats but these effects are not associated with maternal weight loss. Behav. Brain Res. 213: 323–327.


Appendices

Appendix A: Chapter 3 and 4

Information produced by Qiagen regarding the primers used for RT-qPCR. Primers were all bought pre-made and prepared according to manufacturer’s instructions.

Key

<table>
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<th>Transcript</th>
<th>Selected product</th>
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<tr>
<td>SNP</td>
<td>Coding sequence</td>
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SDHA

NM_130428, bp 2277

GAPDH

NM_017008, bp 1306

SHANK3

NM_021676, bp 7391
Appendix B: Chapter 5

Functional gut bath

One of the initial aims of this thesis was to investigate any changes to the gut in offspring following mIA. In an attempt to analyse any changes to gut function in our poly (I:C) mIA model a functional colonic gut assay was performed. Colonic function was particularly interesting as diarrhoea as well as constipation are among the most commonly reported symptoms for ASD patients with comorbid gut problems (Buie et al., 2010; Hsiao, 2014).

The methods for designing and running the functional gut bath were taken from Patel et al., 2014. The experiment was designed based on gut work using the guinea-pig colon and work was carried out at the University of Brighton to confirm the methods required for this experiment. Once the methods were established, male and female offspring from the behavioural study in this chapter were used for measurement of gut motility at adulthood (PD85-90).

The whole colon was harvested from the rat following the adulthood behavioural test battery at ~ PD80 and immediately placed in ice cold Krebs buffer solution. The Krebs buffer was made using the recipe listed in Table 32. It was oxygenated for an hour before use with 95% O₂ and 5% CO₂ to reach a pH 7.4. The faecal pellets used for the experiment were collected prior to the experiment from the rat’s home cage and dried overnight in an oven. They were subsequently painted to maintain structural integrity in the gut bath.

Table 32: Krebs buffer recipe

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<tr>
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<tr>
<td>CaCl₂</td>
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<tr>
<td>MgCl₂</td>
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</tr>
<tr>
<td>NaH₂PO₄</td>
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</tr>
<tr>
<td>NaHCO₃</td>
<td>25</td>
</tr>
<tr>
<td>Glucose</td>
<td>11</td>
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</tbody>
</table>
The whole colon was loosely pinned in a flow bath, allowing a lateral movement of approximately 0.5 cm about the mid-line and perfused with oxygenated Krebs buffer solution at 37 ± 1 °C at a flow rate of 10 ml min−1. A small (~ 2 mm) incision was made in both ends of the colon and the openings pinned flat to facilitate pellet insertion and its expulsion at the distal end (Figure 67). The colon was then left to stabilise in the gut bath for 15 min. To encourage movement the lumen of the colon was flushed with warm Krebs buffer solution. In all recordings no propulsion of the faecal pellets within the lumen of the isolated colon were achieved. Spontaneous movements of the colon were recorded by video camera however, when placing a pellet in the colon no propulsion was recorded. Testing was performed over the three days where different experimental conditions were applied in attempt to achieve movement of the faecal pellet in the isolated colon. These included;

- Longer equilibration times in the bath (15 min – 1 hr)
- Continuous O₂/CO₂ bubbling of the Krebs solution to better control the pH
- Dropping the temperature to 35 °C in attempt this to reduce a lot of the spontaneous disorganised contractions to leave some that might drive motility
- Inserting the pellet further in to the colon to provide plenty of tissue behind the pellet to drive contraction
- Extending the dissection of the colon to include some of the caecum. This was performed in order to ensure all the tissue involved in the initiating colonic migrating motor complex’s (CMMCs) that drive motility (typically the proximal colon) was included in the preparation.

However, none of the above adjustments were successful in achieving faecal pellet propulsion in the isolated colon. Alternative methods such as the use of a stimulated upright organ bath may facilitate further experiments that are required to determine any changes to gut functionality in this model of mIA.
Figure 67: Colonic gut bath. A) The gut bath used in the preliminary work carried out at the University of Brighton where successful propulsion of a pellet was achieved in the guinea-pig colon. B) Example of the gut bath created for the rat colon where propulsion of a faecal pellet was unsuccessful. (* = proximal end of the colon where the faecal pellet was inserted).
Appendix C

I. Conference abstract publications (as co-author) and teaching


Kowash H., Edye ME, Oladipo JM, Fasolino V, Hager R, Neill JC, Glazier JD. The effect of prenatal maternal immune activation on fetal development in a model investigating the developmental origins of schizophrenia. BNA Festival of Neuroscience, April 2017

Potter HG., Edye ME, Oladipo JM, Fasolino V, Hager R, Neill JC, Glazier JD. The Effect of Maternal Immune Activation on Placental Gene Expression and Mother-Offspring Interactions in Rats. BNA Festival of Neuroscience, April 2017


IV. Teaching

**Teaching assistant** - Manchester Pharmacy School, The University of Manchester 2014-2017
The Valproate rat model of Autistic Spectrum Disorder (ASD): Can changes in neural gut innervation provide new insight into aetiology?

Joanna Dennison1, Rebecca Trekeaven2, Viviana Trezza1, Michela Servadio2, Ping Wang2, Joanna Nelligan3, Jakkiel Miyani4
1 Manchester Pharmacy School and 2 Faculty of Life Sciences, University of Manchester, Manchester, M13 9PT, UK
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The Valproate (VPA) Rat Model

Introduction
Autistic spectrum disorder (ASD) is a neurodevelopmental condition that affects central and peripheral neurodevelopment, neurochemistry and behaviour. Current aetiological understanding is limited; however, symptomatic heterogeneity within patient cohorts supports a complex interaction of genetics and environmental mechanisms (Goyal & Miyani 2014). Alongside the core behavioural symptoms (e.g. impairment in social interaction) recent focus has been given to the presence of compromised gastrointestinal (GI) dysfunction (Arrieta et al. 2010).

Methods
Animal models of ASD that employ a maternal insult allow the study of behavioural and pathophysiological changes in affected offspring. Male Wistar rats from injected dams were used for behavioural testing in adolescence and adulthood. These animals were found to exhibit significantly less and demonstrated greater anxiety in behavioural testing.

Valproate (VPA) Model

Microbiota changes in VPA treated rats

Figure 2: A) Organism tree showing the full cohort of different bacterial families present in the fecal samples of controls and VPA-treated rats (n=5). B) A graph to show the significant reduction in the phylum of acetobacteriaceae found in VPA rats vs. control (* P < 0.01). Student (t-test).

The acetobacteriaceae phylum makes up only a very small proportion of total bacterial phyla.

Neural innervation to the gut

Results
Neural innervation was measured in both small (S) and large intestine (L). Across all sections of S1, no significant difference was found in neural innervation between control and VPA treated rats.

Three equidistant tissue samples from the large intestine were used for the analysis of changes to neural innervation to the large intestine. The average % area stained across these sections revealed significant increase in VPA-treated vs. control rats (Figure 3).

6-nitro staining L: Mucoasa and Muscle

Figure 3 A heat map to show the bacterial species found to be statistically different between control and VPA rats (n=5). 40 species differences were found between the groups. The heat map shows within each treatment group whether the bacterial species was enriched or depleted compared to the mean.

Conclusion
These preliminary data are the first to show that a single prenatal exposure to VPA increases neural innervation to the GI tract. This was only seen in L1, which correlates with the developmental stage at which the VPA insult was administered. Changes were also recorded in the GI bacterial environment at the species level. Increased nervous supply may provide abnormal feedback from the gut to the brain, resulting in the aberrant behaviours produced in ASD. Increased understanding of this gut-nervemicrobiota-brain interaction within ASD is particularly important given the incidence of comorbid GI abnormalities seen in the clinic.

References
Using the valproate (VPA) rat model to understand the interaction between brain and gut within Autistic Spectrum Disorder (ASD)

Joanna Dennison1, Viviana Trezza2, Michela Servadio2, Ping Wang3, Jaleel Myan4, Joanna Neill5

1 Manchester Pharmacy School and 2 Faculty of Life Sciences University of Manchester, Manchester, M13 9PT, UK
3 Department of Science, RomaTre University, Viale Marconi 446, Rome, Italy

The Valproate (VPA) Rat Model

Introduction
Autistic spectrum disorder (ASD) is a neurodevelopmental condition that affects central and peripheral nervous system, neurochemistry and behaviour. Current etiological understanding is limited, however, symptomatic heterogeneity within patient cohorts supports a complex interaction of genetic and environmental mechanisms (Goyal & Myan 2014). Alongside the core behavioural symptoms (e.g. impairment in social interaction) recent focus has been given to the presence of comorbid gastrointestinal (GI) dysfunction (McElhinney et al. 2014).

Methods
Animal models of ASD that employ a maternal insult allow the study of behavioural and pathophysiological changes in affected offspring. Male Wistar rats from injected dams were used for behavioral testing in adolescence and adulthood. These animals were found to vocalise significantly less and demonstrated greater anxiety in behavioral testing.

Histology: Immunohistochemistry was performed with anti beta-ubbin to study changes in neural innervation to the gut. Samples of rectum (Duodenum, Jejunum, Ileum) and large intestine were embedded in paraffin wax and 10µm longitudinal sections were prepared using a microtome. 10µm was chosen to allow for some movement of nerve fibres in the plane of sectioning and achieve a sufficient measure of neural innervation.

Metagenomic sequencing: The microbiome of VPA and control rats was analysed using fecal samples (+/-group). DNA of the bacteria present were extracted, amplified and finally sequenced. Fecal samples were collected in late adolescence and immediately frozen (-80°C).

Results: Neural innervation to the gut

Figure 1: A) Example micrograph (1:1000 dilution) of B) Graphs 1-3: Across all sections of the gut no significant difference was found in neural innervation (measured in the dil and the underlying mucosa) between control and VPA treated rats supporting previous findings. B) Example micrograph (1:1000 dilution) of L. Three equidistant tissue samples from the large intestine were used for the analysis of changes to neural innervation to the large intestine. The average % area stained across these sections revealed no significant change in VPA vs. vehicle-treated rats. C) The data analysis was repeated on these sections with measurements taken from 20 x10 µm to increase the validity and reliability of the analysis. This confirmed no significant change in human neural stain.

Microbiota changes in VPA treated rats

Figure 2: A) Total number of classifications at each taxonomic level that were produced from the metagenomic analysis. No significant change in microbial biodiversity was found. B) A graph to show the significant reduction in the Bacteroides class in VPA vs. vehicle treated rats. C) A graph to show the significant increase in the Ruminococcaceae genus in VPA vs. vehicle treated rats (p < 0.05, unpaired Student’s t-test).

Figure 3: A heat map to show the bacterial species found to be statistically different between control and VPA rats (p<0.05). The heat map shows each treatment group whether the bacterial species was enriched or depleted compared to the base mean.

Conclusions and Future work

Increased understanding of the gut-microbiome-brain interaction within ASD animal model is particularly important given the incidence of comorbid GI abnormalities seen in the clinic.

Neural innervation

Conclusion: The methods used were insufficient to detect any changes in the neural innervation to the GI tract. The neurodevelopmental nature of the chosen insult, and changes seen in the composition of microbiota suggest the analysis methods used were inappropriate to detect hard-wired neural changes.

Future work: Analyse neural innervation to the gut with different methods: protein expression (Western blot), investigate changes to GI functionality, organ bath, fecal motility prep.

Microbiota

Conclusion: At the top-level of analysis changes in microbiota may be overlooked.

Future work: Explore further the changes microbiota at species level in order to determine their functional relevance.

References


Faculty of Medical and Human Sciences
Faculty of Life Sciences
Maternal immune activation: A viable candidate for modelling neurodevelopmental disorders in rats?

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¹ Manchester Pharmacy School and ² Faculties of Life Sciences University of Manchester, Manchester, M13 SPT, UK

Maternal Immune Activation

Background
- Epidemiological evidence suggests an association between maternal infection and the risk of neurodevelopmental disorders, including autism (ASD) and schizophrenia.
- Maternal immune activation (MIA) is a key factor in the development of maternal infection during pregnancy in rodents.
- Studies in rodents have shown early gestational exposure to MIA can produce phenotypes relevant to ASD.
- Further validation of this model is required in early gestational time points (gestational day GD 11) to explore the mechanisms leading to the phenotypic changes produced by exposure to MIA in rodent models of ASD.

Aims and Objectives
- Develop the postnatal model of MIA focusing on the induction of a behavioural phenotype relevant to ASD.
- Validate this model by the analysis of somatic conditions in ASD (e.g. gating behavior).

Methods
- GD 11: Pregnant rats were assigned to control (n=10) and MIA (n=10) groups.
- GD 12-19: MIA group was exposed to intraperitoneal injections of LPS (LPS 5 mg/kg, i.p.) on GD 12, 14, and 16.
- GD 20: Pups were removed from the dams and weighed.
- GD 21: Pups were weaned, and the dams were killed.
- Pups were assigned to control (n=10) and MIA (n=10) groups.
- Pups were exposed to a novel environment on GD 21.
- Open Field Test was used to assess locomotor activity and anxiety-like behaviors.
- Blood samples were collected from the pups on GD 21.
- RNA was extracted from the brain tissue and subjected to quantitative real-time PCR analysis.

Results: Cohort 1

Conclusions and Future work
- Further behavioral studies with increased litter numbers are required to determine the validity of the present findings.
- The results suggest that MIA in the maternal and perinatal period can have long-term effects on offspring, including behavioral changes and alterations in gene expression.
- Further studies are needed to understand the mechanisms underlying these effects and to develop potential interventions for neurodevelopmental disorders.

References

Maternal immune activation: a viable candidate for modelling neurodevelopmental disorders in rats?

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Introduction
- Epidemiological evidence supports an association between maternal infection and the risk of neurodevelopmental disorders in offspring (e.g. auditory spectrum disorder (ASD) and schizophrenia) ¹
- Poly(I:C)-induced maternal immune activation (mIA) in rodents is a key model for the investigation of maternal infection during pregnancy
- Studies in rodents have shown early gestational exposure to mIA can produce phenotypes relevant to ASD ²
- Further validation of this model is required in rats at early gestational time points (gestational day (GD) 12.5) to explore both early neurobiological, neurodevelopmental and behavioural changes in male and female offspring

Aims and Objectives
- Provide a comprehensive validation of the poly(I:C) model of mIA at GD12.5
- Perform behavioural phenotyping
- Explore early neurodevelopmental changes within this model

Methods

Behavioural testing: All offspring from poly(I:C) and saline treated dams were tested in the open field test (OFT) and social interaction test. Social communication was monitored at P30 using 30 cm long wildtype or poly(I:C) pups from the next.

qPCR: miRNA expression was normalised to SDH4, housekeeping gene and expressed as fold change from a selected naive tissue using 2^(-ΔΔCt).

Statistical analysis: For comparisons between offspring of poly(I:C) and saline-treated pups, a nested-ANOVA was performed with litter treated as a random variable. The homoscedasticity (Levene’s) test) and normality (Shapiro-Wilk test) of each data set were tested. To perform litter effects skewing data where nested analysis was not possible, mean values per litter are presented (Figure 6). Student’s t-test was performed on a Mann-Whitney test was used when parametric analysis was not applicable.

Results: Maternal response
- 10 mg/kg poly(I:C) induces a variable immune response in pregnant Wistar rats at GD12.5

Results: Behavioural phenotyping
- 10mg/kg poly(I:C) at GD12.5 reduces risk-taking behaviour of male adolescent offspring in the OFT

Results: Social communication
- mIA induces the change in early communication in poly(I:C) offspring

Results: qPCR
- miRNA expression analysis of Oxim3 (microglial marker) in male offspring frontal cortex at GD21

Results: Morphological changes GD21
- mIA induces a reduction in plastic scit in male offspring

Conclusions
- 10 mg/kg poly(I:C) on GD12.5 reduced risk-taking behaviour in male offspring but did not significantly affect other behavioural parameters
- 10 mg/kg poly(I:C) on GD12.5 reduced significant reduction in female plastic weight but no change to other morphometric parameters
- Expression of Oxim3 (microglia marker) was increased in frontal cortex from male offspring at GD21
- This study provides an in-depth longitudinal examination of this mIA model. This is the first study to use GD12.5 mIA in Wistar rats for investigation of NDDs

References

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Early neurodevelopmental and behavioural consequences of maternal immune activation at GD12.5 in Wistar rats

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Introduction
- Viral infection in pregnancy has been associated with increased risk for the development of autism spectrum disorder (ASD).  
- Poly(I:C) induced maternal immune activation (mA) in rats as a model for the investigation of maternal infection during pregnancy
- Studies in rodents have shown early gestational exposure to mA can produce phenotypes relevant to ASD.
- Further validation of the model is required in rats at early gestational time points (gestational day (GD) 12.5) to explore both early neurodevelopmental and behavioural changes in male and female offspring

Aims
- Provide a comprehensive evaluation of the effects of poly(I:C) treatment at GD12.5 in rats
- Explore early neurodevelopmental changes within this model
- Perform behavioural phenotyping

Methods
- Poly(I:C): 25 µg poly(I:C) or saline (0.9% NaCl) were injected intraperitoneally on GD12.5. 
- Behavioural testing: All offspring from poly(I:C)- and saline-treated dams were tested in the open field test (OFT) and Sucrose preference test (SPT) on postnatal day 42 (P42). 
- qPCR: mRNA expression in front cortex was normalised to CDH1 and GAPDH housekeeping genes and presented as the fold change from the control (Gapdh analysis 2006, Geiger). 
- Statistics: For comparisons between offspring of poly(I:C)- and saline-treated dams, a nested ANOVA was performed with litter treated as a random variable. The homoscedasticity (Levene’s test) and normality (Shapiro-Wilk test) of the data set were tested. For comparison of mean values unequal T-test or Mann-Whitney test was used when parametric analysis was not applicable.

Results: GD21
- mA reduces placental weight in female offspring only with no effect on body or brain weight

Results: PD21
- mA alters expression of Shank3 and Mhps2 in a sex-dependent manner in frontal cortex (FC) at PD21
- mA increases anxiety-like behaviour in male adolescent offspring in the OFT

Conclusions
- mA at GD12.5 in Wistar rats resulted in:
  - A significant reduction in female placenta weight but no change in other maternal parameters or gene expression at GD21
  - A significant treatment- and sex-dependent changes in gene expression in the FC related to synaptic scaffolding protein (Shank3) and number of PSD integrity (Mhps2)
  - A significant treatment- and sex-dependent changes in brain weight at PD21
  - The appearance of a subtle anxiety-like phenotype in male offspring but not in any other behavioural parameter measured (social interaction, play or communication and reproductive behaviour)

This study provides an in-depth longitudinal evaluation of this mA model. This is the first study to use GD12.5 mA in Wistar rats for investigations of ASD.

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